The Mouse Mutant Claw Paw:

Cellular and genetic aspects

Ayser Darbaş

Cover: Calligraphy of the word Schwann cell in Arabic letters, depicted as both myelinated peripheral nerve and DNA-helix, in the form of a flower.

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De muis mutant claw paw: cellulaire en genetische aspecten

Proefschrift

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Promotor:	Prof.dr. F.G. Grosveld
Overige leden:	Prof.dr. C.I. de Zeeuw Dr. G.T.J. van der Horst Dr.ir. N. Galjart
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We entered the house of realization,
we witnessed the body.

The whirling skies, the many-layered earth,
the seventy-thousand veils,
we found in the body.

The night and day, the planets,
the words inscribed on the Holy Tablets,
the hill that Moses climbed, the Temple,
and Israfil's trumpet we observed in the body.

Torah, Psalms, Gospel, Quran-
what these books have to say,
we found in the body.

Everybody says these words of Yunus
are true. Truth is wherever you want it.
We found it all within the body.

The drop that became the sea
Yunus Emre
Translated
by Kabir Helminski and Refik Algan

Contents

List of abbreviations

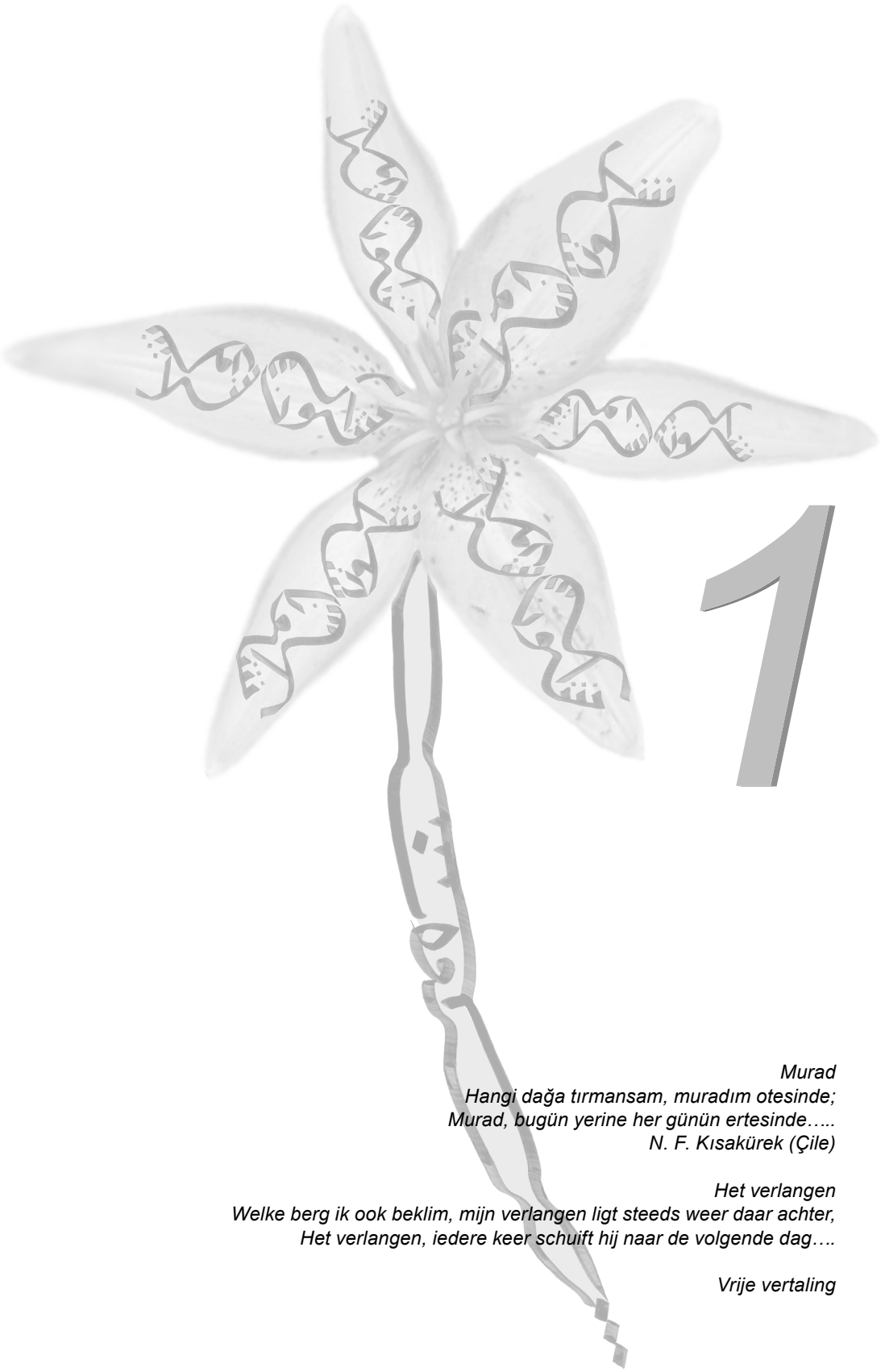
AMC	arthrogryposis multiplex congenita
ATP'ases	Enzyme that catalyses a process involving the hydrolysis of ATP (adenosine triphosphate)
BAC	bacterial artificial chromosome
cAMP	cyclic AMP
caspr	contactin associated protein
CH	congenital hypomyelination
clp	*claw paw*
CMD	congenital muscular dystrophy
CMT	Charcot Marie Tooth
CMTX	X-linked form of Charcot Marie Tooth
CNS	central nervous system
CRP-2	cysteine rich protein 2
dhh	desert hedgehog
DNA	deoxyribonucleic acid
DRP2	dystrophin related protein 2
DSS	Dejerine-Sottas Syndrome
dy/dy	laminin deficient mouse
E	Embryonic day
EAR	Epilepsy associated repeat
Egr-2	Early growth response gene
ENU	N-ethyl-N-nitrosurea
ErbB2/ErbB3	Neuregulin receptors which dimerize
EST's	expressed sequence tags
Fxyd3	Regulator of Na+/K+ ATP'ase
Fxyd7	Regulator of Na+/K+ ATP'ase
GAP-43	growth associated protein-43
GFAP	glial fibrillary acidic protein
GGF	glial growth factor
HMSN	hereditary motor and sensory peripheral neuropathy
HPRT	hypoxanthine phosphoribosyl-transferase
ISE	immature Schwann cell enhancer element in the Krox20 locus
KIF1B beta	An isoform of kinesin superfamily of molecular motor proteins
Krox-20	a zinc finger transcription factor
L1	Immunoglobulin related adhesion molecule
LacZ	ß-galactosidase
Lgi-1	leucine rich glioma inactivated-1
LRR	leucine rich region
MAG	Myelin associated glycoprotein
MBP	myelin basic protein
MOBP	Myelin oligodendrocyte basic protein
MSE	myelinating Schwann cell enhancer element in the Krox20 locus
Nav1.2	sodium channel found in immature axons
Nav1.6	sodium channel found in mature axons
NF	neurofilament
NGF-R	nerve growth factor receptor
NrCAM	neural cell adhesion molecule
Oct-6	a POU domain transcription factor
OSP	Oligodendrocyte specific protein
P	Postnatal day
PAC	P1 phage based artificial chromosome
P2	fatty acid binding protein
PDZ motif	Postsynaptic density protein, the drosophila *discs large* tumor suppressor and the tight junction protein ZO-1
PLP	proteolipidprotein
PMP-22	peripheral membrane protein-22
PNS	peripheral nervous system
POU domain	DNA binding domain first found in Pit-1, Oct-1/2, Unc-86
P-zero	Myelin protein zero
QKI	Quaking I
RNA	ribonucleic acid
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SCE	Schwann cell specific enhancer in the Oct-6 locus
Scn1b	voltage gated sodium channel beta1 subunit
STAR	signal transduction activator of RNA metabolism
STS	Sequence tagged site
WD	Wallerian degeneration
YAC	yeast artificial chromosome

General introduction and aim of this thesis

The overall aim of the experimental work presented in this thesis was to unravel the genetic and cell biological mechanisms that govern myelination in the peripheral nervous system (PNS) in order to understand the relationship between congenital hypomyelination and joint contractures, as observed in claw paw *(clp/clp)* mice [1]. This mouse mutant faithfully models a neurogenic form of congenital arthrogryposis observed in humans [2-7].
The two specific aims of this thesis are: To define in which cell type of the developing nerve *clp* gene function is required and to further define the cell biological and developmental defects in *clp/clp* mice.

The myelin sheath is a highly organized structure, formed by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Myelin formation and homeostasis depends on axonal signals, the nature of which remains poorly understood [8-12]. The *clp/clp* mouse is an animal model for congenital dysmyelination of the peripheral nervous system. Other mouse mutants showing dysmyelination of the PNS include *trembler, dystrophic* and *quaking* mice. *Trembler* and *dystonia* have abnormal myelination exclusively in the peripheral nervous system, similar to *clp/clp*. In contrast, *quaking* exhibit hypomyelination in both the central and peripheral nervous system [13]. However, while in *clp/clp* mutant mice the posture abnormality is detectable within 2 days after birth [1], the other mice exhibit limb abnormalities in the second postnatal week [13].
Reciprocal signaling between axons and Schwann cells is required for myelination. Therefore, the pathology of dysmyelination alone does not reveal whether axonal signals or glial signals are impaired.
Several experimental approaches have been developed to unravel the interdependence of axon and glia in the pathogenesis of dysmyelination.
Schwann cell differentiation can be monitored during development using *in vitro* culture experiments and nerve regeneration experiments [14]. Although nerve regeneration does not mimic nerve development in each single detail [15, 16], it is considered a reiteration of development and can be used as an experimental model to study myelination.
Grafting experiments have been useful to distinguish between axonal or Schwann cell defect in *trembler*, *dystrophic* and *quaking* mouse mutants [13]. Therefore, we used reciprocal grafting experiments between wildtype and *clp/clp* mice, to identify the cell type (neuron, Schwann cell or both) underlying the claw paw phenotype. In addition, we show that *clp/clp* nerves are delayed at the radial sorting stage of nerve development, probably contributing to the previously described congenital hypomyelination phenotype.

Myelination and Schwann cell differentiation was further studied in the *clp/clp* mouse through analysis of the expression patterns of two pivotal transcription factors involved in myelination, namely Oct-6 and Krox-20. In this thesis I further describe our efforts to identify the *clp* mutation through positional cloning.
In summary: Chapter 1 gives a general introduction to regulation of PNS myelination.
Chapter 2 will introduce the experimental work.
Chapter 3 describes the transplantation experiments performed between *clp/clp* and its littermates.
Chapter 4 describes that expression of myelin proteins involved in polarisation are delayed in *clp/clp* mice.
In chapter 5 we make a start with the positional cloning of the clp gene using molecular biological approach.
Chapter 6 contains concluding remarks and future perspectives.



1

Murad
Hangi dağa tırmansam, muradım otesinde;
Murad, bugün yerine her günün ertesinde…..
N. F. Kısakürek (Çile)

Het verlangen
Welke berg ik ook beklim, mijn verlangen ligt steeds weer daar achter,
Het verlangen, iedere keer schuift hij naar de volgende dag….

Vrije vertaling

Chapter 1

Introduction to myelination

Myelin structure

Myelin sheath

Peripheral nerves mainly consist of axons insulated by a layer of myelin. Myelin was first described some 150 years ago by Virchow [17-19]. Many large caliber axons (>0.7 μm) in vertebrates are surrounded by a myelin sheath [9, 20]. The myelin sheath function is frequently compared with that of the plastic insulation around an electric wire. Its principal roles are indeed to reduce the capacitance of the internodal axolemma and preventing dissipation of the action potential. However, myelin also has very important other functions including structural, mechanical and trophic support to the axon [8, 9].

Myelin is found in both the central (CNS) and peripheral nervous system (PNS) (See Figure 1).

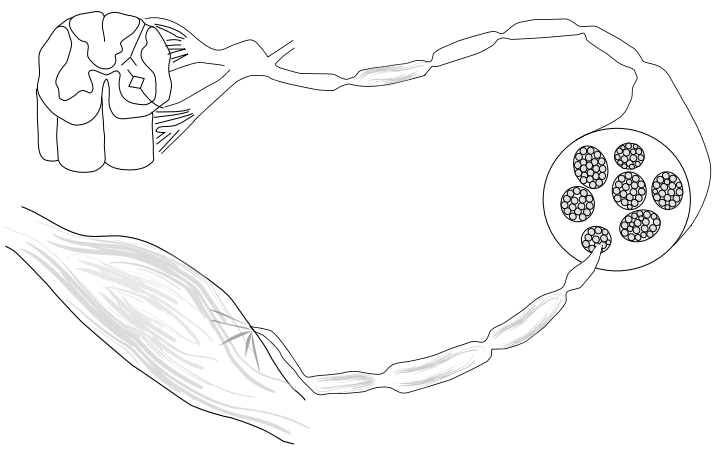


Figure 1. A schematic view of the locomotor apparatus, including central, peripheral nervous system and muscle. Myelin is formed in both the central and peripheral nervous system. Schwann cells form myelin around axons in the peripheral nervous system. Impulses are generated at the axon hillock of sensory neurons in the dorsal root ganglia and conducted rapidly in a saltatory fashion, jumping from node to node, along the length of the axon. Adapted from Ho, Annu. Rev. Neurosci. 1998

However, the cell type forming this sheath is different in PNS and CNS. Oligodendrocytes are the myelinating glial cells in the CNS and Schwann cells in the PNS. While oligodendrocytes form a myelin sheath around multiple axons, only a single axon is myelinated by a Schwann cell. Despite the fact that each Schwann cell has the potency to form a myelin sheath, unknown axonal signals determine whether myelination occurs [8, 21, 22]. Non-myelinating Schwann cells ensheath multiple axons and provide insulation of slow-conducting nerves (C-fibres) [23]. While oligodendrocytes are not associated with a basal lamina, the latter has a critical role in myelin formation by Schwann cells [24, 25]. Although the myelin sheath plays the

same role and has similar biochemical properties in both CNS and PNS, important differences between the myelin sheath in the two systems exist. We will focus on myelination in the peripheral nervous system.

Establishment of proper contact between the future myelinating Schwann cell and an axon involves the polarization of the cell and elaboration of a basal lamina [22]. Next the Schwann cell plasma membrane starts to wrap itself spirally around the axon repetitively 5 to 100 times [26], depending on axonal diameter [27, 28]. The different structural regions that can be recognized in mature myelin are depicted in Figure 2.

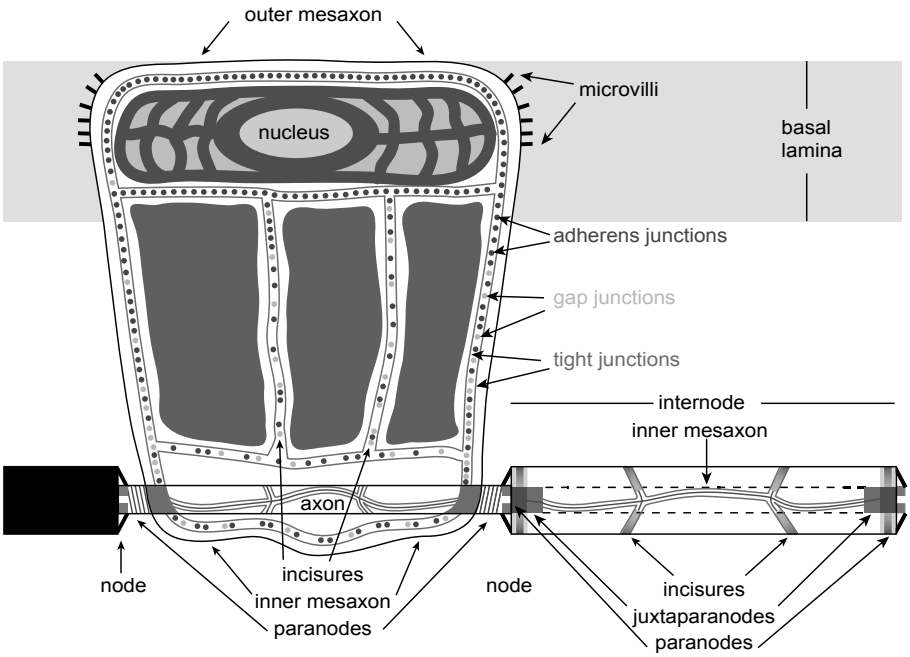


Figure 2. Schematic drawing showing an unrolled myelinating Schwann cell highlighting regions of compact and non-compact myelin. This figure also shows the position of tight junctions (green), gap junctions (orange) and adherens junctions (blue) found in the myelinating Schwann cell. The paranodal and juxtaparanodal regions are depicted in blue, red and green respectively. With permission from the author Steve S. Scherer, J Peripher Nerv Syst 2002 and Blackwell publishing

Two main types of myelin exist in one and the same internode: compact and non-compact myelin [8, 22]. The latter is found in the structurally and functionally distinct domains called Schmidt-Lantermann incisures (funnel shaped interruptions in compact myelin) and paranodal loops (lateral borders of the myelin sheath) [29]. Furthermore, the myelin sheath is interrupted at regular intervals along the length of the axon at the nodes of Ranvier. However, the basal lamina of adjacent Schwann cells are fused, forming a continuous tube of extracellular matrix material enclosing

the entire myelinated axon [29]. The space under the basal lamina at the node of Ranvier is referred to as the nodal gap. It contains interdigitating Schwann cell microvilli and extracellular matrix molecules [8]. The section between two nodes of Ranvier is referred to as the internode.

Although myelin in the CNS and PNS is very similar, overlapping but not identical sets of myelin proteins are found in these two systems [11, 22] (see Figure 3).

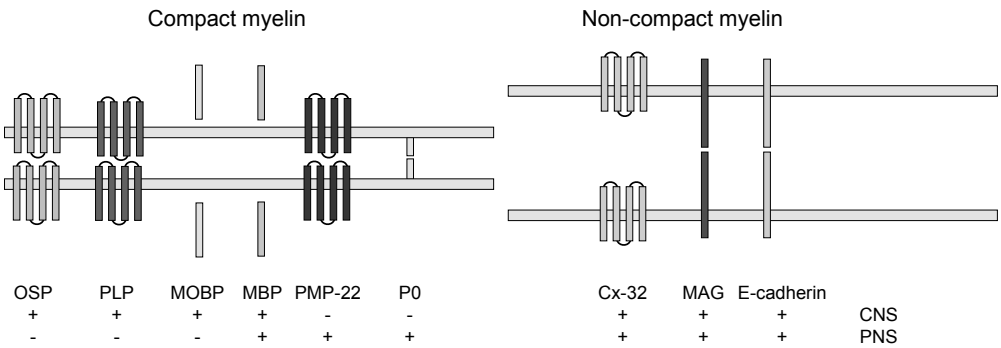


Figure 3. The proteins found in compact and non-compact myelin in both central and peripheral nervous system are depicted. Compact myelin in the peripheral nervous system contains P0, PMP22, MBP, whereas in central nervous system myelin oligodendrocyte basic protein (MOBP), oligodendrocyte specific protein (OSP), proteolipidprotein (PLP), and MBP is found. Connexin-32, myelin associated glycoprotein and E-cadherin are found in non-compact myelin of both peripheral and central nervous system. Adapted from Steve S. Scherer, J Peripher Nerv Syst 2002

Myelin protein zero (P-zero), myelin basic protein (MBP) and peripheral membrane protein-22 (PMP-22) are components of compact myelin [22]. Whereas P-zero and PMP-22 are exclusively found in the PNS, myelin basic protein is found in both the PNS and CNS. Conversely, proteolipidprotein (PLP), myelin oligodendrocyte basic protein and oligodendrocyte specific protein are exclusively found in compact myelin of the central nervous system. Myelin associated glycoprotein (MAG) and E-cadherin are components of non-compact myelin in both CNS and PNS [8].

Nodes of Ranvier

The myelinating glial cells are wrapped around the axon, except at the nodes of Ranvier, which are regularly spaced small gaps enriched with channels.

Clustering of sodium channels and potassium channels in the nodal and juxtaparanodal axolemma respectively, effectively restricts generation and regeneration of the action potential to the nodal region and results in a saltatory mode of impulse propagation along the myelinated nerve fiber. It is generally believed that this clustering of ion channels results from specific axon glia interactions that determine

the development and maintenance of the nodal cyto-architecture [10, 22, 29, 30] (Figure 4).

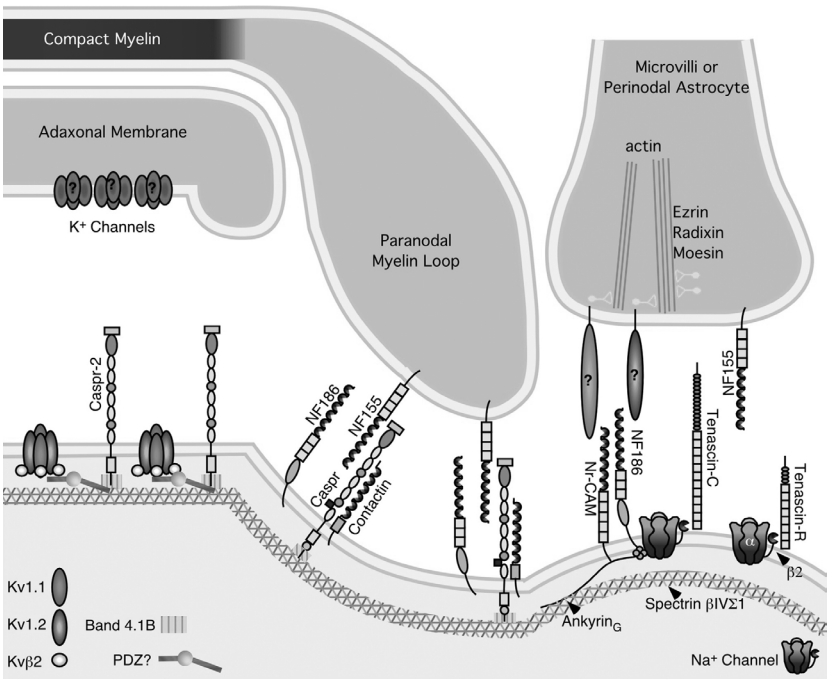


Figure 4. A schematic drawing of nodes, paranodes and juxtaparanodes, including the possible molecular interactions. With permission from the author Steve S. Scherer, J Peripher Nerv Syst 2002 and Blackwell publishing

Voltage gated sodium channels are composed of a central pore-forming α-subunit and two auxillary β-subunits [31]. In addition to voltage gated sodium channels, sodium calcium exchangers and (regulators of) sodium/potassium ATP'ases [30] are involved in maintaining and restoring the action potential. These channels interact with other nodal membrane molecules, such as neurofascin 186 and tenascin, and cortical proteins such as ankyrin-G and Spectrin IVB (see Figure 4) [30, 32]. Sodium channels are able to form complexes with intracellular and transmembrane proteins, stabilize the membrane through linkage with extracellular matrix proteins and are involved in intracellular signaling [30].

As mentioned earlier, it is generally thought that the clustering of ion channels results from interaction with the glial cell. However, there is evidence to suggest that this clustering results from axon intrinsic properties. In particular, it was shown that clustering of sodium channels occurs in dendrites of retinal ganglion cells in the absence of glial cells [33]. In addition, internodal distances are constant along

individual axons and ankyrin-G clustering occurs prior to sodium channel clustering implicating axonal properties [10]. On the other hand, sodium channels can be seen to cluster at the progressing hemi-node during development, as if the sodium channels are being mopped up by the myelinating glia cell [11, 34].

Thus, specific glial-axon interactions orchestrate the development and maintenance of the nodal, paranodal and juxtaparanodal domains. These interactions are mediated through a host of adhesion molecules, some of which are known. The correct cytoarchitecture of the node of Ranvier is essential for proper, saltatory impulse propagation [30].

The paranodes and juxtaparanodes

The saltatory propagation of action potential does not only require intact nodes, but also paranodes. At the paranodes the lateral loops of the myelin sheath (paranodal loops) touch down on the axolemma to form the axo-glial junction as illustrated in Figure 5, which resemble, structurally and functionally, invertebrate septate junctions [22, 29, 35].

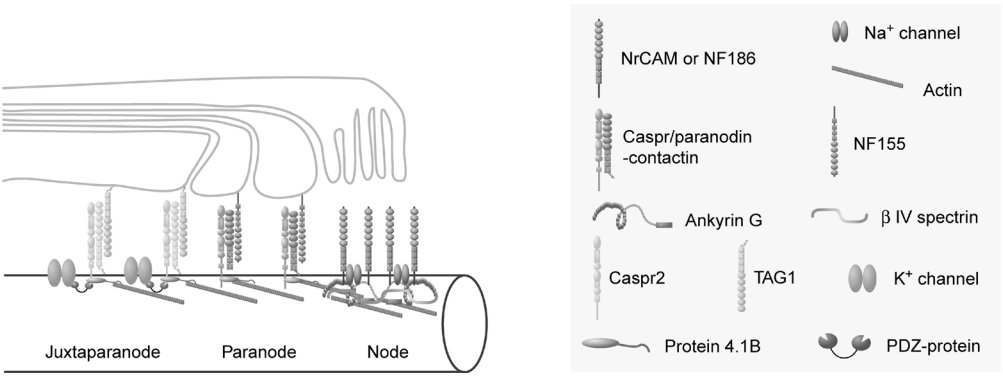


Figure 5. An illustration of axo-glial junction is depicted. These septate-like junctions are progressively formed at paranodes with the recruitment of Caspr/contactin and Neurofascin 155. Furthermore the proteins involved in the formation of juxtaparanodes and nodes are also depicted. With permission from the author E. Peles, Current Opinion Neurobiology 2002 and Elsevier.

Although septate junctions are comparable with vertebrate tight junctions, septate junctions allow diffusion of molecules of low molecular mass (5 kDa) into periaxonal space. Similarly diffusion is possible through septate like axo-glial junction into the periaxonal space, allowing signalling between glia and axon [10, 22, 24, 29, 35]. Adhesion of the paranodal loops to the axonal membrane occurs via adhesion molecules present in both the axonal and Schwann cell membrane. Molecules which are involved in the forming of paranodes are, at the axonal side, Contactin [36, 37], Contactin associated protein (Caspr) and, at the glial side, Neurofascin 155 [38]

(figure 4). Indeed, homozygous deletion of the Contactin gene disrupts attachment of the paranodal loops to the axolemma, resulting in paranodal loops pointing away from the axolemma. As a consequence, potassium channels directly juxtapose sodium channels resulting in altered physiological properties of the nerve fiber and reduced conduction velocities [36].

During development, the sodium channel subtypes expressed at the node undergo a transition from Nav1.2 (immature nodes) to Nav1.6 (mature nodes).

In Caspr deficient mice, paranodal loops detach and retract, the nodes are widened and immature sodium channels (Nav1.2) are found in the CNS, whereas only minor differences in the paranode are found in the PNS. In addition, the transition of sodium channel isoforms from Nav1.2 to Nav1.6 is transiently delayed in PNS, whereas this transition does not occur in CNS of Caspr-deficient mice. Furthermore, potassium channels in the PNS are localized at the paranodes instead of juxtaparanodes (as in contactin-deficient animals), whereas in the CNS a transient mislocalization is observed in caspr-deficient mice [39].

In summary, paranodes regulate the transition of channel subtypes and provide a barrier for lateral diffusion of ion channels [34]. Therefore, paranodal junctions prevent the dispersion of channels and maintain the proper high concentration of sodium channels at the nodes and potassium channels at the juxtaparanodes [39].

Schmidt-Lantermann incisures

Recent work has elucidated that membranes of both Schmidt-Lantermann incisures and paranodes are similar, containing tight junctions, adherens junctions and gap junctions [40]. While at the paranodes adhesion molecules provide tight axo-glial interactions, Schmidtt-Lantermann incisures enable rapid cytoplasmic communication from the outermost (abaxonal) to innermost (adaxonal) cytoplasmic compartment of the Schwann cell via gap junctions (see Figure 6). It was hypothesized that the presence of gap junctions in the Schmidt–Lantermann membranes provides a direct radial pathway for metabolites and signal exchange, thus short cutting the circumferential route [41, 42]. To determine whether these channels indeed provide a radial pathway across the myelin sheath, thus diminishing the molecular transport distance, dye transfer studies were performed on freshly isolated myelinated fibers. The Schmidt-Lantermann incisures were stained when the dye front moved down the myelin sheath and this diffusion could be blocked with a drug interfering with the opening of gap junctions [41, 42]. Consistent with this observation, it was suggested that in patients suffering from the X-linked form of Charcot-Marie Tooth (CMTX), where mutations are found in connexin-32, this radial pathway is impaired resulting in longer transport routes and hence demyelination and axonal loss. Surprisingly dye studies performed on myelinating Schwann cells of connexin-32 null mice did not provide the

expected answer. Instead they showed a similar pattern to the wildtype, suggesting that one or more other gap junction protein are present and fullfill this function [29, 41, 42]. Although the suggestion that connexin mutations might impair the radial pathway is a plausible explanation for demyelinating neuropathies within some families, it does not clarify axonal neuropathies in other families. Some authors have suggested a misclassification of the disease [43]. Others have proposed that abnormal properties of functional channels, for example altered voltage gating [44], may underlie the pathology of CMTX. Nevertheless connexin-32 is one of the gap junction proteins found at paranodes and Schmidtt-Lantermann incisures, providing a link between the abaxonal and adaxonal cytoplasmic compartment within the Schwann cell [30].

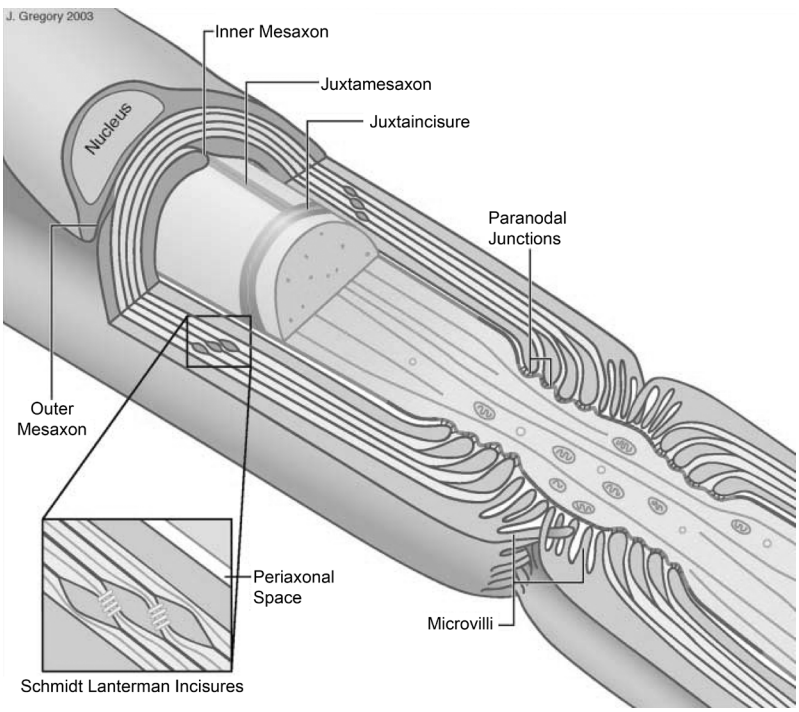


Figure 6. A schematic picture of a peripheral myelinated nerve fibre. Both longitudinal and cross sections are illustrated, demonstrating the different regions such as paranodes, juxtaparanodes, microvilli, Schmidtt-Lantermann incisures. Furthermore, the inner mesaxon and outer mesaxon is depicted. With permission from the author James L. Salzer, Neuron 2003

Basal lamina

The differentiation of Schwann cells into myelin and non-myelin forming cells involves the polarisation of the cellmembrane into an adaxonal (directly contacting

the axolemma) and an abaxonal domain. As part of this process, a basal lamina is deposited by the Schwann cell at it's abaxonal surface. The formation of a basal lamina is important for subsequent cellular differentiation [25, 45, 46], in particular myelination, as myelination is delayed in its absence [47].

The basal lamina is continuous along the length of the myelinated fiber and non-myelinated Remak fiber. Major components of the basal lamina are the laminins. Laminins provide an essential function in assembling the other components of basal lamina into a tight network. These other components include molecules such as type IV collagen, fibronectin, glypican, N-syndecan and entactin [8, 48]. In addition, laminins are important ligands for cell surface receptors such as the integrins and dystroglycan [8, 48], thus linking the basal lamina to essential processes such as cellular proliferation, differentiation, migration and morphology [47, 48].

Each laminin molecule consists of three subunits, α, β and γ chains. Laminin-2 (α2β1γ1) also referred as merosin (s-laminin) is a major constituent of basal lamina in muscle and peripheral nerve. In the peripheral nerve the basal lamina is linked to the actin cytoskeletal network via dystroglycan, dystrophin related protein (DRP2) and L-periaxin (myelin protein required for maintenance of myelin integrity) as indicated in Figure 7 [26, 49].

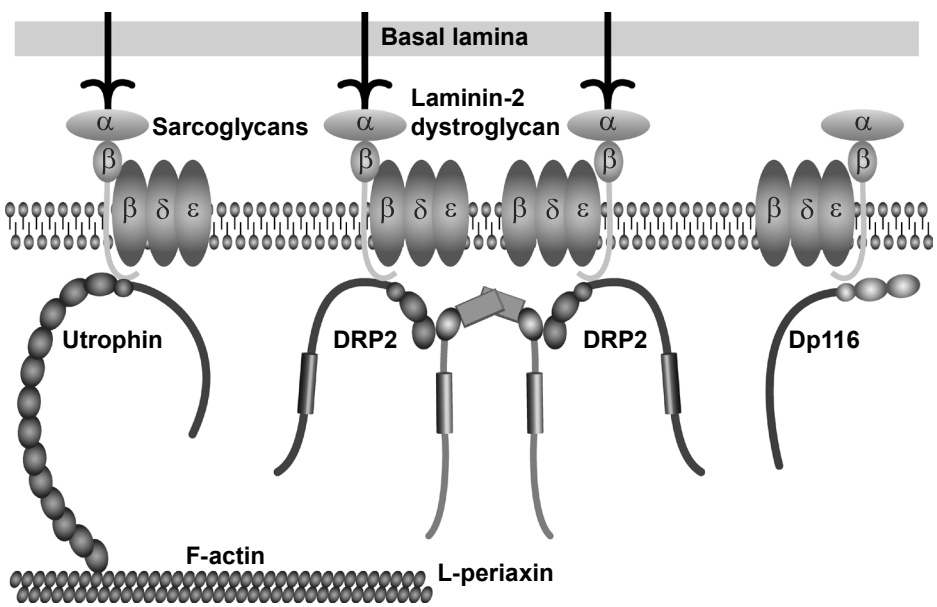


Figure 7. A schematic picture of the Schwann cell basal lamina and the possible molecular interactions. Laminin-2 is linked to α- and β-dystroglycan which in turn is linked to dystrophin related protein and periaxin. With permission from the author Steve S. Scherer, J Peripher Nerv Syst 2002 and Blackwell publishing

Myelination of the peripheral nervous system

Initiation of myelination; axonal components

During embryonic development axons navigate through the embryonic environment to reach their target organs. A part of establishment of such a complex network as the peripheral nervous system is the directed growth of the axon to its target. Pathfinding of migrating axons and the forming of connections in the developing nervous system is controlled via attractive, repulsive and permissive cues. For example neurotrophins, ephrins and extracellular matrix molecules (laminins) are associated with axonal guiding. Either they act via specific receptors or bind to specific cell adhesion molecules. Integration of these attractive and repulsive signals occurs at the tip of the growing axon, referred to as the growth cone, resulting in cytoskeletal changes of the axon and hence in neuronal polarity [50]. In addition to extracellular matrix molecules and diffusible molecules, it has been shown that also neuronal activity plays a role in axon guidance as analysed in Xenopus spinal neurons. It was demonstrated that electrical stimulation of neurons could turn attractive impulses into repulsive ones and vice versa [51].

Axonal outgrowth precedes and regulates Schwann cell survival and differentiation. Genetic and cell culture experiments have demonstrated that survival of Schwann cell precursors depends on axonally derived factors encoded by the Neuregulin1 gene [52-55]. This gene encodes, through alternative splicing, a number of proteins that can be grouped in three structurally related isoforms, called type I to III. The neuregulin isoform that acts as a cell survival factor for Schwan cell precursors is the ‘sensory and motor neuron derived factor’ (SMDF). The effects of Neuregulin isoforms are mediated through the hetero-dimeric receptor ErbB2/ErbB3 which is expressed on the Schwann cell membrane [52, 53]. In Neuregulin1 knock-out mice, Schwann cell precursors do not survive. Schwann cell precursors also die in ErbB2 and ErbB3 [56] knock-out mice. While it is unlikely that neuregulins play a role in the initiation of myelination, recent evidence establish a role for neuregulins in the extent of myelination, that is the number of myelin wraps elaborated around the associated axon [57]. It has been known for a long time that myelin is only formed around axons with a diameter larger than 0.7 μm and that the number of myelin wraps correlates with axonal diameter [58]. With the identification of neuregulin as one important regulator of myelin thickness, a significant step forward in understanding myelination has been made.

Axon calibre largely depends on neurofilaments (NF) and microtubules, major components of the axonal cytoskeleton [59]. While microtubules are found in immature axons, maturation of axonal calibre is associated with neurofilament and

in particular with phosphorylation of neurofilaments [60]. The phosphorylation state of neurofilaments, and thus the diameter of the axon, depends on myelination [61]. Therefore, reciprocal signalling between the Schwann cell and the axon determines axonal calibre and myelin thickness.

Despite extensive studies, it is still unclear how axons initiate the myelination program. But a correlation with action potentials [62], secretion of diffusible molecules and axonal diameter has been made.

Schwann cell development and differentiation

Schwann cell development and differentiation has been studied extensively, using modern and classic experimental approaches, including cell culture, nerve surgery, transgenesis and gene knock-out technology. Schwann cells develop from the neural crest, a transient migratory population of pluripotent embryonic cells that originates from the dorsal aspect of the neural tube. These migrating neural crest cells invade and populate the outgrowing embryonic nerve bundle and differentiate into Schwann cell precursors. These Schwann cell precursors further differentiate through a series of well-defined stages to give rise to the two mature Schwann cell phenotypes observed in the adult peripheral nerve tissue; the myelinating and non-myelinating Schwann cell. Neural crest cells do not only give rise to Schwann cells but also to neurons of the peripheral and enteric nervous system, endocrine cells, bone, cartilage, connective tissue, pigment cells and additional glia cell types such as the satellite cells of sensory neurons and the glia cells of the enteric nervous system [63, 64].

Schwann cell precursors migrate and proliferate along the outgrowing nerve bundles. These cells subsequently invade the bundles with their cytoplasmic extensions and segregate fibers into individual axons or families of axons, in a process referred to as radial sorting. While a prospective non-myelinating Schwann cell ensheaths multiple lower calibre axons, a prospective myelinating Schwann cell segregates axons until a one to one ratio is achieved.

Three major transitional stages can be distinguished in the differentiation of a Schwann cell; the transition of a migrating crest cell to a Schwann cell precursor (around embryonic day 9/10 E9/10), from precursor to immature Schwann cell (which occurs around E13 in mouse and E15 in rat) and finally the reversible formation of mature myelinating and non-myelinating Schwann cells (around birth and the first postnatal week) [65] (figure 8). These different stages are defined by a number of morphological criteria, expression of marker proteins and growth factor dependence in culture [21, 66]. For example, Schwann cell precursors express the low affinity neurotrophin receptor p75 and depend on Neuregulin for their survival in culture. Immature Schwann cells can be distinguished from precursors on the basis of expression of growth associated protein-43 (GAP-43) and their independence of

neuregulin for survival in vitro. Premyelinating and promyelinating Schwann cell are characterized by the expression of Oct-6 and, one day later, by expression of Krox-20 [67].

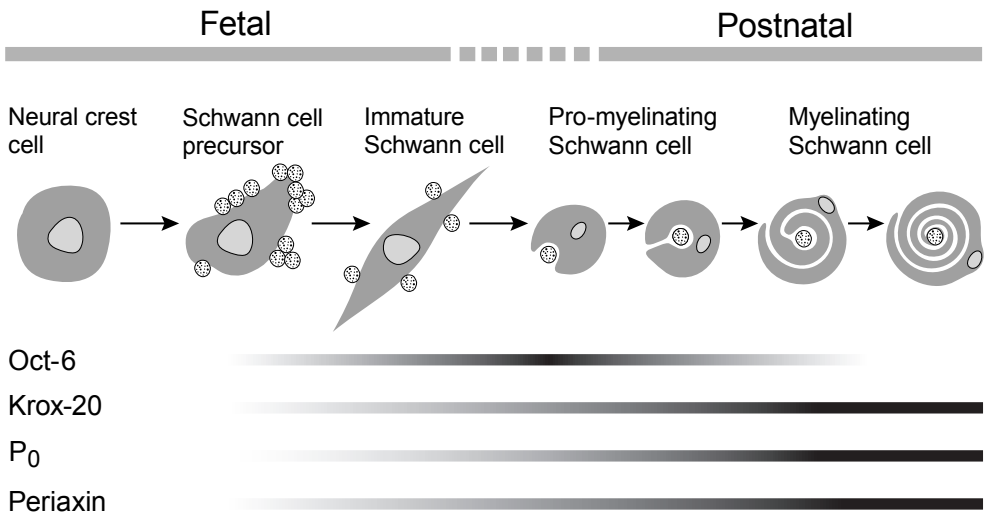


Figure 8. A schematic picture of Schwann cell differentiation and temporal expression of a number of relevant genes. Among these proteins, Oct-6 and Krox-20 are transcription factors and periaxin and P0 are myelin proteins.

In order to gain a deeper understanding of the molecular interactions between neurons and Schwann cells, it is important to identify the molecular components of the myelination program and to study the interaction of these components with each other and other factors that drive myelination. Among the genes involved in the differentiation of Schwann cells, the transcription factors Oct-6 and Krox-20 are extensively studied and have been shown to play important roles in the genetic cascade of myelination. Expression of both factors requires direct axonal contact.

Initiation of myelination; Schwann cell components

Oct-6

Transcription factors are also referred to as *trans*-acting factors. This term refers to the fact that transcription factors regulate expression of other genes, through binding to cis-acting sequences, such as enhancers, silencers and promoters present in the target gene locus [68]. Transcription factors are in general modular in stucture and can conveniently be thought of as consisting of several covalently linked functional

domains. One important functional domain of the transcription factor is the DNA binding domain. DNA binding domains are variations on several structural motifs such as the helix-turn-helix motif found in all types of homeodomain proteins and the zinc-finger domain, a finger-like structural motif that binds a Zn2+ atom. The POU (Pit-1, Oct-1/2 and Unc-86) domain consists of a POU specific domain linked through a short peptide sequence to a homeodomain, specific for this class of proteins. Both the POU-specific domain and the POU homeodomain are structural variations of the helix-turn-helix motif. Thus, the POU domain consists of two DNA binding domains tethered by a short linker. As a consequence, POU domain proteins bind to a bipartite DNA sequence which are variations on the octamer sequence, ATGCAAAT, first described to be an essential part of the immunoglobulin gene promoter and enhancer [69]. Members of the POU domain protein family play important roles in cell specific gene regulation during embryonic development, in particular in the nervous system [69, 70]. One of these POU proteins is Oct-6 (also referred as 'suppressed cAMP inducible POU' (Scip) or Testis 1 (Tst1; official MGI name; Pou3f1) [71]. This transcription factor is expressed in embryonic stem cells, testes, epidermis and in myelinating cells, both oligodendrocytes and Schwann cells [72]. In cultured rat Schwann cells, Oct-6 expression is up-regulated by agents that increase intracellular cyclic AMP (cAMP) concentrations such as Forskolin, a reversible activator of adenylyl cyclase. A similar upregulation was observed for the myelin proteins P-zero and P2. Therefore it was assumed that Oct-6 might be involved in regulation of myelin gene expression. However, it was found that Oct-6 is most abundantly expressed in promyelinating Schwann cells and is rapidly downregulated once myelination starts [69, 72, 73]. Furthermore, cotransfection studies in cultured rat Schwann cells, demonstrated that Oct-6 strongly represses the promoters of the P-zero and MBP myelin genes and $p75^{NTR}$ gene [74, 75]. This suggested an alternative role for Oct-6 as a repressor of myelin gene expression. It was hypothesized that downregulation of Oct-6 results in derepression of myelin gene expression and initiation of myelination. To test this hypothesis, Weinstein and colleagues sought to define a system in which Oct-6 could be specifically inhibited in Schwann cells. Their cotransfection data suggested that a truncated form of Oct-6, essentially consisting of the POU domain, inhibits repression of myelin gene promoters by Oct-6. Therefore, they generated a transgenic mouse expressing the truncated Oct-6 (referred to as ΔSCIP) from a rat P-zero promoter. The logic of this approach is twisted as it is expected that Oct-6 will repress the P-zero promoter. Moreover, it was known that P-zero is already expressed, although at low levels, in Schwann cell precursors and one could therefore expect expression of the transgene well before the beginning of myelination [76]. Nevertheless, transgenic mice were obtained that exhibit premature myelination and hypermyelination of the peripheral nerves [77]. However, their conclusion that indeed Oct-6 serves as

a repressor of myelin genes and that downregulation of Oct-6 initiates myelination contradicts evidence obtained through analysis of the developmental peripheral nerve phenotype in mice in which Oct-6 is homozygously deleted [78, 79]. Mice homozygous for the disrupted Oct-6 allele usually die within 2 hours after birth due to breathing problems, although some survive for several days, weeks or, in rare cases, even for months. Oct-6 mutant mice exhibit delayed onset of myelination of peripheral nerves with a transient arrest of Schwann cell differentiation at the promyelin stage. Myelination of the central nervous system by oligodendrocytes appears normal [78, 79]. This suggests that Oct-6 is not directly involved in myelination, but is required for progression from the promyelin into the myelinating stage of Schwann cell differentiation [78, 80].

The highly dynamic and diverse expression pattern of Oct-6 suggests the existence of a collection of complex enhancers within the Oct-6 locus [81]. In general, cell specific gene expression is governed by tissue specific promoters or other regulatory sequences such as enhancers which are the target of transcription factors and intra- and extracellular signalling pathways. As Schwann cell specificity was found not to be solely associated with the Oct-6 promoter, other regulatory regions were suspected to be involved. Using a deletion mapping approach in transgenic mice, a minimal cis-acting element was characterized, which was required to activate the Oct-6 gene expression in Schwann cells [82]. This element (Schwann cell specific enhancer (SCE)) was found to be sufficient to drive the Oct-6-dependent myelination. Hence, it was expected that deletion of the SCE would result in a Schwann cell specific Oct-6 null allele. To test this hypothesis, mice were generated in which the SCE was deleted. Indeed, deletion of the SCE only affected Schwann cell specific expression of Oct-6 without affecting the expression of Oct-6 in regions such as the hippocampus, brainstem nuclei and retina [15]. As the peripheral nerve phenotype in Oct-6 null mice and ΔSCE null mice is similar, this experiment also formally proves that Oct-6 is cell autonomously required in Schwann cells to drive the differentiation of these cells [15].

In summary, Oct-6 is cell autonomously required in Schwann cells to drive the cells through the promyelin stage of cell differentiation.

Krox-20

Krox-20 (Early growth response gene = Egr-2) is part of a multigene family encoding zinc finger transcription factors, and may play a role in the regulation of cellular proliferation. Krox-20 was identified as an immediate-early response gene that binds DNA in a sequence specific manner and is required for myelination of peripheral nerves. During mouse development Krox-20 is expressed in rhombomeres 3 and 5 (r3 and r5) of the segmented hindbrain, in immature and myelinating Schwann cells, in

hypertrophic chondrocytes and differentiating osteoblasts [83] and in hair follicles. In the adult, Krox-20 gene expression is maintained at low levels in myelinating Schwann cells, in bone and in specific neuronal populations of the cortex [84]. The expression pattern of LacZ (encoded by the b-galactosidase gene that was knocked in the Krox-20 locus) in Krox-20$^{\beta geo/\beta geo}$ mice, indicated that hindbrain segmentation is affected and that expression in boundary cap cells (glial cells) is confined to the motor and sensory roots of cranial and spinal nerves from embryonic day 10.5 (E10.5) till E14.5 [84]. It was remarkable to see that Krox-20 expression was restricted to boundary cap cells till E15.5. A transition occurs at day E15.5 when Krox-20 expression is detected along the peripheral nerve [84].

Thus, one of the questions was how Krox-20 expression is regulated from E10.5 till E15.5. This could be due to axonal factors, for example a soluble factor secreted during that time period. Cell culture experiments were performed in which E12.5 dorsal root sensory neurons were placed in culture together with Schwann cells derived from newborn mice heterozygous for the Krox-20/LacZ allele [67]. Indeed, sensory neurons were capable of inducing Krox-20 expression, suggesting that axons (E12.5) already have a signal to induce Krox-20 expression in Schwann cells. That Krox-20 is integral to the myelination program, was supported by experiments in which Krox-20 was expressed in cultured rat Schwann cells from a viral promoter. Using micro-array analysis of such transfected cells it was shown that Krox-20 induces expression of myelin genes, including P0, periaxin and myelin basic protein [85]. This study shows that Krox-20 influences expression of genes involved in Schwann cell differentiation, such as the cytoskeletal protein dendrin, cell adhesion molecules, laminin, integrin and genes involved in lipid metabolism.

Ghislain and colleagues [86] identified Schwann cell specific regulatory elements within the Krox-20 locus. Two specific cis-acting elements governing Schwann cell specific expression of Krox-20 were identified; the immature Schwann cell element (ISE) and the myelinating Schwann cell element (MSE) [86] (Figure 9). The latter element required the POU domain transcription factor Oct-6 at the time of myelination, consistent with the observation that Krox-20 expression is delayed in Oct-6 mutant Schwann cells. However, the activity of the former element (ISE) does not require Oct-6 [86], indicating that there could be another factor, probably parallel to Oct-6 and Krox-20, which could initiate myelination. Recently Jaegle and colleagues identified Brn-2 as the factor that can partially fullfil Oct-6 function in Schwann cell differentiation [87].

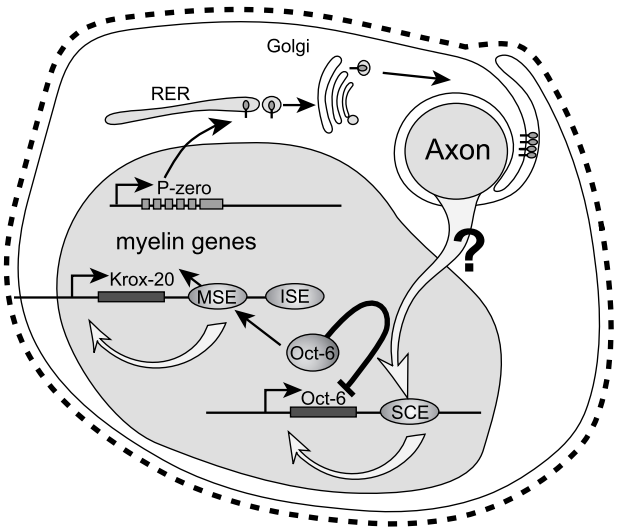


Figure 9. A Schwann cell has surrounded an axon, the presence of a basal lamina indicates myelination. Within the Schwann cell the genetic cascade involved in myelination is depicted. Unknown axonal signals initiate Oct-6 expression, in turn regulating Krox-20 gene expression via the recently identified MSE (myelinating Schwann cell element). Krox-20 is regulating the upregulation of many genes including myelin genes such as P0, periaxin and myelin basic protein. Adapted from W. Mandemakers [116]

Oct-6 and Krox-20 null phenotypes

Analysis of both the Oct-6 and Krox20 mouse mutants was hampered by the early postnatal death of homozygous knock-out pups. While Oct-6 knock-out mice are characterized by a smaller size and exhibit occasional tremors, surviving Krox-20 null mice tremble. The appearance of Oct-6 protein in Schwann cells precedes Krox-20 by 24-48 hours and, as discussed above, probably directly regulates Krox-20 gene expression through interaction with the myelinating Schwann cell element (MSE). In the peripheral nervous system of both Oct-6 and Krox-20 null mice, a one to one ratio of axon and Schwann cell is established and a normal basal lamina is formed. Whereas promyelin figures in Oct-6 null mice diminish in time (arrest is transient), in Krox-20 null mice promyelin figures are maintained. Thus while myelination is transiently delayed in the Oct-6 null mouse, virtually all axons in Krox-20 null mice remain devoid of myelin [78, 79, 84]. See table I. However, it should be kept in mind that Krox20 mice do not survive beyond the third week of postnatal life.

The claw paw mouse

Claw paw phenotype

28 years ago Nelda Blaisdell observed abnormal front limbs within an inbred strain of C57Bl/6J-*obese(ob)* mice [1]. The limbs, predominantly forelimbs, were held flexed at one or more joints, but extended at the elbow [1]. This phenotype was therefore called claw paw *(clp/clp)* [1]. In addition to the limb abnormalities, peripheral nerve abnormalities were detected. These were the only abnormalities found. Other tissues such as skeletal muscle, bone and joints were normal. However, a slight difference was observed in the radial and ulnar bones, which were thinner and straighter in claw paw mice.

Mutant mice could be recognized shortly after birth. The trait was crossed out of the obese background onto a C57Bl/6J and it was established that it was inherited in an autosomal recessive pattern. The severity of the posture abnormality correlated with the course of specific behavioral deficits such as impaired movement (mice rather slither) around the cage and an unfavorable position with respect to the littermates when competing for milk or food [1].

During the early life of *clp/clp* mice no fixed deformities are found, while in time contractures develop [1] (Figure 10).

Figure 10. A photo of a wildtype and *clp/clp* mice at postnatal day 6. Note the limbs, which are held along the thorax.

If *clp/clp* mice survive the first several weeks of age, their condition variably improves and they have a normal lifespan. Contrary to males, who breed poorly, females breed successfully [1]. Based on linkage analysis, the *clp* gene [1] was mapped to chromosome 7, in the vicinity of Gpi-1 locus.

Analysis of *clp/clp* nerves showed a delay in myelination, resulting in hypomyelination and promyelin figures throughout the peripheral nervous system that persist into adulthood [1]. Nerves at brachial and lumbosacral levels were similarly affected and no obvious correlation between limb deformity and nerve pathology was found. However, promyelin fibers were more prominent in those nerves which normally contain many small caliber axons, such as spinal dorsal roots and cranial nerve V [1], than in nerves that normally contain mainly large fibers, such as spinal ventral roots and cranial nerve VII. Additionally, both endoneurial connective tissue and fascicular subdivision of nerves (small fascicles) are increased (Figure 11).

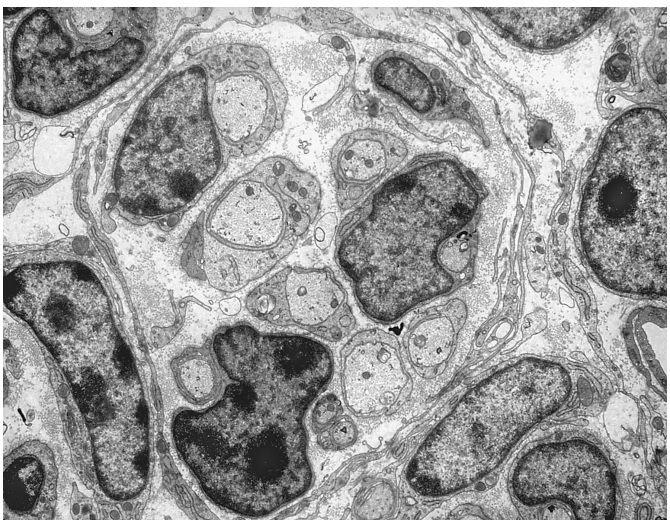


Figure 11. Electronmicroscopic picture of a *clp/clp* peripheral nerve at postnatal day 8. Note the hyperplasia of the endoneurium, minifascicles and promyeline figures.

The absence of onion bulb structures, and other pathological characteristics of demyelinating disease, in *clp/clp* nerves suggest that the presence of promyelin figures results from a developmental defect and not from cycles of de-myelination and re-myelination [1]. In summary, these data indicate that *clp* is a spontaneous mutation on chromosome 7 near the Gpi-1 locus, which causes an autosomal recessively inherited disorder, resulting in limb posture abnormality and delay in myelination within the peripheral nervous system.

Seven years after description of the claw paw phenotype by Henry, this mouse model was selected to study the complex signaling mechanism between axon and Schwann cell during nodal development. Earlier studies on development of the node suggested that formation and localization of the node along the length of the nerve, results from a complex interplay between the axon/neuron and the Schwann

cell (see previous section). The delayed myelination in *clp/clp* mice provided an opportunity to study node formation and sodium channel clustering in the absence of myelin during early postnatal development [88]. Since *Mag* expression precedes sodium channel clustering at the node, nodal development was visualized via whole mount immunohistochemistry of nerve fibers, with antibodies against *Mag* and sodium channel [88]. While in nerve fibers from P14 heterozygous mice, sodium channels were concentrated at the nodes diffuse staining along the *clp/clp* nerve fibers was observed suggestive of delayed myelination and node formation [88]. Sodium channel clustering was restricted to the end of myelinating *clp/clp* Schwann cells. Thus, nodal development is delayed in *clp/clp* nerves. In addition the internodes were short in *clp/clp* nerves [88].

The defect in *clp/clp* appears to involve early Schwann cell axon interactions. Since the gene for *Mag* is located on mouse chromosome 7 in the vicinity of Gpi-1 (*clp*) and *Mag* is assumed to be involved in early axo-glial interactions, it represented a likely candidate gene. However, *Mag* was normally expressed at the mRNA level in *clp/clp* nerves and no altered form of *Mag* was found [89]. Furthermore, the *Mag* knock-out phenotype did not resemble the claw paw phenotype [90, 91] as it displayed only subtle ultrastructural abnormalities of myelin without affecting peripheral nerve development. It is therefore very unlikely that *clp* is an allele of the *Mag* gene.

In a more recent study *clp/clp* nerves were used as a dysmyelinating mouse model and compared with Oct-6 null mutants in the search for genes regulated by Oct-6. In situ hybridization of *clp/clp* nerves revealed reduced mRNA expression of myelin associated genes, including dendrin (a protein of unknown function found in dendrites of the neuron), CRP2 (LIM-domain protein, binds to components of actin cytoskeleton) and P2 (fatty acid transport protein). However, mRNA expression of P0 seems to be unaffected, although the nerves are thinner [92].

Table 1 Mouse models

Gene	Axon Schwann cell	Mutation	Mouse #	Limb abnor-malities	effect on myelination	Other experimental findings	fenotype
claw paw [1, 88] Lgi4	Schwann cell and axon	autosomal recessive	7	yes	hypomyelination, promyelin figures, minifascicles, delayed nodal forming, shorter internodes	reduced expression of P2, dendrin, CRP2, MAG, Scn1b, Fxyd3	Limb abnormalities clearly detectable at postnatal day 2, already present in utero No tremors, convulsions
Oct-6 [77]	Schwann cell	transgene		No	hypermyelination and aberrant figures		
Oct-6 [79]	Schwann cell	knock-out	4	No	transient delay in myelination and promyelin figures p16 A defect in the nucleus of the lateral olfactory	IH:reduced levels of P0 and MBP; normal laminin, MAG and periaxin	null mice do not survive due to breathing problems
Oct-6 [78]	Schwann cell	knock-out	4	No	EM p1: actively ensheathing, 1:1 ratio of axons and Schwann cells normal basal laminae	Reduced mRNA levels of P0, MBP, PMP-22 and MAG	smaller size, occasional tremors in the second week
Krox-20 [84]	Schwann cell	knock-out	10	no	Virtually all axons were devoid of myelin 1:1 relation with axons ensheathing Schwann cell No demyelination normal basal laminae	10,5 lacZ expression in boundary cap cells 14,5 confined to motor and sensory neurons 15,5 lacZ expression along the nerves reduction P0, P2 and MBP;S-100 and MAG not affected mRNA: slight reduction in MAG and P0 differences in morphology of the nuclei and increase in density.	Surviving mice tremble
Periaxin [93]	Schwann cell	knock-out	7	no	extensive hypermyelination	light microscopy 6 weeks: focal thickenings	Normal till 6 weeks, when lifted by tail (4-6 weeks) clasping of hindlimbs
					By 8 months, naked or thinly myelinated axons	(tomacula) and infoldings of internodal myelin	slight tremor
					supernumerary Schwann cells;Onion bulbs	Western: MAG, P0 and MBP normal	6-9 months of age unsteadiness in mutants' gait,
					surrounded by redundant basal laminae		great difficulty to supporting themselves on their hindlimbs > 6-9 months lost weight rapidly, inability to feed, labored breathing

Table 1 Mouse models

Gene	Axon Schwann cell	Mutation	Mouse #	Limb abnor-malities	effect on myelination	Other experimental findings	fenotype
KIF1B beta [94]	axonal	knock-out	4	yes	Progressive axonal neuropathy		posture abnormality similar to *claw paw*
p0 [95]	Schwann cell	knock-out	1	No	Severe hypomyelination and axonal degeneration	Upregulation of N-CAM, NGF receptor, MAG, PLP, tenascin, these are normally downregulated	characteristic behavioral phenotype at postnatal week 3, abnormalities in motor coordination, tremors and occasional convulsions
p0 [96]	Schwann cell	transgene		No	At p28:hypomyelination, pro-myelin figures and sorting problems 1:1 ratio is established; some cells are arrested while aberrant myelin is formed		Increasing severity of behavioural, electrophysiological and morphological phenotype parallels increasing copy number
Dhh [97]	Schwann cell	Knock-out [98]	15	No	hypomyelination epi and perineurium less compacted, less collagen in epineurium, in some places almost absent		
PMP-22 Trembler	Schwann cell	duplication	11	no	hypomyelination, poorly compaction, demyelination and onion bulb formation		affected animals develop tremor, quadriparesis, and seizures
ß1-integrin [99]	Schwann cell	Conditional knock-out	8	No	Few myelinated axons, Promyelin figures and impaired sorting of axons, the latter are surrounded by a ring of perineurial cells.		In the first 2 weeks progressive muscular weakness, evolving to hindlimb paralysis by 3-5 months. Tremor, wide based gait, muscular atrophy after 1 month
γ-1 laminin [100]	Schwann cell	Conditional knock-out	1	No	Very few axons were myelinated and sorting failed.		Hind leg weakness is observed by 4 weeks, manifesting as difficulty in walking, unsteadiness in gait and inability in using the hind leg to grasp. By 3 months most of the affected mice had almost completely paralysed hind legs and muscular atrophy. Severe tremor and motor defects were in some mutants also observed in the front limbs

Table 2 Human diseases

Gene	Axon Schwann cell	Mutation	Human #	inheritance	Disease	effect on myelination	fenotype
Lgi4 [101]	Schwann cell and axon	Polymorphisms	19	Autosomal recessive	Childhood absence epilepsy		
Egr-2 (homologue of Krox-20) [102]	Schwann cell	Missense mutation	10	Autosomal recessive	Congenital hypomyelination	Hypomyelination and severe neuropathy	Congenital onset, delayed motor milestones, decreased nerve conduction velocities
Periaxin [103]	Schwann cell	Loss of function	19	Autosomal recessive	Dejerine Sottas syndrome	Demyelination, increased connective tissue, onion bulb, occasional tomacula formation with focal myelin thickness; Abnormal paranodal myelin loops, focal absence of septate junctions.	Delayed motor milestones during the first year of life, scoliosis in puberty, slowed nerve conduction velocity and reduced compound muscle action potential and impaired sensory nerve action potential
KIF1B beta [94]	axonal	Loss of function mutation	1	Autosomal dominant	CMT 2A	Axonopathy rather than demyelinating	
p0 [104]	Schwann cell	Frameshift mutation	1	De novo mutation	Congenital hypomyelination	Large axons with little to no compact myelin and few basal lamina onion bulbs	At birth hypotonia, arthrogryposis; lack of sensory and motor responses via neurophysiological tests Cranial nerve dysfunction
p0 [105]	Schwann cell	Missense	1		Dejerine –Sottas syndrome	Severe hypomyelination, Schwann cell hyperplasia, onion bulbs consisting of multilayered processes of Schwann cell cytoplasm	Delayed motor milestones, severe kyphoscoliosis and the onset of peripheral sensory impairment. Cranial nerve involvement.
Dhh [106]	Schwann cell	Homozygous missense mutation in exon 1	12	Autosomal recessive	HMSN with gonadal dysgenesis	Marked decrease in myelinated fibres, minifascicles, perineurial cells	
PMP-22 [109]	Schwann cell	duplication	17	Autosomal dominant	CMT-1A	Lack of small- and large diameter axons at an early stage, demyelination followed by axon loss.	Motor milestones normal, onset of disease in the first decade, lower limb areflexia, nerve enlargement, slowed nerve conduction velocities in both motor and sensory axons.

Comparison with other mouse mutants and human diseases

A better understanding of myelination in health and disease can be achieved by comparison of different mouse models for myelination disorders. In table 1 we have compared claw paw with other mouse mutants. Similar to Oct-6 and Krox-20 mutant mice, *clp/clp* mice are characterised by congenital hypomyelination and delayed onset of myelination in the PNS indicating that the *clp* gene is part of the genetic hierarchy that controls myelination and includes the Krox20 and Oct-6 genes.

Although promyelin figures are observed in Oct-6 null, Krox-20 null and P-zero null mice, limb abnormalities characteristic for *clp/clp* are not observed in these mutants. Instead posture abnormality is observed postnatally in mice lacking the kinesin protein KIF1B. However, the observed progressive neurodegeneration and impaired central nervous system myelination characteristic for KIF1B mutant, is absent in *clp/clp* mice.

The genes involved in dysmyelination in mouse have human homologues involved in different sorts or neuropathies in humans (table 2).

In humans any disruption resulting in decreased fetal mobility, either intrinsic or extrinsic to the developing fetus, could give rise to arthrogryposis multiplex congenita [2, 3, 7]. Only a few within this heterogeneous group are neurogenic in origin and some are associated with Schwann cell defects [4, 5].

The heterogeneous group of human diseases referred to as Charcot Marie Tooth (CMT) or hereditary motor and sensory peripheral neuropathy (HMSN) (table 2) is a general name for both sensory and motor neuropathies. Its inheritance is diverse but mainly autosomal dominant. The peripheral neuropathy can be due to neuronal (axonal) damage, Schwann cell defect (demyelinating) or both. Since disruption of the myelin layer affects saltatory propagation and other axonal characteristics, a Schwann cell defect is generally associated with reduced nerve conduction velocities, whereas a neuronal defect does not initially affect the conduction velocity. Thus, based on electrophysiologic parameters, CMT is classified as either an axonal or demyelinating neuropathy. Although this classification is clinically very useful, it does not contribute much to the understanding of the cellular and genetic disease mechanisms that underlie the neuropathy. For example, based on clinical, genetic and electrophysiological characteristics it is not possible to distinguish between two types of severe neuropathies in children: Dejerine-Sottas (DSS) and congenital hypomyelination (CH).

Congenital hypomyelination is a very rare form of neuropathy, which presents at birth with profound hypotonia, areflexia, contractures [107] and little or no development of peripheral myelin [104, 108, 109]. Usually these patients die because of feeding difficulties or respiratory distress. However, some do survive and spontaneous improvement of the motor function with increasing age is reported

by several authors [44, 110-113]. In humans, CH is associated with mutations in P0 [104, 114], EGR-2 [102], PMP22 [115] and MTMR2. Dysmyelination of the central and peripheral nervous system is observed in most cases. Mutations in Egr-2 or P0 are also found in children diagnosed with Dejerine-Sottas syndrome. Neuropathology is the only tool for clinicians to distinguish CH from DSS syndrome. Reduced myelination of peripheral nerves and absence of onion bulbs (found in DSS) characterize CH syndromes [104, 107]. Taken together, the absence of onion bulbs, reduced myelination and progressive clinical improvement in these patients is strikingly similar to the neuropathology and disease course observed in *clp/clp* mice. Similarly, *clp/clp* mice exhibit hypomyelination throughout the peripheral nervous system, absence of onion bulbs and improvement of the disorder when these mice survive the first several weeks. Therefore claw paw can be considered a mouse model for hereditary peripheral neuropathy in particular CH, which is associated with arthrogryposis.

These findings emphasize once more the dependence and intimate cellular interactions of Schwann cells and neurons, predominantly during development, but also in adult life. In the previous pages, I have tried to illustrate the interdependence of axons and Schwann cells and the influence of one cell type on the other. Understanding the claw paw pathogenesis could shed light on the processes involved in congenital hypomyelinating syndrome and arthrogryposis multiplex congenita. To elucidate the claw paw phenotype we asked ourselves two main questions:

1) Which cell type underlies the claw paw phenotype (Schwann cell, neuron or both), and is Oct-6 expression during Schwann cell differentiation impaired in *clp/clp* nerves
2) Which gene is affected in this mouse model.

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2

Wie wil vormen en gevormd wil worden,
ontloopt de klappen niet.
Roemi (Juwelen)

Chapter 2

Introduction to grafting experiments

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Introduction to grafting experiments

Nerve grafting is a surgical procedure in which a nerve segment is transplanted from one animal to the other. Nerve grafting experiments can create a situation in which the regenerating axons of the host contact genetically distinct Schwann cells and thus could provide insight in the cell autonomy of genes that affect neuron-Schwann cell interactions.

Axonal damage via crush, transection, ischemia or inflammation results in a process referred to as Wallerian degeneration (WD). WD is a process in which the axon and its associated myelin disintegrate distal to the site of the insult. As a consequence of WD axonal changes, Schwann cell and other neural cell responses are observed.

Axonal changes

Although the signal that initiates WD is unknown, there is evidence that a neuronal factor is essential for the maintenance of the myelinating phenotype of the Schwann cell [1-3]. Due to this axo-glial disruption, distal and proximal nerve stumps retract. Axoplasm leaks out, axons fragment and disappear. This process occurs in an interval of many hours to a few days. The structural breakdown of the axon directly (within 24 hours) impairs the generation and conduction of nerve action potentials. It is believed that intra-axonal calcium concentrations rise after nerve transection throughout the distal stump [4, 5]. Furthermore, nerve transection results in accumulation of transported molecules on both sides of the lesion, involving both anterograde and retrograde transport. Because of disruption of the established neuron-Schwann cell relationship, myelin sheaths break up, starting at the nodes, into so called ovoids. Macrophages, recruited to the site of the lesion, remove myelin ovoids (myelin is a potent inhibitor for neural outgrowth) and axonal debris by phagocytosis and hence create a new environment for regeneration [2, 3, 6, 7].

Subsequently, within a few hours after injury, axons give rise to sprouts, each tipped with a growth cone. The growth cone actively searches for a suitable matrix and environment, the so-called Schwann tubes (Schwann cells and their basal laminae=endotubes). One of these sprouts will grow towards the target via these Schwann tubes. Hence, successful axonal regeneration depends on the interaction between axons and glial cells [8] within Schwann tubes. These observations raised a number of clinically important questions such as; are these endotubes required during regeneration and if so which components are active in promoting axon elongation. Several studies have revealed that axons prefer endotubes with Schwann cells in situ and that in the absence of Schwann cells, extracellular matrix molecules promote regeneration only modestly.

Schwann cell changes

After axonal damage, expression of late myelin genes is downregulated within 12 hours in Schwann cells and immature, non-myelinating markers are upregulated. Within the first 2 weeks following nerve transection, Schwann cells divide. Both Schwann cells and macrophages have cleared most of the myelin debris after 3 weeks. The basal lamina remains intact while the Schwann cell dedifferentiates and proliferates.

Although extracellular matrix molecules such as laminin promote neurite outgrowth *in vitro*, regeneration is much better in the presence of Schwann cells. Schwann cells express in addition to other immature markers, cell adhesion molecules such as NCAM, L1, N-cadherin. Several *in vitro* experiments have shown that growth cones use L1 and N-cadherin to extend on Schwann cells and β integrins to grow on Schwann cell extracellular matrix (laminin) [1, 6]. In fact, myelin regeneration observed after WD, partly reproduces the steps of normal myelin development. Regeneration studies are useful to study both Schwann cell and neuronal contribution to myelination.

Changes in other endoneurial cells

The endoneurial fibroblasts respond dramatically and proliferate similarly to Schwann cells within the first week. Each fibroblast surrounds partially a degenerating nerve fiber and weeks after axotomy fibroblasts may form a partial basal lamina, adopting a perineurial cell phenotype. In extreme cases, fibroblasts divide the nerve into mini-fascicles. Furthermore, they appear to produce nerve growth factor, which provides an attractive environment for regeneration. Thus after transection, the process of Wallerian degeneration results in a favorable environment for invasion by regenerating sprouts. Additionally, the denervated Schwann cell, within the original Schwann cell basal lamina, provides a favorable pathway for regenerating axons.

Through nerve grafting, a situation can be created, where a genetically distinct Schwann cell is introduced at the site of nerve transection. Since Schwann cells dedifferentiate and remain in their basal lamina, the grafted segment contains Schwann cells distinct from the host. Aguayo and colleagues have used grafting experiments to investigate the pathogenesis of *trembler, quaking* and *dystrophic* mice. For both *trembler* and *quaking* they identified a Schwann cell autonomous defect, while for *dystrophic* mice they found normal myelination in each experimental setting, implicating that the underlying defect in the *dystrophic* mouse was more complex [9].

Four different experimental settings are required to interpret results of these grafting experiments.

1. A baseline of regeneration in wildtype: engraft a wildtype nerve segment into a wildtype host
2. To have a baseline of regeneration in mutant mice: engraft a mutant nerve segment into a mutant host.
3. To study mutant Schwann cells: engraft a mutant nerve segment into a wildtype host
4. To analyze mutant axons: engraft a wildtype nerve segment into a mutant host

Introduction to cloning experiments

How to find a gene

To find something you have to know what it is. Already in 1933 Demerec asked himself what is a gene? [10]. In his paper, published in Journal of Heredity, he writes that the obtained information about genes is indirect. Via analysis of inheritance, something could be said about obvious characters, their relative position to each other on a chromosome. He concludes that genes are organic molecules, either individually or in a complex they govern life processes of cells and thus of the organism [10]. Nowadays we can read in Molecular Biology of the Cell [11] that genes are regions of DNA that control a discrete hereditary characteristic, usually corresponding to a single protein or RNA. This definition includes coding and non-coding DNA sequences, encompassing the entire functional unit. Although much is changed, the principle idea remains the same.

How to search for a gene?

Several strategies to link genotype and phenotype have been developed over the years. These strategies are generally referred to as forward genetics and reverse genetics.

1. Forward genetics; from phenotype to genotype. Genes causing a particular phenotype are identified using a positional cloning approach [12].
2. Reverse genetics; from genotype to phenotype. These strategies aim to assign function to a particular gene sequence through study of its primary sequence relationship with other genes, expression pattern and, most importantly, mutagenesis experiments [12].

Positional cloning approach

Since ages humans create maps to define their position either on land, sea, or in the air. These efforts have resulted in the creation of topographical maps, sea guides, highway maps, etc. What about our genome? Are there landmarks in the genomic landscape to define our orientation throughout the desert of A, G, C, T's?? [13] Our genome is mapped with different kinds of maps, including genetic, physical and transcript maps [14]. These maps could be generated thanks to either inheritance pattern, markers dispersed throughout nucleotides, restriction sites or expression profile. Each of them further defines the region of interest [13].

To summarize the strategy of positional cloning: genetic mapping (region on a chromosome where the disease gene is localised is defined by linkage and recombinant analysis), physical mapping (identification and isolation of genomic clones (e.g. YAC, P1, BAC or cosmid) that are located in the disease region, construction of contigs and assembly of a restriction map and transcript mapping (isolation of transcripts for candidate genes) [15].

A positional cloning strategy first defines a certain DNA segment in the genome, small enough to be directly sequenced completely to identify relevant mutations or to be used in complementation experiments to ultimately identify the gene or gene mutation underlying the phenotype of interest.

Release of human genome databases

There are public and private databases. The private database from Craig Venter, named Celera genomics, used a shotgun sequencing approach together with the sequence data coming out of the public sequencing consortium (international human genome sequencing consortium or IHGS) to build a draft of the human genome sequence [16, 17]. Data from the worldwide public sequencing effort are accessible through the websites of NCBI, UCSC and Ensembl [18]. All these databases have their shortcomings and have to be treated with suspicion. The NCBI and UCSC database used the already available sequence data set as a starting point for a complete assembly using sequences from overlapping BAC clones.

A large and growing number of genomes have been sequenced to date and made publicly available through the Ensembl, NCBI, UCSC and Celera databases. Most importantly these databases provide detailed annotations of the genome sequence with links to other databases containing information on gene structure, function, phenotypes and human diseases. Although the data of Celera and the others largely overlap some discrepancies between the datasets remain. Databases are far from perfect, but they are enormously helpful to select candidate genes between two non-recombinant markers. In addition, they enable comparison between species, giving an idea about the number of genes, their orientation, their intron-

exon boundaries, protein information and which markers and clones cover the critical region of the genome. STS (anonymous markers), EST's (expressed sequence tags) and Riken cDNA sequences, known genes, known proteins and predicted genes and proteins are shown [18]. The amount of information you get is overwhelming, making it difficult to select the relevant information.

Candidate genes

What are the characteristics of a candidate gene? In principle, any gene in a region defined by markers that segregate fully with the phenotype and are flanked by markers showing minimal recombination in a large set of meiotic chromosomes, should be considered a candidate gene. To select candidates for further study one needs to define a number of criteria. These criteria will of course depend to a large extent on the details of the phenotype.

A first selection could be based on expression of the candidate genes. It is reasonable to discard candidate genes that are not expressed in the tissue of interest. However, this criterion should be approached with great care as cell non-autonomous effects could play an important role in the phenotype. It is therefore important to establish the cell autonomy of the mutation, if possible (see chapter 3). Clearly a literature search is required to collect all the available data on the potential candidate genes. This data might give you an idea about the expression of the gene, about tissue specificity, about expression pattern in a certain differentiation stage in a particular celltype etc. Furthermore data of human patients or known phenotype in other species where a mutation is identified in this particular gene can be used to compare. The ultimate identification of the mutation underlying the phenotype will require genetic complementation (in case of recessive traits), creation of transgenic or cell culture models (dominant traits) or gene knock out models.

If the mutation is in a suspected gene regulatory element other approaches such as bandshift and reporter gene expression studies might be performed. For example preaxial polydactyl is a disorder that is associated with a point mutation in a regulatory element 1 Mb away, resulting in ectopic Sonic hedgehog expression and as a consequence supernumerary preaxial digits [19].

Furthermore different mutations in the same gene can result in distinct phenotypes [20]. Recently this issue was studied in families with patients having either complex neurocristopathy or a more restricted phenotype known as Waardenburg-Shah syndrome. Mutations in the Sox-10 gene (a transcription factor) have been found within those families. The severity of disease is associated with stability of mRNA. Unstable mRNAs are degraded by the non-sense mediated mRNA decay pathway, resulting in less severe phenotype. In contrast stable mRNA is translated into a mutant protein with a more severe phenotype [21].

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3

Gaye
Perdenin ardı perde, perdenin ardı perde,
Her siper aşıldıkça gaye obür siperde….
N. F. Kısakürek (Çile)

Het doel
In iedere onthulling is een volgende vraag verhuld,
na het bereiken van een beoogde doel, is slechts een deel overbrugd

Vrije vertaling

Chapter 3

Cell autonomy of the mouse *claw paw* mutation

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Cell autonomy of the mouse *claw paw* mutation

Aysel Darbas,[a,1] Martine Jaegle,[a,1] Erik Walbeehm,[b] Hans van den Burg,[c] Siska Driegen,[a,c] Ludo Broos,[a] Matthijs Uyl,[a] Pim Visser,[a] Frank Grosveld,[a] and Dies Meijer[a,*]

[a] *Department of Cell Biology and Genetics, ErasmusMC, Erasmus University Medical Center, 3000DR Rotterdam, Netherlands*
[b] *Department of Reconstructive Surgery, ErasmusMC, Erasmus University Medical Center, 3000DR Rotterdam, Netherlands*
[c] *Department of Neurosciences, ErasmusMC, Erasmus University Medical Center, 3000DR Rotterdam, Netherlands*

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Abstract

Mice homozygous for the autosomal recessive mutation *claw paw* (*clp*) are characterized by limb posture abnormalities and congenital hypomyelination, with delayed onset of myelination of the peripheral nervous system but not the central nervous system. Although this combination of limb and peripheral nerve abnormalities in *clp/clp* mice might suggest a common neurogenic origin of the syndrome, it is not clear whether the *clp* gene acts primarily in the neurone, the Schwann cell or both. In the work described here, we address this question of cell autonomy of the *clp* mutation through reciprocal nerve grafting experiments between wild-type and *clp/clp* animals. Our results demonstrate that the *clp* mutation affects the Schwann cell compartment and possibly also the neuronal compartment. These data suggest that the *clp* gene product is expressed in Schwann cells as well as neurones and is likely to be involved in direct axon–Schwann cell interactions. Within the Schwann cell, *clp* affects a myelin-related signaling pathway that regulates periaxin and Krox-20 expression, but not Oct-6.

Keywords: Myelination; Schwann cell; Arthrogryposis; POU factors; Periaxin

Introduction

Congenital limb posture abnormalities in humans and other vertebrates occur under a variety of circumstances and clinically manifest as a singular abnormality or as part of a syndrome. In clinical practice, such abnormalities are described as arthrogryposis multiplex congenita (AMC; arthron = "joint" and gryposis = "bent"). It is generally accepted that any condition resulting in reduced fetal movement will lead to joint contractures in newborns (Drachman, 1971; Hageman and Willemse, 1983; Hall, 1997). In principle, such conditions could be extrinsic or intrinsic to the developing fetus. An example of the former is provided by experiments in which chick or rat embryos were paralyzed by injection with curare, causing limb posture abnormalities at birth (Del Torto et al., 1983; Drachman and Coulombre, 1962; Moessinger, 1983). Additionally, maternal antibodies that functionally inhibit the fetal acetylcholine receptor cause arthrogryposis in human fetuses (Jacobson et al., 1999; Matthews et al., 2002; Polizzi et al., 2000).

Intrinsic causes of reduced fetal movement can be classified as either myogenic and/or neurogenic in origin. In several clinical cases, AMC was found associated with congenital hypomyelination of the peripheral nerves suggesting a common aetiology (Boylan et al., 1992; Charnas et al., 1988; Seitz et al., 1986). In the peripheral nervous system, myelin formation and maintenance of the peripheral nervous system by Schwann cells is dependent on continuous reciprocal interactions between the axon and Schwann cell (Fields and Stevens-Graham, 2002). Mutations that affect this dialogue by disabling function in the Schwann cell, the axon or in both result in dys- or demyelinating neuropathies as in the human hereditary motor and sensory neuropathies (Suter and Scherer, 2003). Limb posture abnormalities, mostly pes cavus, and distal muscle wasting frequently develop in these patients as consequence of axonal loss.

Congenital limb posture abnormalities are observed in mice homozygous for the murine autosomal recessive mutation *claw paw* (*clp*) (Henry et al., 1991). In affected

* Corresponding author. Department of Cell Biology and Genetics, ErasmusMC, Erasmus University Medical Center, Dr Molewaterplein 50, PO Box 1738, 3000DR Rotterdam, Netherlands. Fax: +31-10-408-9468.
E-mail address: d.meijer@erasmusmc.nl (D. Meijer).
[1] These authors contributed equally to this study.

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animals, the forelimbs are flexed at one or more of the joints (shoulder, wrist or digital joints) but extended at the elbow, resulting in the forelimbs pointing in the direction of the hindlimbs. These abnormalities are visible within 2 days of birth. More severely affected animals also show involvement of one or both hindlimbs. Pathological examination of affected animals demonstrates severe congenital hypomyelination with delayed onset of myelination of the peripheral nerves with central nerves unaffected. Persistently blocked myelination is observed in a fraction of the smaller axons (Henry et al., 1991). Additionally, it was found that the mean internodal length is greatly reduced for all axonal size classes in *clp/clp* nerves when compared to heterozygous or wild-type animals (Koszowski et al., 1998). The *clp* gene has not been identified yet, although mapping studies have placed the gene close to the *Gpi* locus on mouse chromosome 7 (Henry et al., 1991). No mutations were found in the *myelin-associated glycoprotein* (*MAG*) gene, close to the *Gpi* locus, excluding this gene as a *clp* candidate (Niemann et al., 1998). The pathology of the *clp/clp* mouse is strikingly similar to the clinically described cases of AMC mentioned above. Hence, *clp/clp* mice provide an opportunity to study the basis of the relationship between arthrogryposis and hypomyelination.

It has been hypothesized that the *clp* gene is part of the neurone–glia signaling system that governs myelination initiation during development. However, it is not known what cellular and molecular components of this signaling system are affected by the *clp* mutation. To further characterize this mutant mouse, we set out to determine whether the gene acts in the neurone, the Schwann cell or both, through reciprocal nerve graft experiments. Additionally, we studied the interaction of the *clp* gene with many known Schwann cell autonomous regulatory genes involved in myelination including the transcription factors *Oct-6* and *Krox-20* (Topilko and Meijer, 2001). Our results indicate that *clp* function is required within the Schwann cell as well as in the neurone. Upregulation of the promyelin transcription factor Oct-6 is not affected by *clp*, while the subsequent Krox-20 activation is delayed. Additionally, embryonic periaxin expression is absent or reduced in *clp/clp* Schwann cells and postnatal expression is delayed. Taken together, these results suggest that *clp* is involved in neurone–glia interactions and furthermore that, within the Schwann cell, *clp* is involved in a pathway that regulates periaxin and Krox-20 expression. This *clp*-dependent pathway is parallel and non-redundant to the myelin-related Oct-6-dependent pathway.

Materials and methods

Animal surgery

Nerve crush experiments were performed on young adult F2 animals from a C57BL6J/Balbc-*clp* heterozygous intercross. Affected and non-affected mice were anesthetized by inhalation of halothane and placed on a heating pad. The left sciatic nerve was exposed and crushed for two times 15 s at mid-femoral level using No.5 Biology forceps. Mice were sacrificed 4 weeks after the operation through trans-cardiac perfusion with a solution of 1% gluteraldehyde/3% paraformaldehyde in 100 mM cacodylate (pH 7.4). The operated and contralateral nerve was isolated for light and electron microscopic analysis.

Transplantation experiment

Reciprocal nerve grafting experiments were performed between wild type and wild type (wt:wt), wild type and *clp/clp* (wt:clp), *clp/clp* and wild type (clp:wt) and between *clp/clp* and *clp/clp* (clp:clp). All animals were of C57BL6J background, obviating the use of immune-suppressants to prevent graft rejection. Operations were performed on two animals at a time, both animals serving as nerve donor and recipient. Two mice of appropriate genotype were anesthetized through inhalation of halothane and placed on a heating pad. The left sciatic nerve was exposed in both animals. Approximately 7.5 –10 mm of nerve was excised and placed in the same orientation in the host animal. Nerve and muscle tissue were kept wet by regular application of a drop of sterile phosphate-buffered saline (PBS). The nerves were sutured at both the proximal and distal anastomosis site with two 10-0 black filament sutures. The retracted muscles were placed back in position and the skin was sutured. All surgical manipulations were done under a microscope. Animals were transferred to a clean cage and allowed to recover under a heating lamp. Animals were carefully monitored for wound healing on a daily basis. Four weeks after transplantation, animals were sacrificed.

Perineurium permeability

A 1% Evans Blue (Sigma) solution in 5% bovine serum albumin (fraction V)/PBS (pH 7.4) was dialyzed overnight in PBS. The sciatic nerves of an adult claw paw animal and a wild-type littermate were dissected out and placed immediately in 4 ml of Evans blue albumin (EBA) solution at room temperature. After a 30- or 60-min incubation period, nerves were briefly washed in PBS and fixed in 4% paraformaldehyde for 2 h. Nerves were cryoprotected in 30% sucrose/PBS, frozen and sectioned at 24 μm. Sections were mounted in Vectashield® (Vector laboratories) and viewed immediately under a fluorescence microscope.

Quantitative RT-PCR

Total RNA was extracted from sciatic nerves using RNA-Bee (Tel Test Inc.). First-strand cDNA was generated using Superscript II (Invitrogen) reverse transcriptase and oligo-dT as primer. The following primers were used to amplify *dhh* and *cyclophilin* transcripts: dhh forward, CATGTGGC-

CCGGAGTACGCC; dhh reverse, CGCTGCATCAGCGGCCAGTA; cyclophilin forward, GGTCAACCCCACCGTGTTCTTCGACAT; cyclophilin reverse, GGACAAGATGCCAGGACCTGTATGCT. Annealing temperature for dhh primers: 60°C, annealing temperature for cyclophilin primers 68°C. Aliquots were taken from the reaction with two cycle intervals and amplification products were analyzed on a 1.8% agarose gel.

Light/electron microscopy

Anesthetized animals were sacrificed through transcardial perfusion with PBS (pH 7.4) for 3 min followed by fixative [3% PFA (Sigma); 1% glutaraldehyde (Sigma) in 100 mM cacodylate buffer, pH 7.2] for 10 min. Sciatic nerves were isolated, fixed overnight in the same fixative at 4°C. The next day, nerves were rinsed with 0.2 M cacodylate buffer, osmicated in 1% osmium tetroxide solution and embedded in Epon. Sections (1 μm) of Epon-embedded sciatic nerves were mounted on glass slides and stained with methylene blue. Ultra-thin cross sections from nerve grafts at mid-graft level were cut and uranyl acetate and lead citrate stained for electron microscopic analysis (Philips CM100).

Nerve conduction velocities

Nerve conduction velocities were measured on the tail nerve of anesthetized animals. Animals were placed on a thermo-comfort heating pad at 38°C. Recording and stimulation electrodes were made of 300-μm tungsten needles with a 10-μm tip. The stimulus frequency and stimulation period was 1 Hz and 0.1 ms, respectively. Impedance of the recording electrode was 100 and 25 kΩ/kHz for the stimulation electrode.

Immunohistochemistry

Rabbit polyclonal antibodies used in this study are directed against Oct-6 (Jaegle et al., 2003), Periaxin (Scherer et al., 1995) and Krox-20 (1:400). The Krox-20 antibody was raised against the unique amino-terminal portion of the protein (amino acids 1–180). Animals were immunized with 6xHis-tagged Krox-20^{1-180} protein expressed in *E. coli* (M15 strain) and purified on Ni^{2+}-NTA-agarose beads (QIAGEN). Mouse monoclonal antibodies used were against P-zero (1:1000) (Archelos et al., 1993) and neurofilament medium chain (1:200, hybridoma 2H3, Developmental Studies Hybridoma Bank).

Embryos and tissues were isolated, fixed in a mixture of methanol, acetone, acetic acid and water (35:35:5:25) for 1 h at 4°C, embedded in paraffin and sectioned at 7 μm. After dewaxing, tissue sections were hydrated and blocked in 1% BSA, 0.05% Tween-20 in PBS. Rabbit antibodies and mouse monoclonal antibodies were incubated simultaneously in PBS/0.05% Tween-20 overnight at room temperature. Oregon Green-conjugated goat anti-rabbit IgGs (Molecular Probes) and Texas Red-conjugated goat anti-mouse IgGs (Molecular Probes) were subsequently used as secondary antibodies. The tissue was viewed using a Leica fluorescence microscope.

Results

Morphological examination of clp nerves and comparison with Oct-6 mutant nerves

Our interest in the *clp* mouse mutant was initially raised by the similarities in peripheral nerve phenotypes between claw paw mice and *Oct-6* transcription factor mutant mice (Bermingham et al., 1996; Henry et al., 1991; Jaegle et al., 1996). Both mice are characterized by delayed initiation of myelination of their peripheral nerves. The similarities between these phenotypes suggested several possible interactions between the *Oct-6* and *clp* genes. For example, it is possible that the *clp* gene product is involved in those signaling pathways that activate *Oct-6* or, *clp* could be a major regulatory target of Oct-6. Alternatively, *clp* could act in a non-redundant pathway parallel to Oct-6. To begin to answer these questions, we first compared nerve morphology in both mutant and wild-type mice at several postnatal stages of development. Previously, nerve morphology in *clp/clp* animals has been described only from postnatal day 14 (P14) onwards (Henry et al., 1991). Therefore, we extended this analysis to include earlier stages of nerve development. Fig. 1 shows a comparison of representative transverse sections through the sciatic nerve of animals of different genetic background at different stages of postnatal development.

The sorting of prospectively myelinated axonal fibers by Schwann cells during late fetal development culminates in the promyelin stage of cell differentiation in which a Schwann cell has established a one to one relationship with its axon. These cells will exit the cell cycle, elaborate a basal lamina and initiate myelination. The majority of myelin-competent cells transit through this stage during the first week of postnatal life. This is evident in transverse section of the wild-type sciatic nerve at birth, in which many promyelin cells can be observed with some cells already actively myelinating (Fig. 1A, panel a). By P8, a majority of cells is actively myelinating and at P16 almost all myelin-competent cells are in an advanced stage of myelination (Fig. 1A, panels d and g). In contrast to wild-type, *clp/clp* Schwann cells are still at a very immature sorting stage at birth (Fig. 1, panel b). Most Schwann cells are found at the periphery of naked axon bundles, with cellular extensions pioneering into the bundles. This configuration of Schwann cells and naked axon bundles is typically observed in embryonic nerves around mouse embryonic day 17 (E17) (Feltri et al., 2002). To illustrate this developmental stage of *clp/clp* Schwann cells more graphically, we traced the

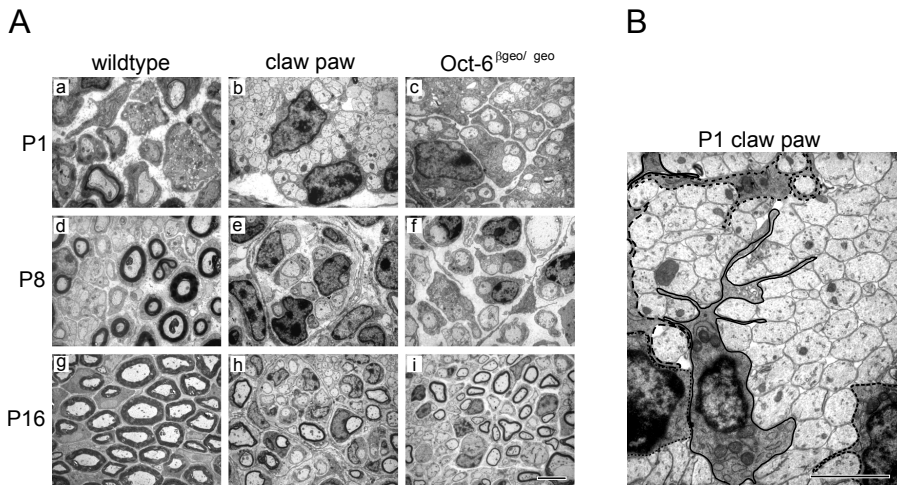

Fig. 1. Abnormal development of peripheral nerves in *clp/clp* and *Oct-6* mutant mice. (A) Shown are representative micrographs of transverse sections of sciatic nerves from wild-type, *clp/clp* (claw paw) and *Oct-6* mutant (Oct-6$^{\beta geo/\beta geo}$) animals at P1 (a, b and c), P8 (d, e and f) and P16 (g, h and i). At P1, axonal sorting is well advanced in wild-type nerves, with many axons in a 1:1 relationship with Schwann cells and few in an early myelinating stage (a). In contrast, axonal sorting in *clp/clp* nerves is in a very early stage with large groups of unsorted axons (b). Sorting in Oct-6 mutant nerves is well advanced with most cells in a late sorting and promyelin stage (c). At P8, the majority of myelin-competent Schwann cells in wild-type nerves are actively engaged in myelination (d), while in *clp/clp* nerves Schwann cells have now adopted a promyelin configuration (e). At this time, most Oct-6 mutant Schwann cells are still at the promyelin stage of differentiation (f). At P16, myelination is well advanced in wild-type nerves (g). In contrast, only a fraction of *clp/clp*, and Oct-6 mutant, Schwann cells have progressed to the myelinating stage of differentiation with many cells still at the promyelin stage (h and i). Scale bar: (a–c) 1.6 μm; (d–f) 2.8 μm; (g–i) 5 μm. (B) Axonal sorting is delayed in nerves of *clp/clp* (claw paw) mice. In this representative micrograph, *clp/clp* Schwann cells are just beginning to send out processes in the axon bundles. The outline of sorting Schwann cells is traced with a colored pencil. Each color represents an individual Schwann cell. Scale bar: 2 μm.

outline of several cells in a P1 nerve highlighting the cytoplasmic extensions of the sorting Schwann cell (Fig. 1B). One week later, these axon bundles are sorted out and a majority of Schwann cells have acquired a promyelin configuration (Fig. 1, panel e). Over the next week, many promyelin cells initiate myelination, such that by P16, a large number of myelin figures are observed. However, a substantial number of Schwann cells are still at the promyelin stage of cell differentiation, suggesting that this transition occurs at a lower rate in *clp/clp* Schwann cells than in wild-type Schwann cells (Fig. 1, panel h). By P32, the majority of *clp/clp* Schwann cells are actively meylinating with only few cells remaining in the promyelin configuration (data not shown and Henry et al., 1991). In contrast to *clp/clp* Schwann cells, *Oct-6* mutant Schwann cells transit through the sorting stage of development normally, but then stall at the promyelin stage for several days before initiating myelination (Fig. 1A, panels c, f and i). Thus, *clp/clp* Schwann cells appear strongly inhibited in the sorting stage of nerve development and to a lesser extent in the promyelinating to myelinating transition.

One other remarkable difference between *clp/clp* nerves and *Oct-6* mutant nerves (and wild-type nerves) is the extensive fasciculation of the former. Division of the endoneurium by flattened, perineurial-like cells is already apparent at P8 in *clp/clp* nerves (Fig. 1A, panel e). As illustrated in Fig. 2A, these cells contain, like perineurial sheath cells, many caveolae and secrete a basal lamina (arrows and arrowheads, respectively, in Fig. 2A, panel a). The number of cell layers that make up the perineurium and the morphology of the perineurial cells appear normal in *clp/clp* nerves at P16 and adult (Fig. 2A, panel b, and data not shown). The hyper-fasciculation phenotype observed in *clp/clp* peripheral nerves is reminiscent of that observed in *dhh* mutant animals (Parmantier et al., 1999). Therefore, it is possible that this particular aspect of the claw paw phenotype results from a failure to express *dhh* in the developing nerve. We therefore examined expression of *dhh* mRNA in *clp/clp* and wild-type nerves at P12 using quantitative PCR normalized to *cyclophilin* expression. We found that *dhh* is expressed in P12 *clp/clp* nerves, albeit at reduced levels (Fig. 2B). It is therefore possible that the extensive subdivision of *clp/clp* nerves by perineurial sheath cells is a consequence of reduced *dhh* expression. It has been hypothesized that the hyperfasciculation of *dhh* mutant nerves results from a functionally defective perineurium (Parmantier et al., 1999). A major function of the perineurium is to prevent the invasion of cells and macromolecules from the surrounding tissue into the endoneurium (Thomas et al., 1993). This nerve–tissue barrier function of the perineurium is compromised in *dhh* mutant animals as evidenced by the penetration of dye loaded serum albumin into the endoneu-

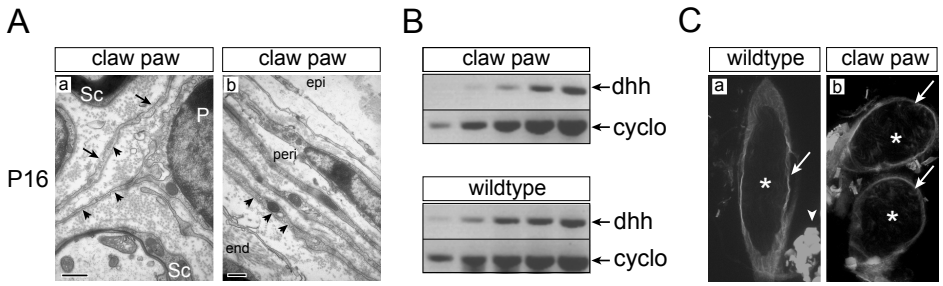

Fig. 2. Morphological and functional characterization of the perineurium of claw paw nerves. (A) Perineurial-like cells subdivide the endoneurium of claw paw nerves (panel a). This electron micrograph shows the typical flattened extensions of perineurial-like cells studded with numerous caveolae (arrows) and the basal lamina produced by these cells (arrowheads). Schwann cells (Sc) and the nucleus of a perineurial cell (P) are indicated. The number of cellular layers and the morphology of the perineurial cells appear normal in the developing perineurium of claw paw nerves (panel b). The epineurium is poorly defined at this stage and later stages. Scale bar: 500 nm. (B) *dhh* mRNA expression is reduced in claw paw nerves. Expression levels of *dhh* were estimated using quantitative RT-PCR during the exponential phase of the reaction and normalized against cyclophilin (cyclo), a housekeeping gene expressed at rather constant levels. Samples were taken with two-cycle intervals. It is estimated that *dhh* mRNA levels are 4- to 16-fold lower in claw paw nerves at P12. (C) The nerve–tissue barrier function of the perineurium is intact in claw paw nerves. Following submersion of dissected sciatic nerve of wild-type (panel a) or claw paw nerve (panel b) for 1 h in EBA solution, EBA is found associated with the epi- and perineurial sheath of the nerve (arrows). EBA did not penetrate the endoneurium of wild-type or claw paw nerves (asterisk). However, strong EBA staining is seen within muscle tissue lying next to the nerve (arrowhead).

rium (Parmantier et al., 1999). We similarly tested the integrity of the tissue–nerve barrier in claw paw nerves. After 1 h exposure to a Evans blue albumin solution ex vivo, nerves were processed for fluorescence microscopy. No dye complex had penetrated the endoneurium of wild-type or claw paw nerves (Fig. 2C, panels a and b, respectively) demonstrating that, in contrast with *dhh* mutant nerves, the nerve–tissue barrier in claw paw mice is intact. Thus, *clp/clp* nerves resemble *dhh* mutant nerves in their extensive subdivision into smaller fascicles by perineurial sheath cells but have, unlike *dhh* mutant nerves, a functionally intact perineurium.

Activation of Oct-6 expression in clp/clp nerves

As previous work has demonstrated that the transcription factors Oct-6 and Krox20 are major cell-autonomous regulators of the Schwann cell myelination program, it is possible that the delayed myelination phenotype in *clp/clp* nerves can in part be explained by a failure to activate Oct-6 and subsequently Krox-20, in *clp/clp* animals (Ghislain et al., 2002). We therefore examined expression of Oct-6, Krox-20 and the myelin proteins P-zero and periaxin in *clp/clp* animals at P8 and P16 by immunohistochemistry and compared it with the expression of these proteins in wild-type and *Oct-6* mutant Schwann cells (Fig. 3). At P8, most *clp/clp* Schwann cells are, like most *Oct-6* mutant cells, at the promyelin stage of cell differentiation (see Fig. 1) and will commence myelination in the course of the next 2 weeks. As shown in Fig. 3A, Oct-6 is expressed in Schwann cells of *clp/clp* animals at P8. This is in agreement with previous mRNA expression data (Bermingham et al., 2002). As reported earlier, Oct-6 is an upstream regulator of Krox-20 and hence, *Oct-6* mutant Schwann cells do not express Krox-20 (Ghazvini et al., 2002; Ghislain et al., 2002). Despite the expression of Oct-6 in *clp/clp* Schwann cells, Krox-20 is not expressed at P8, but eventually is activated in *clp/clp* nerves (and *Oct-6* mutant nerves) when myelination proceeds (Ghazvini et al., 2002 and Fig. 3B, panel a). As expected, P-zero expression is very low in both mutant mouse strains at P8, as the majority of Schwann cells have not yet produced compact myelin (Fig. 3A, panels h and i). However, at P16, *clp/clp* nerves show strong P-zero immunoreactivity in myelin-forming Schwann cells (Fig. 3B, panel b). Interestingly, the myelin protein periaxin appears reduced or misexpressed in *clp/clp* Schwann cells at P8. This is in contrast to promyelin-arrested *Oct-6* mutant Schwann cells in which periaxin is expressed at near normal levels (panels j, k and j). However, once *clp/clp* cells form compact myelin, high levels of periaxin are observed in these cells (Fig. 3B, panel c). Thus, in contrast to *Oct-6* mutant Schwann cells, periaxin protein does not accumulate in promyelin-arrested *clp/clp* Schwann cells suggesting that the *clp* mutation affects activation of periaxin expression or subcellular localization and/or processing.

To distinguish between these possibilities, we further examined periaxin expression at late fetal stages of development in *clp/clp* and wild-type animals. Periaxin is first expressed in the nuclei of embryonic Schwann cells at E14. By E17, periaxin expression shifts to the cell membrane and by E18, periaxin is no longer found in the nucleus (Sherman and Brophy, 2000). We examined periaxin expression in *clp/clp* Schwann cells of embryonic nerves at E17, the stage at which claw paw animals can first be recognized. No periaxin is detected in *clp/clp* nerves at this stage (Fig. 4A, panel d). In contrast, wild-type Schwann cells express periaxin in the nucleus and/or in the cytoplasm (Fig. 4A, panel c). Thus, the *clp* mutation affects onset of periaxin expression at this embryonic stage and subsequently, also the subcellular localization of the periaxin protein.

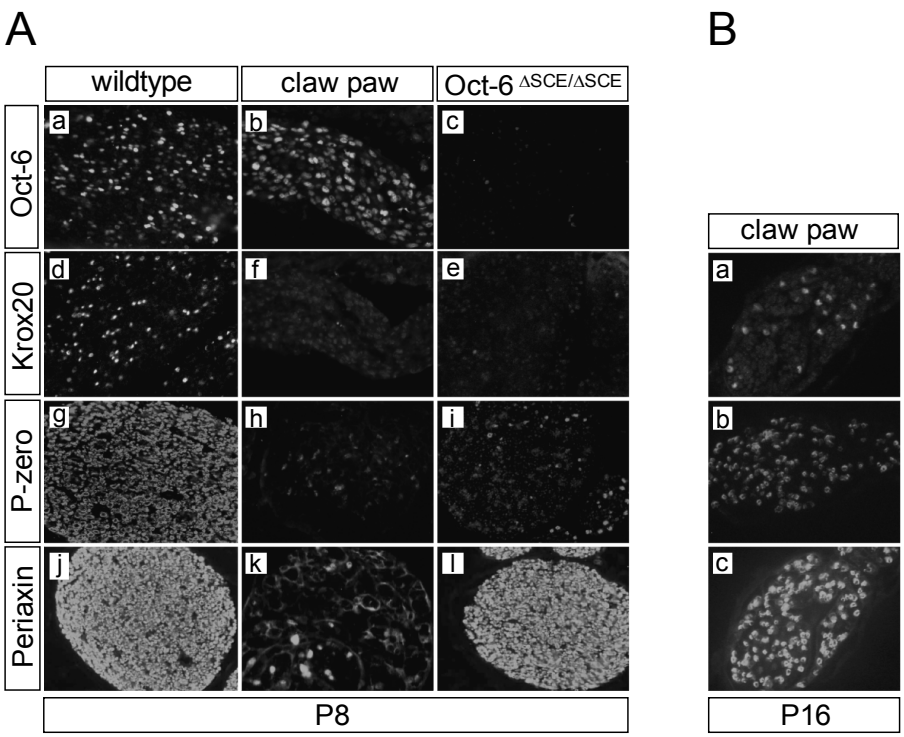

Fig. 3. Developmental expression of Oct-6, Krox-20, P-zero and periaxin in *clp/clp* and *Oct-6* mutant nerves. (A) Transverse sections of sciatic nerves of *clp/clp* (claw paw), *Oct-6* mutant (Oct-6$^{\Delta SCE/\Delta SCE}$) and control (wild-type) mice at P8 were stained for Oct-6 (a–c), Krox-20 (d–e), P-zero (g–i) and periaxin (j–l). In the control nerve, myelinating Schwann cells express Oct-6, Krox-20 and the myelin proteins P-zero and periaxin (a, d, g and j). At P8, most *clp/clp* Schwann cells have adopted a promyelin configuration and express Oct-6 (b) but not the myelination-associated transcription factor Krox-20 (f). Some cells express appreciable levels of P-zero. These cells are probably in an advanced promyelin stage as there is no evidence of compact myelin yet at this stage (h). Very low cytoplasmic periaxin expression is observed in many, but not all, cells (k). Few cells exhibit high level of periaxin expression. No nuclear periaxin staining is observed at this stage. Like *clp/clp* Schwann cells, *Oct-6* mutant Schwann cells do not express Oct-6 or Krox-20 at this stage (c and e). The few cells that have initiated myelin formation are strongly positive for P-zero (i). In strong contrast to *clp/clp* Schwann cells (k), *Oct-6* mutant cells express already high levels of periaxin at this stage (l). Magnification ×40. (B) Myelin formation is evident in *clp/clp* nerves at P16. An appreciable number of Schwann cells have initiated myelination and express Krox-20 (a). High levels of P-zero and periaxin are present in Schwann cells that have formed compact myelin (b and c). Magnification ×40.

We next examined whether Oct-6 is also delayed in its onset of expression. Oct-6 protein normally starts to accumulate in the nuclei of immature Schwann cells of the sciatic nerve at late fetal stages and reaches maximum levels in promyelin cells during the first postnatal week of development. As *clp/clp* Schwann cells at P1 morphologically resemble Schwann cells in E16–E17 wild-type embryonic nerves, we examined Oct-6 expression in P1 *clp/clp* nerves. At this stage, we found that *clp/clp* Schwann cells already express appreciable levels of Oct-6 (Fig. 4B, panel b), demonstrating that the clp gene product does not affect activation of Oct-6 expression. In contrast to wild-type Oct-6-positive Schwann cells, Oct-6-positive *clp/clp* Schwann cells do not express appreciable levels of P-zero protein (Fig. 4B, panels c and d).

Taken together, these data demonstrate that the *clp* mutation affects nerve maturation through a pathway that includes activation of periaxin, but not Oct-6, and thus strongly suggest that during Schwann cell differentiation, periaxin and Oct-6 are regulated through distinct signaling pathways.

Nerve conduction velocities of clp nerves are reduced

The delayed maturation of peripheral nerves in *clp/clp* animals results in reduced internodal lengths for all diameter classes and thus, in the absence of axonal loss, increased Schwann cell numbers. In addition, myelin thickness is reduced in some, but not all, *clp/clp* animals and no decrease in mean axon diameter was reported (Henry et al., 1991;

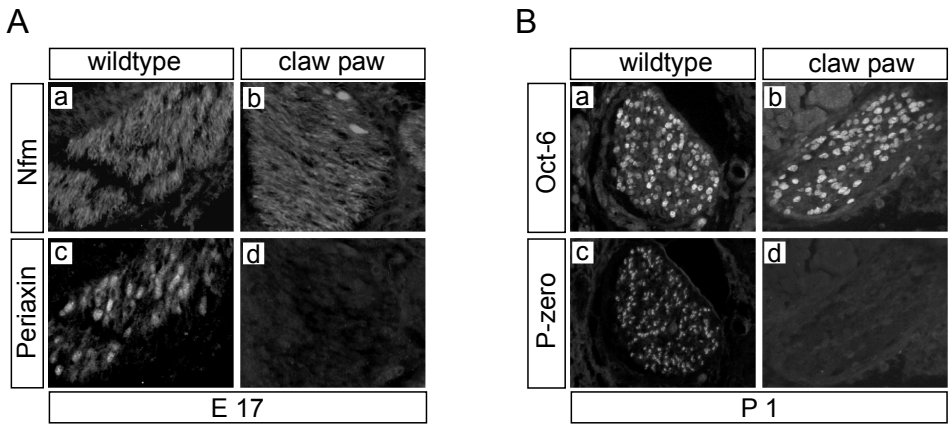

Fig. 4. Expression of periaxin and Oct-6 in *clp/clp* Schwann cells. (A) Embryonic sciatic nerve is visualized by expression of the neurofilament medium chain in the axonal fibers (Nfm; a and b). Embryonic Schwann cells within the nerve express periaxin in their cytoplasm or nucleus (c). In contrast, no periaxin expression is observed in nerves of claw paw embryos at this stage of development (d). (B) Oct-6 is expressed in all Schwann cells in newborn wild-type sciatic nerves (a). Also, Schwann cells in sciatic nerves of *clp/clp* animals express Oct-6 (b). Other than wild-type Schwann cells, *clp/clp* Schwann cells uniformly express high levels of Oct-6. In wild-type nerves, many Schwann cells are at the promyelin configuration and some have initiated myelination and thus express high levels of P-zero (c). In contrast, *clp/clp* Schwann cells appear at an early sorting stage at P1 (see Fig. 1) and do not express detectable levels of P-zero protein (d).

Koszowski et al., 1998). The mean internodal length in every axonal diameter class in *clp/clp* animals is approximately half of that of wild-type animals. Intuitively, and on theoretical considerations, it is to be expected that propagation of action potentials will be slowed when the mean internodal length drops well below its optimum (Brill et al., 1977; Goldman and Albus, 1968; Rushton, 1951). To assess nerve conduction velocity (NCV) in *clp* and wild-type animals, we measured compound action potentials (CAP) of the tail nerve (Fig. 5A). The mean NCV in wild-type and heterozygous mice was 35.3 m/s (SD 1.8). In contrast, the mean NCV in *clp/clp* mice was 23.9 m/s (SD 2.2), a reduction of 35% (Fig. 5B). A similarly reduced conduction velocity was observed for motor nerves within the sciatic nerve of *clp/clp* animals as compared to wild type (data not shown). Reduced NCV in the tail nerve of claw paw mice shows little variance, and this variance does not correlate with age or severity of the behavioral phenotype. Thus, it is possible that the reduction in NCV in *clp/clp* animals can be accounted for, at least in part, by the reduction in mean internodal length.

Response to nerve injury

Although the claw paw phenotype has been described as a developmental abnormality, it is not clear from these observations whether the phenotype results from an intrinsic defect in one or more components of the developing nerve or through defects in other organs of the developing fetus. Studying the damage response of the nerve in the adult animal allows examination of Schwann cell–axon interac-

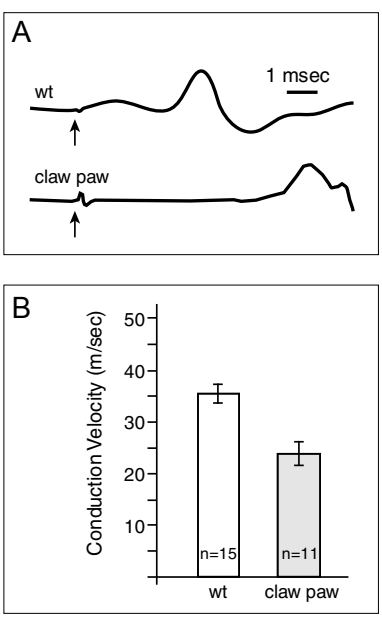

Fig. 5. Nerve conduction velocities are reduced in *clp/clp* nerves. (A) Representative recordings of compound action potentials of the tail nerve of wild-type (wt) and *clp/clp* (claw paw) animals. Arrows indicate the stimulation artefact. (B) Mean conduction velocity for wild-type (wt) and *clp/clp* (claw paw) nerves. The number (*n*) of animals from which recordings were obtained in each group is indicated. The error bar represents standard deviation. The difference in mean NCV between *clp/clp* and wild-type animals is significant ($P < 0.00001$, Student's *t* test).

tions that are largely a reiteration of developmental events (Scherer and Salzer, 1996). Following nerve transection, axons and myelin in the distal nerve segment disintegrate in a process called Wallerian degeneration. Schwann cells revert back to an immature pre-myelinating phenotype, proliferate and participate with macrophages in the removal of myelin and axonal debris. These reactive Schwann cells create an environment that stimulates axonal regeneration of the transected neurones. Upon restoration of axonal contact, Schwann cells differentiate into myelin or non-myelin forming cells (Scherer and Salzer, 1996). Therefore, if the *clp* defect resides within the nerve tissue, it is likely that at least part of the defect will be recapitulated during nerve regeneration following nerve axotomy in an adult animal. On the other hand, if the *clp* defect is strictly developmental and not intrinsic to the nerve tissue, it is expected that regeneration will be relatively normal.

To examine the effect of the *clp* mutation on Schwann cell–axon interaction during regeneration, the sciatic nerve of adult *clp/clp* and wild-type mice were crushed and studied morphologically 4 weeks later (Fig. 6). The nerves were sectioned at two levels distal to the injury, 3 and 6 mm, respectively, representing two successive stages of regeneration. In wild-type animals, regeneration is well advanced, with only few remnants of myelin remaining (arrows in Fig. 6A, panel b) and most axons associated with compact myelin. Regeneration of *clp/clp* nerves differs significantly from wild-type nerves in some aspects. First, most large-caliber axons are ensheathed by a single Schwann cell, but only a few of these are associated with a thin compact myelin sheath (Fig. 6A, panels e and f). Second, supernumerary Schwann cells surround large axon–Schwann cell units and are associated with numerous thin axons (Fig. 6B). These structures are enclosed by the original basal lamina (arrows in Fig. 6B), indicating excessive sprouting of the regenerating axon. Third, excessive fasciculation results in a further subdivision of the *clp* nerve (Fig. 6A, panels e and f). Thus, while *clp* axons do grow back into the distal nerve stump, regeneration is clearly disturbed. Some of these regeneration abnormalities, such as delayed myelination and hyper-fasciculation, are also observed during development suggesting that the *clp* defects are caused by a nerve intrinsic defect in one or more components within the developing or regenerating nerve.

Nerve grafting

To distinguish between a Schwann cell and neuronal defect in the regeneration of *clp/clp* nerves, we performed nerve grafting experiments. Nerve transplantation experiments create a situation in which Schwann cells, genetically distinct from the host, differentiate in association with host-derived neurons (Aguayo et al., 1977; de Waegh and Brady, 1991; Scaravilli and Jacobs, 1981). We performed nerve transplantation operations in which a wild-type host was engrafted with a segment of a wild-type (wt:wt) or *clp/clp* (clp:wt) sciatic nerve segment. Also, the converse set of transplantations was performed in which a *clp/clp* host was engrafted with a wild-type (wt:clp) or *clp/clp* (clp:clp) sciatic nerve segment. The poorly developed epineurium of *clp/clp* nerves as compared to that of wild-type nerves made the grafting of the segments particularly demanding on the technical level. Four weeks after the operation, the transplanted nerves were isolated and examined microscopically. Each nerve was serially sectioned and examined at 250-μm intervals. Representative sections from the middle of the graft and proximal and distal to the anastomosis site are shown in Fig. 7. In the wt:wt transplantation, regenerat-

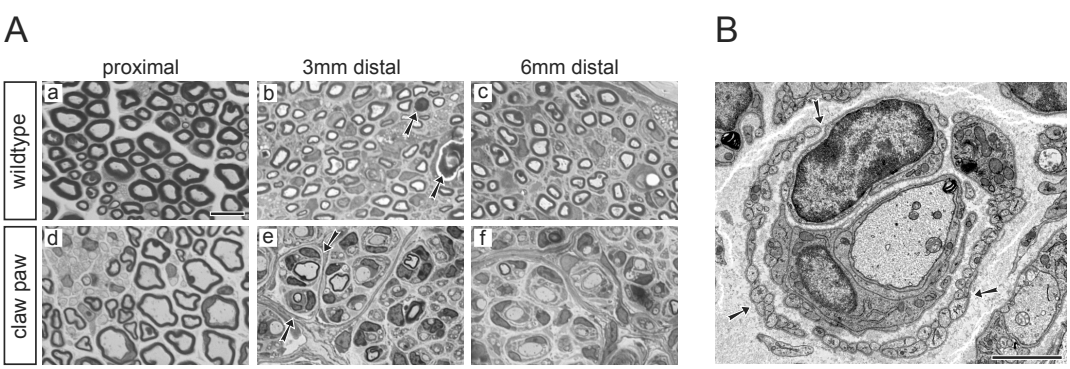

Fig. 6. Abnormal response to nerve injury in *clp/clp* mice. (A) Light micrographs of cross sections of sciatic nerves of young adult wild-type and *clp/clp* (claw paw) animals 4 weeks after nerve crush. The myelin sheath in *clp/clp* nerves is thinner than in wild-type nerves, as described earlier (a and d). In wild-type nerves, regeneration is well advanced, both at 3 and 6 mm distal of the nerve crush (b and c), with only little myelin debris remaining (arrows in panel b). All regenerated axons are myelinated. Regeneration in *clp/clp* nerves is delayed with only few regenerated axons myelinated. This is even more pronounced at 6 mm distal to the lesion, corresponding to a later time point in regeneration (f). Excessive fasciculation is evident in the regenerating nerve. A layer of epineural cells enclosing a group of regenerating fibers is indicated with arrows (e). Scale bar: 10 μm. (B) In this electron micrograph of a regenerating *clp/clp* nerve, supernumerary Schwann cells are associated with regenerating axons. In addition to the large central axon, excessive numbers of axon sprouts (red asterisks) are observed within the regenerating unit that is enclosed by one basal lamina (arrows). The cross section is through three Schwann cells. Scale bar: 2 μm.

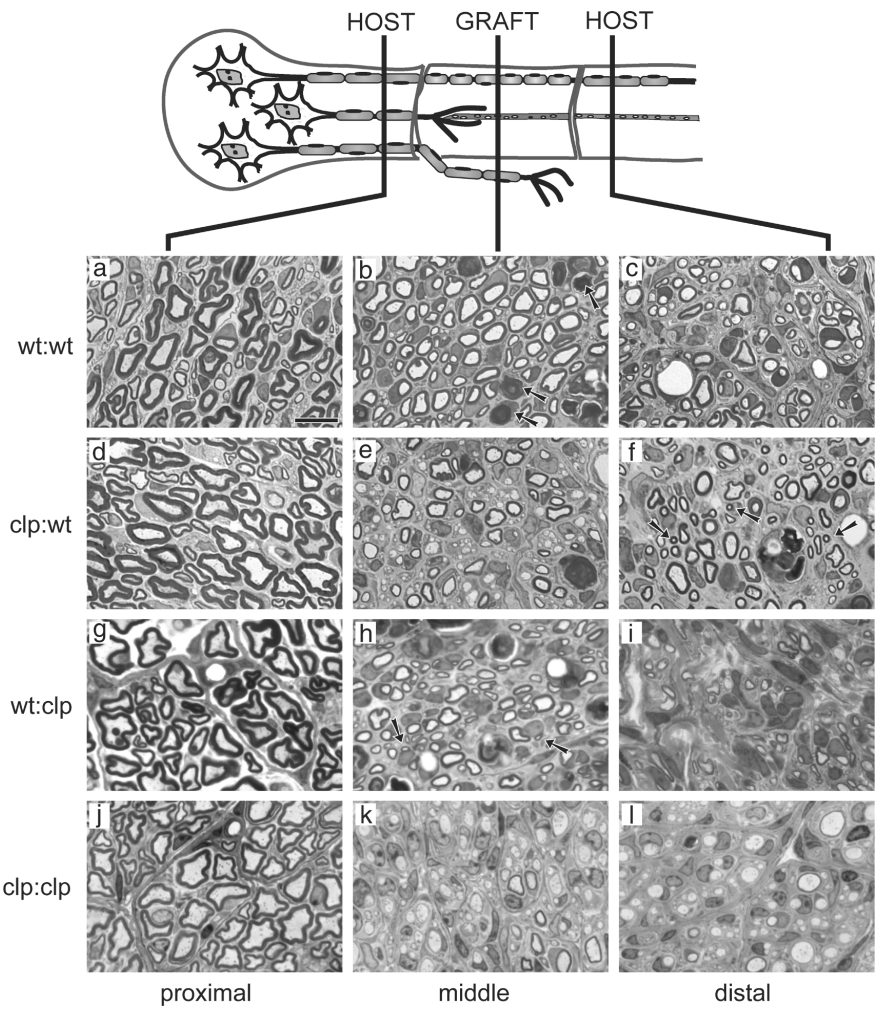

Fig. 7. Nerve grafting experiments reveal Schwann cell and neuronal expression of the *clp* defect. Light micrographs of semi-thin cross sections of sciatic nerve at three different levels of the engrafted nerve 4 weeks after the operation. The position of the sections relative to the engrafted segment is schematically depicted in the diagram above the micrographs. At the level of the engrafted segment and distal to this segment, three different configurations are expected: regenerated axons myelinated by graft-derived Schwann cells (blue in diagram); Schwann cell tubes (blue) that do not get re-innervated by host neurones and host axons that do not enter the nerve graft and are associated with host-derived Schwann cells (gray in diagram) that migrate with the regenerating axons. Donor and recipient genotypes (donor/recipient) are listed to the left. Non-innervated Schwann cell tubes with myelin debris are indicated with arrows in panel b. Thinly myelinated axons that have re-innervated the distal nerve segment of the wild-type host are indicated in panel f with arrows. Arrows in panel h also point to regenerating wild-type axons that are myelinated by *clp/clp* Schwann cells. For further details, see text. Scale bar: 10 μm.

ing axons have grown through the graft and entered the distal nerve stump. Wild-type Schwann cells in the graft and distal nerve stump have formed compact myelin around regenerated axons (Fig. 7, panels b and c). Schwann cell tubes (reactive Schwann cells within their basal lamina: bands of Bunger) that were not re-innervated still contain some myelin debris (arrows in panel b). In the clp:wt transplantation, wild-type axons do regenerate through a *clp/clp* nerve graft into the distal nerve stump (Fig. 7, panels e and f). Moreover, in contrast to wild-type Schwann cells, *clp/clp* Schwann cells have only myelinated the larger axons in the graft and many axons are still in an early ensheathing

or promyelin configuration (panel e). Smaller axons that have navigated through the graft and have entered the distal nerve stump are normally myelinated (arrows in panel f). These observations suggest that at least part of the delayed myelination phenotype in *clp/clp* animals is Schwann cell autonomous.

Transplantations of wild-type nerve segments into *clp/clp* hosts were less successful as ligations to the distal nerve stump did not hold. Nevertheless, *clp/clp* axons did enter the wild-type nerve graft and were myelinated by the wild-type Schwann cells within the graft (Fig. 7, panel h). Also smaller-caliber claw paw axons were myelinated (arrows in panel h). Distal to the second anastomosis site, regrowing axons did not enter the distal nerve stump, but were found associated with a large blood vessel (not shown). Most likely, graft-derived perineural cells and Schwann cells accompany these axons (panel i). Notwithstanding, these data suggest that wild-type Schwann cells receive appropriate signals from *clp/clp* neurones to myelinate them. Finally, the clp:clp transplantations show that *clp/clp* axons do grow through the grafted segment into the distal nerve stump. Consistent with the results in the *clp/clp* nerve crush experiment (Fig. 6), regenerating *clp/clp* axons are associated with supernumerary Schwann cells and only few axons are thinly myelinated (Fig. 7, panels k and l). We also observe a further increase in the extent of fasciculation. This increased fasciculation was less pronounced in the clp:wt and in wt:clp transplantations. Therefore, this specific aspect of the claw paw phenotype arises mainly from interaction of *clp/clp* axons with the *clp/clp* endoneurium. This is also true for the delayed myelination phenotype, as it is much more severe in the clp:clp transplantation than in the clp:wt and wt:clp transplantations. Thus, while the former two grafting experiments (wt:clp and clp:wt) suggest a Schwann cell autonomous effect of the *clp* mutation on myelination, the nerve crush and clp:clp transplantation suggest that there is also an important Schwann cell non-autonomous, most likely neuronal, aspect to the *clp* mutation.

Discussion

We have studied the cellular and molecular basis of the congenital limb abnormality manifested by claw paw mutant mice. Our findings demonstrate that the *clp* mutation affects both the axonal and endoneural/Schwann cell compartment, resulting in axonal sorting and myelination defects. At the molecular level, *clp* affects a signaling pathway that is parallel and non-redundant to the Oct-6-dependent pathway, suggesting that axonal signals that drive Schwann cell differentiation and myelination are transduced through multiple parallel pathways. These findings suggest several possible roles for the *clp* gene and, additionally, define a set of requirements for *clp* candidate genes, to be identified in the ongoing positional cloning effort.

What process in nerve development is affected by the clp mutation?

The development of peripheral nerve tissue can be conveniently thought of as an ordered series of cellular transitions that result from several complex interactions between Schwann cells, axons, mesenchymal cells and extracellular components (Jessen and Mirsky, 1999; Webster, 1993). The first transition occurs when migrating neural crest cells associate with outgrowing axon bundles and acquire Schwann cell precursor characteristics (Jessen and Mirsky, 1991; Jessen et al., 1994). These cells proliferate, migrate with the growing axons and, in a second transition, differentiate into immature Schwann cells that invade the axon bundles and start the process of radial sorting of nerve fibers. This process culminates in larger axons singled out by individual Schwann cells (promyelin stage) which, in a third transition, will go on to myelinate their axon and multiple smaller axons associated with Schwann cells to form the so-called Remak fibers of non-myelinated axons. During this last phase, immature Schwann cells exit the cell cycle and elaborate an extracellular matrix. The peri- and epineural layers that surround and protect the nerve differentiate from the surrounding mesenchyme in a process that requires Schwann cell-derived desert hedgehog signaling (Bunge et al., 1989; Du Plessis et al., 1996; Parmantier et al., 1999).

The major defects in the developing and adult peripheral nerves of claw paw mutant mice were originally described as a general delay of myelination initiation and the persistence of promyelin fibers in adult animals (Henry et al., 1991). On the basis of these morphological observations, Henry et al. hypothesized that the *clp* defect affects the complex signaling between axon, Schwann cell and extracellular components that control the transition of promyelin Schwann cells into myelinating cells. Molecular studies have shown that this transition is mediated by the activity of the transcription factors Oct-6 and Krox-20 acting consecutively in the Schwann cells (Ghislain et al., 2002). Our finding that *clp* does not affect activation of Oct-6 expression but that Krox-20 activation is delayed suggests that *clp* acts either downstream of Oct-6 or in a second non-redundant pathway.

Several observations presented here argue in favor of the second possibility. Morphological examination of nerves of newborn claw paw animals revealed that Schwann cells are still at an early sorting stage of development while wild-type and *Oct-6* mutant Schwann cells are at the end of the sorting stage. This early sorting stage observed in P1 *clp/clp* nerves would be appropriate for embryonic nerves in E16–E18 wild-type embryos. Sorting and ensheathment of axons by Schwann cells critically depends on rearrangements of the Schwann cell cytoskeleton. Deletion of the β1 integrin or the laminin γ1 gene in Schwann cells results in impaired sorting of axon bundles, suggesting that these rearrangements are mediated through interactions between the extracellular matrix laminins and their integrin receptors (Chen

and Strickland, 2003; Feltri et al., 2002; Saito et al., 2003). The similarity in ultrastructural abnormalities observed in newborn *clp/clp*, β1 integrin and laminin γ1 mutant nerves, suggests that clp acts in these pathways. In contrast to laminin γ1 null Schwann cells, *clp/clp* Schwann cells are not permanently blocked at the sorting stage. It is therefore likely that a *clp* redundant function exists or that the *clp* allele is a hypomorph.

In addition, *clp/clp* nerves exhibit hyperfasciculation, a phenotype resembling that observed in *dhh* mutant mice, suggesting the possibility that this specific aspect of the claw paw mutant phenotype results from downregulation of dhh expression. In agreement with this suggestion, we found that *dhh* mRNA is expressed at reduced levels in *clp/clp* nerves (at P12). However, in contrast to *dhh* mutant animals, the nerve–tissue barrier function of the perineurium in claw paw animals is not affected (Fig. 2). As the biological effects of hedgehog proteins are known to be dose-dependent, it is tempting to speculate that the differences in phenotype between *dhh* mutant and *clp/clp* nerves reflect differences in *dhh* expression levels in the developing nerves of these two mutant animals.

Nuclear periaxin expression in embryonic Schwann cells, and the later accumulation of periaxin in promyelin cells at P8, is not observed in claw paw nerves at these stages (Figs. 2 and 3), although periaxin does eventually accumulate in Oct-6 and Krox-20 expressing myelinating *clp/clp* Schwann cells (Fig. 2). It has recently been suggested that a dual mechanism of periaxin regulation exists in which Krox-20 amplifies an earlier Krox-20 independent activation of the periaxin gene (Parkinson et al., 2003). Our results suggest further that the early Krox-20 independent activation of periaxin is regulated through a *clp*-dependent pathway. Alternatively, it is possible that *clp* Schwann cells at E17 represent an earlier differentiation stage at which periaxin is not expressed yet (they will have to be very early Schwann cell precursors, as E14 Schwann cell precursors express periaxin in their nuclei, (Sherman and Brophy, 2000). It is unlikely that the absence of detectable levels of periaxin in *clp* Schwann cells contributes significantly to the peripheral nerve phenotype in claw paw mice, as peripheral nerve development is morphologically normal in *periaxin* null mice (Gillespie et al., 2000).

Thus, in contrast to what has been suggested earlier, the *clp* mutation affects nerve development at a stage well before the promyelin-myelinating transition. The particular defects suggest that *clp* is involved in axon–Schwann cell interactions that drive the invasion and sorting of immature Schwann cells and the subsequent elaboration of a basal lamina and myelination initiation.

On the nature of the claw paw mutation

In the absence of knowledge about the mutation underlying the claw paw phenotype, we do not know what mechanism could account for the peripheral nerve developmental defect and the behavioral defect in claw paw animals. However, on the basis of the observed developmental defects, the recessive nature of the *clp* mutation and in particular the results of regeneration and nerve transplantation experiments, we can speculate on possible scenarios. Although the morphological abnormalities observed in the nerve of claw paw animals suggest that the *clp* mutation affects primarily Schwann cells, the transplantation and nerve regeneration experiments suggest that *clp* function might also be required in the neurone and possibly the endoneurial fibroblasts. It is therefore likely that the *clp* gene product is expressed and functioning in both Schwann cells and neurones and that the *clp* mutation is not a gain of function mutation. These suggestions are compatible with *clp* functioning in, or regulating, homo- or heterophilic interactions between axons and Schwann cells. For example, it is possible that the claw paw protein is involved in bringing adhesion molecules to the cell surface or that claw paw itself is a cell surface protein directly involved in cell–cell interactions. Alternatively, it is possible that the *clp* mutation is a regulatory mutation affecting expression of genes involved in the abovementioned interactions. These impaired axon Schwann cell interactions at an early stage could have repercussions for nerve activity during fetal stages and affect the normal spontaneous movement of the fetus, important for proper posture development.

In summary, we have provided evidence that the mouse *clp* mutation affects the Schwann cell compartment and possibly the neuronal compartment of the regenerating and, by extension, the developing nerve, suggesting that the *clp* gene is expressed in Schwann cells and neurones and is involved in direct axon–Schwann cell interactions. Furthermore, the *clp* mutation affects early activation of periaxin expression and Krox20 expression in Schwann cells, but does not affect the signaling pathway that activates Oct-6 expression, suggesting that axonal signals that initiate myelination are transduced through multiple parallel, non-redundant pathways.

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4

Het hart vindt rust bij woorden die waar zijn
zoals een dorstig man rust vindt bij water.
Roemi (Juwelen)

Chapter 4

claw paw: transient arrest of Schwann cell polarisation?

Aysel Darbas[1], Mehrnaz Ghazvini[1], Marko Piirsoo[1, 3], Smiriti Raghoenath[1], Siska Driegen[1], Martine Jaegle[1], Frank Grosveld and Dies Meijer[1,4]

Departments of [1]Cell biology and Genetics, Erasmus University Medical Center, 3000 DR Rotterdam, Netherlands; [2]present address: National Institute of Chemical Physics and Biophysics, Tallinn, Estonia; [3] corresponding author

Department Of Cell biology and Genetics
Erasmus University Rotterdam
P.O. Box 1738
3000 DR Rotterdam
Netherlands
E-mail: d.meijer@erasmusmc.nl
Tel. +31-10-4088250
Fax. +31-10-4089468

Chapter 4

Abstract

Mice homozygous for the autosomal recessive mutation *claw paw* (*clp/clp)* exhibit limb posture abnormalities and congenital hypomyelination of the peripheral nervous system but not the central nervous system. Within the peripheral nervous system promyelin figures are observed, indicating a myelination delay. During differentiation, Schwann cells become polarized and myelination is initiated at the end stages of Schwann cell polarization. The process of polarization is characterized by expression of laminin and the myelin proteins MAG, P-zero and periaxin. We have previously shown that expression of P-zero and periaxin is delayed in *clp/clp* Schwann cells. However, the precise cellular localization of periaxin remained unclear. Periaxin is indirectly linked to laminin, an essential component of the basal lamina. While periaxin could not be detected in the nuclei or cytoplasm of embryonic *clp/clp* Schwann cells, periaxin was found to be present in myelin when it first appears during the second and third postnatal week of development. Additionally, we found that laminin expression was delayed. We hypothesize that the *clp* mutation impairs Schwann cell polarization, resulting in delayed entry into the promyelin and myelinating phase of cell differentiation.

Introduction

Dysmyelination in claw paw

Claw paw *(clp)* mice are characterized by congenital limb contractures and dysmyelination of the peripheral nervous system (PNS) [1]. Several studies have tried to clarify the claw paw phenotype [2, 3] and some have excluded mutations in genes involved in myelination, like *Mag*, as causative of claw paw [4]. A majority of Schwann cells in *clp/clp* nerves are at the promyelin stage of cell differentiation at postnatal day 14 (p14) [1, 2]. This stage is characterized by the upregulation of *Mag*, downregulation of neural cell adhesion molecule (NCAM), and immature pattern of sodium channel clustering at the nodes [2]. In *clp/clp* nerves, P-zero mRNA expression is found at normal levels at p1.5 [3], while expression of the fatty acid binding protein (P2), of a cytoskeletal protein (dendrin) and the cysteine rich protein 2 (CRP-2) are delayed [3].

The axonal signals that govern myelin gene expression and initiate myelination remain largely uncharacterized [5]. Normally, myelin protein constituents start to be synthesized by the Schwann cell after it contacts one single large calibre axon at the end of the radial sorting process [6-8]. The transcription factors Krox-20 and Oct-6 are both important regulators of the myelination process. Despite similarities in peripheral nerve pathology between claw paw and Oct-6 null and Krox-20 null

mutants, limb abnormalities are not observed in the latter two mutant mice [9-11].

Limb posture abnormalities are an important feature of newborn mice in which the gene encoding the kinesin protein KIF1B β is homozygously deleted. KIF1B β is involved in anterograde axonal transport of synaptic vesicles and mitochondria, hence in cellular polarization. In these KIF1B β -/- mice an ATP binding site of the Kif1B β protein is disrupted, affecting both axonal transport and polarisation [12]. Disruption of KIF1B β results in progressive nerve degeneration and central nervous system abnormalities [12]. This differs from claw paw, where central nervous development is unaffected and the claw paw phenotype improves over time. However, the limb posture abnormalities become fixed contractures in severely affected mice [1]. Fixed contractures have not been described in KIF1B β mice.

Congenital contractures (arthrogryposis) in humans can be a feature of neuromuscular impairment during development. In addition, arthrogryposis has been associated with congenital hypomyelination [13]. Mutations in P-zero [14] , Krox-20 or PMP-22 have been described as cause of central and peripheral hypomyelination [15-18]. Impaired myelination throughout peripheral nervous system, in particular Schwann cell arrest, has been reported in these disorders [19-23]. Therefore, the study of the *clp/clp* dysmyelination serves as a model for human diseases.

Myelination proceeds from Schwann cell differentiation and polarization

Prior to myelin synthesis, Schwann cell become polarized. Cellular polarization creates cellular asymmetry, through site-specific expression of proteins along its cell surface. Thus, typical epithelial cells have a basal and apical membrane, each with specialized function. Similarly, Schwann cells have an adaxonal membrane (facing the axolemma) and an abaxonal membrane (facing the endoneurium). One of the characteristics of Schwann cell polarization is the formation of a basal lamina at the abaxonal surface. Although current evidence suggest that basal lamina formation critically depends on direct axonal contact, some studies suggest a diffusible neuronal factor can induce a patchy basal lamina [24, 25]. One of the constituents of the basal lamina is the extracellular matrix molecule laminin [26, 27].

Within the Schwann cell an intracellular shift of *Mag* [26, 28], P-zero [26] and periaxin [8, 29, 30] is observed during Schwann cell differentiation. Therefore, the expression of *Mag* [30], P-zero [26] and periaxin [30, 31] proteins is considered an indicator of Schwann cell polarisation.

The periaxin gene encodes at least two proteins; a full length form, L-periaxin, and a truncated isoform, S-periaxin. Both proteins have a PDZ motif (post-synaptic density protein PSD-95, Drosophila discs large (dlg) tumor suppressor gene and the tight junction-associated protein ZO-1), found in a growing family of proteins. PDZ domain proteins might organize and transduce signals at sites of cell-cell contact.

Since these proteins shuttle between the cell surface and the nucleus it was believed that some PDZ domain proteins were transmitting signals between the cell surface and the nucleus. In fact L-periaxin is targeted to the nucleus of embryonic Schwann cells, whereas in mature myelinating Schwann cells it is localized at the plasma membrane [32]. As a cytoskeletal linker protein [33], periaxin is linked to laminin via dystrophin-related protein (DRP2), which in turn is associated with dystroglycan [29, 34].

In peripheral nerves, axons are individually and collectively ensheathed by respectively Schwann cells (endoneurium) and perineurial cells. Both endoneurial and perineurial basal lamina consists of laminins. Laminins consists of α, β and γ chains, each encoded by distinct genes. Laminin-2 (α2β1γ1), also referred as s-laminin or merosin, is predominantly found in muscle and peripheral nerve [35]. The role of laminin in Schwann cell myelination has been highlighted by studies on the dy/dy mouse. In the laminin defective, *dy/dy* mutant mouse, mRNA expression levels of the laminin gene are reduced. Similar to patients suffering from congenital muscular dystrophy (CMD), these mice exhibit muscular weakness [36, 37]. The spinal nerves of *dy/dy* mouse are characterized by a reduced number of myelinated axons, atypical patterns of Schwann cell ensheathment and amyelination. In addition, the basal lamina around Schwann cells is discontinuous. Abnormalities in peripheral nerves are probably due to the absence of this extracellular matrix molecule. Contrary to expectations, laminin deficient mice do myelinate [38]. Apparently, in the absence of laminin myelination is merely delayed. One additional feature of *dy/dy* Schwann cells is the lack of polarization [39].

Schwann cell polarization in claw paw

Previous studies of Oct-6 null mice, have revealed an arrest at the promyelin stage of Schwann cell differentiation with normal expression of both laminin and periaxin, [10] but delayed Krox-20 expression [40]. In *clp/clp* mutant mice expression of P-zero and periaxin is delayed in addition to Krox-20 expression [41]. In the present study, we describe the expression of laminin in *clp/clp* nerves by immunohistochemistry. In addition, basal lamina formation is studied by electron microscopy in order to elucidate the sequence of events underlying initiation of myelination.

Materials & Methods

Immunohistochemistry

Wildtype and *clp/clp* nerves were isolated at postnatal day 8 (P8) and at embryonic day 17½ (E17.5). Nerves were fixed in 35% methanol, 35% acetone, 5% acetic acid and 25% H_2O. After dehydration and paraffin embedding, 7-µm sections were cut and mounted on gelatin coated microscope slides.

Antibody incubations were performed in PBS/0.05% Tween-20/1% BSA. Primary antibody was used at a dilution 1:10000 (periaxin); neurofilament medium chain (1:200, Hybridoma 2H3, Developmental Studies Hybridoma Bank) and undiluted supernatant of the s-laminin (Clone C4), and incubated overnight at room temperature. After extensive washing, sections were incubated for 2 hours at room temperature with oregon green coupled goat anti-rabbit secondary antibody and with texas red goat anti-mouse secondary antibody (Molecular Probes).

Electron microscopy
Wild type (Oct-6 $^{+/\Delta SCE}$) and claw paw animals were perfused with PBS for 3 minutes followed by fixative (3% PFA (Sigma); 1% glutaraldehyde in 100 mM cacodylatebuffer pH 7.2) for 10 minutes. Sciatic nerves were isolated, washed with cacodylate buffer, osmicated in 1 % osmium tetra-oxide and embedded in Epon. Semithin sections (1µm) of Epon embedded sciatic nerves were mounted on glass slides and stained with methylene blue. Thin cross sections from the sciatic nerves were cut and uranyl acetate and lead citrate stained for electron microscopy.

Results

Adaxonal periaxin expression and delayed laminin upregulation in postnatal *claw paw* nerves

Similar to Oct-6 null mutant nerves, *clp/clp* nerves display congenital hypomyelination with many Schwann cells at the promyelin stage one week after birth. In Oct-6 null nerves P-zero protein expression is delayed, while upregulation of periaxin and laminin is normal. We studied the expression of these proteins in *clp/clp* nerves at postnatal day 8. We collected sciatic nerves of wildtype, and *clp/clp* nerves and myelination was analysed morphologically by light microscopy. Expression of periaxin (Fig 1) and laminin (Fig 2) was studied by immunohistochemistry. As shown in wildtype nerves, (Fig 1A) a ring-like structure characteristic for myelin is observed. At postnatal day 8, the staining pattern of *clp/clp* nerves does not structurally resemble myelin, (Fig 1B). We further analysed the expression of periaxin by confocal microscopy. This analysis revealed periaxin expression at the adaxonal membrane in both wild type and *clp/clp* nerves, consistent with its normal expression pattern (Fig 1C, D).

Since laminin expression was unaffected in Oct-6 null mouse [10], we studied laminin expression in wild type nerves (Fig 2A) and *clp/clp* nerves (Fig 2B) at postnatal day 8 and 16.

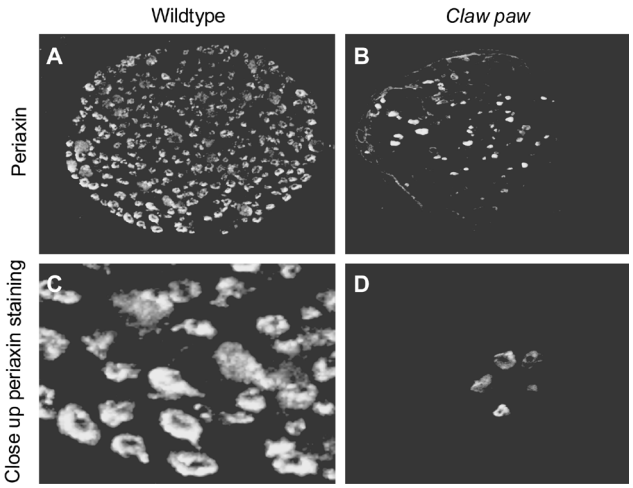

Figure 1. Adaxonal expression of periaxin in both wildtype and *clp/clp* mice. Transverse sections of P8 sciatic nerve from wildtype (A, C), and *clp/clp* (B, D), animals stained with an periaxin antibody and visualized using confocal microscopy.

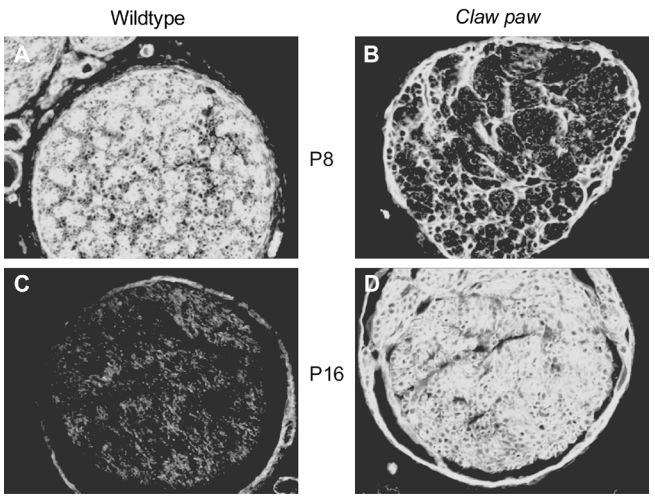

Figure 2. The expression of laminin was analysed by immunohistochemistry in both wildtype and *clp/clp* sciatic nerves, at two distinct postnatal stages (p8 and p16).
Axons are individually and collectively ensheathed with a basal lamina in wildtype nerves at both postnatal days 8 (2A) and 16 (2C). Conversely *clp/clp* axons are predominantly collectively ensheathed at P8 (2B), eventually at P16, axons are individually ensheathed (2D).

Laminin expression is aberrant in *clp/clp* nerves with characteristic staining of minifascicles (perineural cells also elaborate a basal lamina). These minifascicles are a characteristic feature of *clp/clp* nerve [1] (Fig 2B). Endoneurial laminin associated with the Schwann cell basal lamina is eventually found in *clp/clp* mice at postnatal day 16 (Fig 2D). We further analysed the formation of the basal lamina by electron microscopy.

Whereas in control nerves (Oct-6 +/ΔSCE), an extracellular matrix is clearly visible at P8 (Fig 3 A, C), *clp/clp* nerves deposit an extracellular matrix later (Fig 3 B, D).

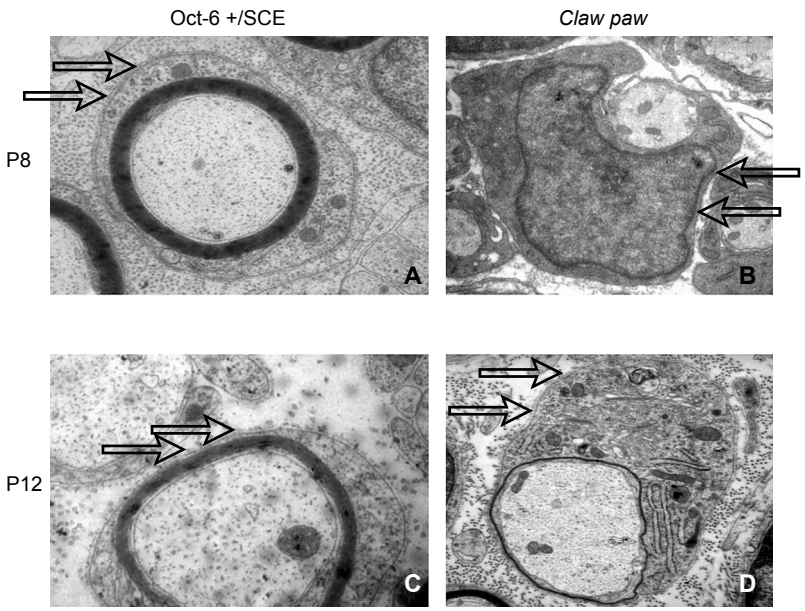

Figure 3. The formation of a basal lamina was analysed with electronmicroscopy in both wildtype (Oct-6 +/ΔSCE) and *clp/clp* sciatic nerves at two different postnatal stages (P8 and P12).
Axons are ensheathed with a basal lamina in both genotypes, indicated with arrows. Myelination is well advanced at both P8 (3A) and 12 (3C). In contrast, *clp/clp* axons are just secreting a basal lamina at P8 (3B), eventually at P12, axons are individually ensheathed and myelination commences (3D; arrows).

Delay of laminin expression in developing nerves of *claw paw*

Previous studies have shown that expression of P-zero is not affected in sciatic nerves of E18 Oct-6 null embryos [9], while at P8 expression of P-zero is reduced in Oct-6 null nerves [9, 40]. This means that embryonal stages of Schwann cell development are not affected by the Oct-6 mutation. In contrast, in *clp/clp* nerves delayed expression of P-zero is observed [41]. The induction of high levels of P-zero gene expression in differentiating Schwann cells is correlated with the initiation of myelin formation and the formation of a basal lamina [42].

To determine the effects of the *clp* mutation on the expression of laminin, we used antibodies directed against laminin and neurofilament to study laminin expression in tissue sections of E17 *clp/clp* nerves (Fig 4). Histology showed that while in control nerves individually and collectively a basal lamina was formed around axons, (Fig 4A,C) the expression of s-laminin (merosin) was reduced and restricted to the perineurium in *clp/clp* nerves (Fig 4B, D). These data indicate that expression

of laminin, which is dependent on axonal contact, is delayed in embryonic stages of developing *clp/clp* nerves.

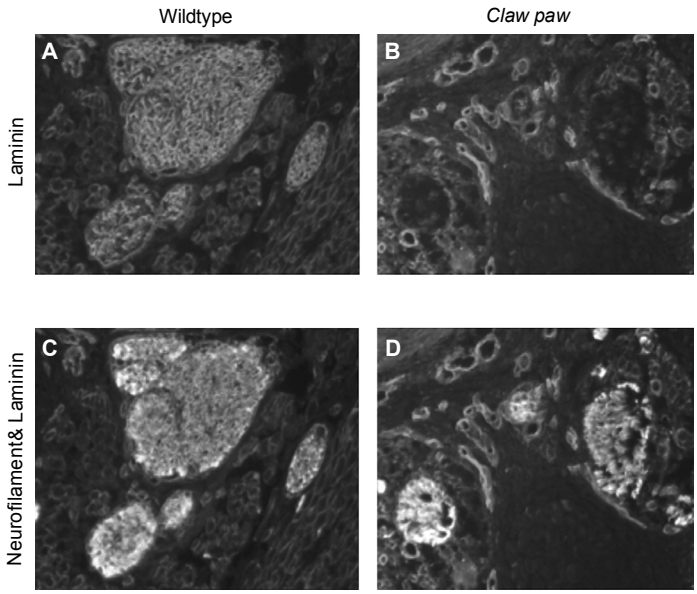

Figure 4. Wildtype nerves are compared with *clp/clp* nerves at embryonic day 17 ½ (E 17 ½). Wildtype nerves express neurofilament (4C), and laminin (4A) antigens. But *clp/clp* nerves only express neurofilament (4B) and initiate laminin (4D) expression.

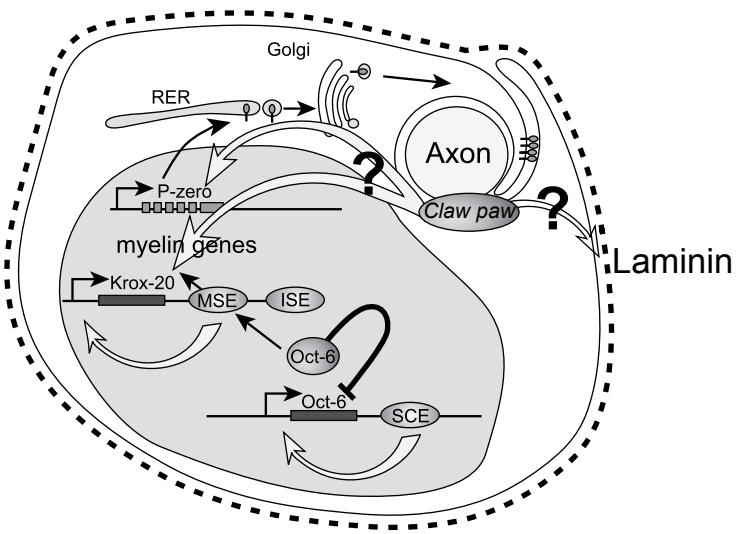

Figure 5. Schematic representation of the cascade induced by *clp* when myelination is initiated. Myelination is under control of continued axonal signaling. One important target of this signaling is the *clp* gene, whose function is probably required for axo-glial interactions that govern cellular polarization. Important targets of *clp* signaling are Krox-20, P-zero, periaxin and laminin, as upregulation of these genes are delayed in the absence of *clp*. (Adapted from [45])

Discussion

Claw paw: transient arrest in Schwann cell polarisation?

The delayed expression of *Mag* [2], P-zero, laminin and periaxin, all proteins involved in polarization, implies that delayed myelination in *clp/clp* mice is associated with impaired Schwann cell polarization. Evidence for a transient arrest of Schwann cell polarization in *clp/clp* nerves comes from the following observations: 1) abnormal and delayed formation of a basal lamina, 2) delayed expression of *Mag* [2] and P0 [41], 3) delayed expression of periaxin during embryonal development as far as its nuclear expression is concerned [41].

We have previously shown that in *clp/clp* nerves Oct-6 expression is unaffected during pre- and postnatal development. However, expression of Krox-20 and myelin proteins such as *Mag* [2], P-zero and periaxin is delayed [41]. The results of immunohistochemistry experiments on nerve sections indicate that the *clp* gene acts either downstream or, more likely, parallel to Oct-6 and upstream of Krox-20 [41]. Myelin formation is controlled by axon-associated cues that regulate a genetic program of gene expression involving the transcription factors Oct-6 and Krox20 (see figure 5). These intra-cellular signaling pathways converge, at least in part, on the Oct-6 Schwann cell enhancer some 10kb downstream of the transcriptional start site. In turn, Oct-6 regulates Krox-20 expression, probably directly via the Krox-20 myelinating schwann cell enhancer (MSE). Krox-20, a key transcription factor, regulates a set of genes including periaxin, P-zero and laminin [43]. However, early embryonic expression of periaxin is independent of Krox-20 [44]. Periaxin belongs to the family of PDZ-domain proteins, which are considered to transmit regulatory signals between the cell surface and the cell nucleus [32] and to be regulated by a nuclear localization signal. Indeed, periaxin is found in nuclei of Krox-20 null embryonic Schwann cells [44]. The significance, if any, of nuclear L-periaxin expression remains to be elucidated [32]. As initial myelin formation appears relatively normal in periaxin homozygous null mice, it is unlikely that the lack of periaxin expression in embryonic *clp/clp* nerves is causally involved in the radial sorting and myelination delay in these mice. The basal lamina is normally formed in Krox-20 and Oct-6 null nerves. In contrast, basal lamina formation appears delayed in *clp/clp* nerves. Since the Schwann cell, and not oligodendrocytes secrete a basal lamina, the delayed basal lamina formation could explain why in *clp/clp* mice mainly the PNS is affected and not the CNS. Apparently, the *clp* gene acts in a pathway which influences expression of the extracellular matrix molecule laminin. Basal lamina formation is a result of axo-glial communication by both axonal signaling and a soluble factor [24, 25]. Hence, the *clp* gene might be required during axo-glial communication or alternatively it is a soluble factor.

Normally, periaxin and *Mag* precedes P-zero [31] protein expression. The

latter is considered normal in terms of mRNA transcription levels in *clp/clp* nerves at p 1.5 [3]. If we assume that mRNA expression in *clp/clp* mouse mutants is normal, this would indicate that the *clp* gene influences unknown posttranscriptional control mechanisms.

From our experiments, insight is achieved with respect to localization of periaxin expression and basal lamina formation in the *clp/clp* mutant. *Clp* acts most likely parallel of Oct-6 and upstream of Krox-20, periaxin and laminin [41] (Figure 5). *Clp/clp* and Oct-6 null mouse mutants are similar in that both exhibit a transient defect in Schwann cell differentiation. Whereas an Oct-6 mutation transiently arrests the Schwann cell in myelination, *clp* probably does so by disrupting radial sorting and cell polarization.

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5

Koşu
Hakikat degişiyor daha bitmeden cümle
koşuyorum yetişmek için bütün gücümle
N. F. Kısakürek (Çile)

Het Hardlopen
De werkelijkheid verandert nog voordat de zin eindigt,
ik loop zo hard als ik kan om bij te blijven.

Vrije vertaling N. Başalan

Chapter 5

Positional cloning of *claw paw*

Aysel Darbas[1], John Bermingham[2], Martine Jaegle[1], Mehrnaz Ghazvini[1], An Langeveld[1], Frank Grosveld and Dies Meijer[1,3]

Departments of [1]Cell biology and Genetics, Erasmus University Medical Center, 3000 DR Rotterdam, Netherlands;[2] McLaughlin Research institute, Great Falls, Montana 59405;
[3] corresponding author

Department Of Cell biology and Genetics
Erasmus University Rotterdam
P.O. Box 1738
3000 DR Rotterdam
Netherlands
E-mail: d.meijer@erasmusmc.nl
Tel. +31-10-4088250
Fax. +31-10-4089468

Chapter 5

Positional cloning of *claw paw*

Abstract

The *claw paw* (*clp*) mouse mutant exhibits striking nonfixed limb posture abnormalities and congenital hypomyelination throughout the peripheral nervous system but not the central nervous system. Following a previous report, describing the linkage of *clp* locus on mouse chromosome 7, we have undertaken a positional cloning approach to identify the *clp* gene. Genetic mapping through linkage analysis resulted in refinement of the region in the vicinity of the *Gpi-1* locus to an area of approximately 737 kb. In this area 41 defined and/or predicted genes and proteins were localized when this study was initiated. Among these genes, three were selected for further study. These genes, Scn1b, Fxyd3 and Fxyd7, were found to be expressed in Schwann cells. No mutations in the ORF or splice junctions were detected in any of these genes excluding them for now as *clp* candidate genes. However, reduced mRNA expression of at least three genes was observed in *clp* nerves, suggesting that *clp* might function as a regulator of PNS genes.

Introduction

Claw paw (clp/clp) mice with abnormal front paws (flexion at one or more joints) were noticed in 1977 within a litter of C57Bl/6J-*obese* inbred strain [1]. Subsequently matings with unrelated non-*obese* C57Bl/6J mice were established to discard the unwanted *obese* gene. The genetic defect showed autosomal recessive inheritance [1]. The severity of the phenotype was very variable. In some animals only one of the forelimbs is affected (flexion of a paw), while more severely affected animals show abnormalities in both forelimbs (forelimb held along the thorax) and one of the hindlimbs [1].

If *clp/clp* mice survive the first several weeks of age, which mainly depends on the severity of the phenotype, their condition improves over the months except that the non-fixed posture abnormality develop into a fixed contracture [1].

Thus, the autosomal recessive *clp/clp* mouse mutant exhibits nonfixed limb posture abnormalities, which are detectable at late embryonic stages and the first postnatal days. Ultrastructural studies have shown that small caliber axons are blocked in myelination and the Schwann cells are arrested in the promyelin stage [1]. Minifascicles are observed in the peripheral nerve similarly to the *desert hedgehog (dhh)* knock-out phenotype [2]. In addition, nodal frequency in *clp/clp* mouse is altered, while sodium channel clustering has not yet occurred at P14 [3], leading to decreased nerve conduction velocity [4].

Congenital contractures have been reported, in patients with congenital hypomyelination, a subset of hereditary motor and sensor neuropathy (HMSN) [5-7]. Only in very few cases is the genetic cause of the hypomyelination known. Congenital posture abnormalities occur in the heterogeneous group of arthrogryposis multiplex congenita (AMC), where virtually any condition resulting in decreased fetal movement can cause fixed contractures [8-10]. Among these conditions, only a few are linked to impairment of peripheral nerve myelination, without abnormalities of myelination in the CNS [11, 12].

Several mouse mutants, including *trembler* [13], *dystonia* [14] and *quaking* [13] exhibit dysmyelination in the peripheral nervous system. In contrast with these mouse mutants, *clp/clp* mutants exhibit limb abnormalities in addition to dysmyelination [1]. Whereas no central dysmyelination is reported for both *trembler* and *dystonia*, CNS myelin abnormalities are observed in the *quaking* mouse mutant. *Trembler* mutants are models for human peripheral neuropathies, in particular CMT1A [6, 15, 16], *dystonia* and *quaking* are not associated with a particular human disease.

The *KIF1B beta* (kinesin protein involved in anterograde axonal transport of synaptic vesicle precursors and mitochondria) [17] exhibits, similarly to *clp,* congenital limb posture abnormality in homozygous (lethal) and heterozygous pups. However, in contrast to *clp*, *KIF1b beta* gene null mutations affect central nervous system development. In addition, affected mice gradually acquire a staggering gait after one year of age [17]. In this respect the *KIF1B beta* null mutants show signs of progressive degeneration, a feature not present in *clp/clp* mice.

The *clp/clp* mouse can therefore be considered a good model for studying early stages of PNS myelination processes and to elucidate the relationship between congenital contractures and PNS dysmyelination.

It is nowadays very well established that myelination results from interactions between constituent cells (axons and Schwann cells) of the peripheral nervous system [18]. Myelinating Schwann cells are involved in regulation of axonal properties and mediate for example spacing of sodium channels, which cluster in the nodes of Ranvier during development [19]. Conversely, axonal signals are involved in the initiation of myelination. Therefore mouse mutants with impaired myelination, either spontaneously arosen or engineered, help us to understand the interactions between axons and their surrounding glial cells (Schwann cells) [16]. Extensive research over the last decade has elucidated some of the mechanisms and molecules involved in the interactions between axons and glial cells.

Two transcription factors, Oct-6 (POU domain transcription factor also referred as SCIP/Tst-1) and Krox-20 (a zinc finger transcription factor also known as Egr-2) have been shown to be pivotal in initiation of myelination. While Oct-6 is required for timing

[20, 21] and acts genetically upstream of Krox-20, the latter seems to be integral to the myelination program [22]. Hence, absence of the Oct-6 gene result in a transient arrest of myelination, while disruption of Krox-20 gene is associated with a permanent block of myelination, at least for the time period studied (up to postnatal day 15) [20-23].

The similarity between the ultrastructural findings (arrest in the promyelin stage) in the peripheral nervous system of *clp/clp* [1] and the reported findings in Oct-6 [20, 21] and Krox-20 [22] implied that *clp* could fit in the already established genetic hierarchy [24]. In order to put *clp* in this hierarchy we aimed at identifying the *clp* gene via positional cloning. Linkage studies placed *clp* gene near the *Gpi-1* locus on chromosome 7 [1].

Our efforts led to a reduction of the area of *clp* gene.

Materials & Methods

Mouse breeding

Heterozygous *clp*/C57Bl6 mice, obtained from the Jackson laboratory were bred and in the progeny *clp* homozygotes were selected for their posture abnormalities at birth detectable within postnatal day 2 (P2). *Clp/clp* homozygote female mice were mated to BalbC (+/+) male mouse, and the resultant +/*clp* F1 progeny was intercrossed. In general the *clp* strain was maintained either by mating homozygote *clp* females with their +/*clp* brothers or by intercrossing +/*clp* individuals. In addition to get more BalbC in the strain from each F1, a *clp* female was again mated with a pure BalbC male. To confirm the genotype “each” *clp* animal was autopsied and characterized by analysis of sciatic nerves which are thinner.

Genotyping

Genotyping was performed by PCR on extracted tail DNA using three different STS markers: one in the vicinity of *clp* (D7MIT155) and the other two on either side (D7MIT77 and D7mit270) to define a region of non-recombinant STS markers. Whenever a recombination was found, the additional markers in between (D7MIT117; D7MIT308; D7MIT25) were tested to define the recombination region. To obtain a detailed version of the recombination region, another set of markers were selected and tested (D7MIT246;D7MIT344;D7MIT343;D7MIT155;D7MIT224;D7MIT308;D7MIT79;D7MIT225;D7MIT78;D7MIT25).

Dna fluorescent in situ hybridisation

Fiber FISH analysis was used to identify the relative localization of D7MIT155 and D7MIT224 (the non-recombinant STS markers) to each other on mouse chromosome 7. The following double linked YAC clones containing these non-recombinant markers were obtained from: WC-930 clone 309-G-9 and clone 193-B-10 (D7mit 155) http// www.informatics.jax.org/searches/contig.cgi?537 and WC-931 clone 421-B-8 (D7mit 224) http//www.informatics.jax.org/searches/contig.cgi?2028. YAC DNA was isolated, the presence of STS marker in this particular clone confirmed and the DNA was used to hybridise with mouse genomic DNA.

FISH analysis was performed as described by Mulder et al.[25]
The probes were labeled with biotin and digoxygenin and immunochemically detected with fluorescein and Texas red. The DNA was counterstained with DAPI.

Expression analysis

Total RNA was isolated of sciatic nerves, heart and testis, spinal cord and muscle of 8-11 days, using TRIzol reagent (Gibco BRL). RT-PCR was performed with primers generated for cDNA of Scn1b (forward TGGAGGTGGATTCCGATACC; reverse GCCACAAGCCATATGGTCAAC), Fxyd3 (forward AACCACCTCTCAGCCTGTTGA; reverse ACAAAGAGCCTGCTACCACGA) and Mag (forward GCCACGGTCATCTAT GAGAGTCAGC; reverse GGTGCCCCAGAGATTCTGAATTCGG).

Mutation analysis

Each exon of the selected genes (Scn1b, Fxyd3, Fxyd7, see results section) within the *clp* area was amplified separately using primers complementary to flanking intronic sequence. DNA sequences can be found in the appendix. After confirming the PCR product size, the product was send to a sequencing company Baseclear for sequencing.

Results

The *clp* gene had previously been mapped to mouse chromosome 7 near the *Gpi-1* locus (Fig 1A). Several STS markers surrounding the *clp* locus were selected (Fig 1B). To increase the resolution of the map around *clp*, crosses were expanded to a total number of 190 progeny *clp/clp* mice (380 meiotic chromosomes). After having established an initial order for these STS markers on a panel of 120 meiotic chromosomes, several markers could be discarded, as their recombination rate (distance) was too high (Fig 2). Genotyping an additional 130 *clp/clp* animals (260 meiotic recombination events) resulted in a genetic map with 4 non-recombinant STS

markers (D7mit343, D7mit155, D7mit224, D7mit308) flanked at the centromeric site by D7mit344 (1 recombination) and at the telomeric site by D7Mit79 (1 recombination) (Fig 2).

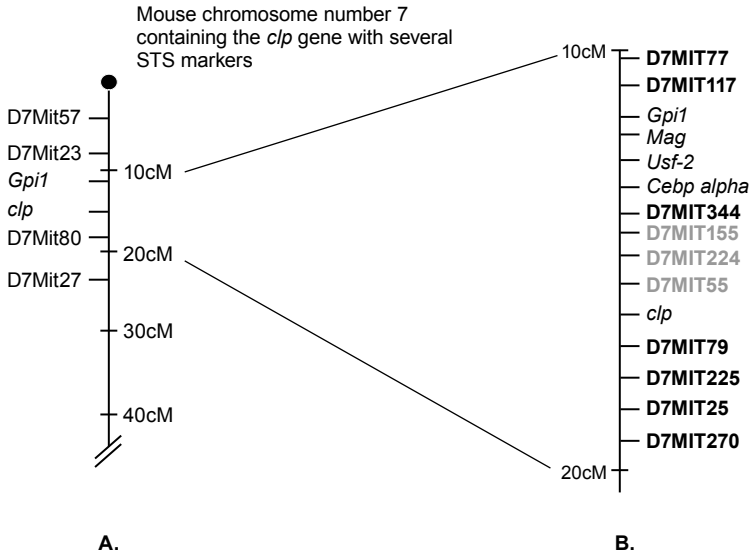

Figure 1. (A) Genetic map of the *clp* locus at the start of our study (B) Selection of STS markers in the vicinity of *clp* locus. Furthermore, genes localized on chromosome 7 are shown. Gray color indicates no recombination found with this specific STS marker with linkage analysis. Bold indicates STS markers where one or more recombinations have been found.

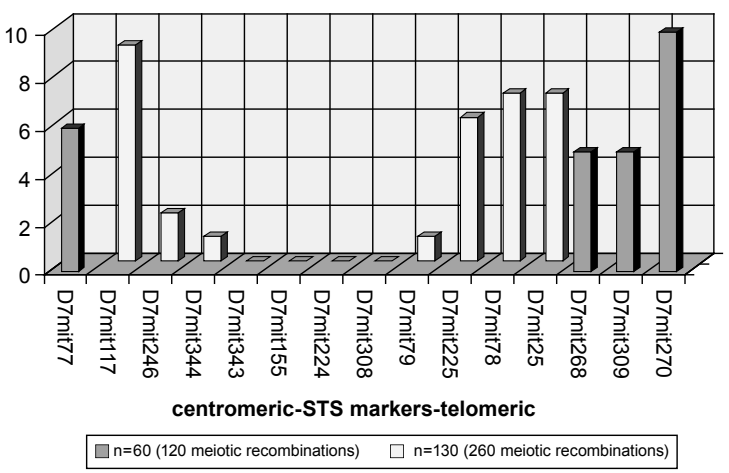

Figure 2. Recombination scores
Recombination was scored by selection of Balb/C alleles for a specific STS marker in a *clp/clp* animal. As *clp/clp* originates from C57Bl/6J background while the inheritance is autosomal recessive, any Balb/C allele, in a *clp/clp* mutant, for a marker indicates that this specific STS marker is not linked to *clp*.

YAC clones were ordered to determine the size of the insert via pulsed field gel electrophoresis. After having confirmed the presence of the STS marker in the YAC DNA, we wanted to know whether these YAC clones contained overlapping stretches of DNA. Fiber FISH analysis revealed that the YAC clones were overlapping with the STS marker D7mit 224 containing YAC clone telomeric from the YAC clone containing the STS marker D7mit155 (Fig 3), consistent with information in the mouse genome informatics database. After release of the mouse genome draft sequence (2002), we pursued our research by looking for candidate genes instead of working with YAC clones.

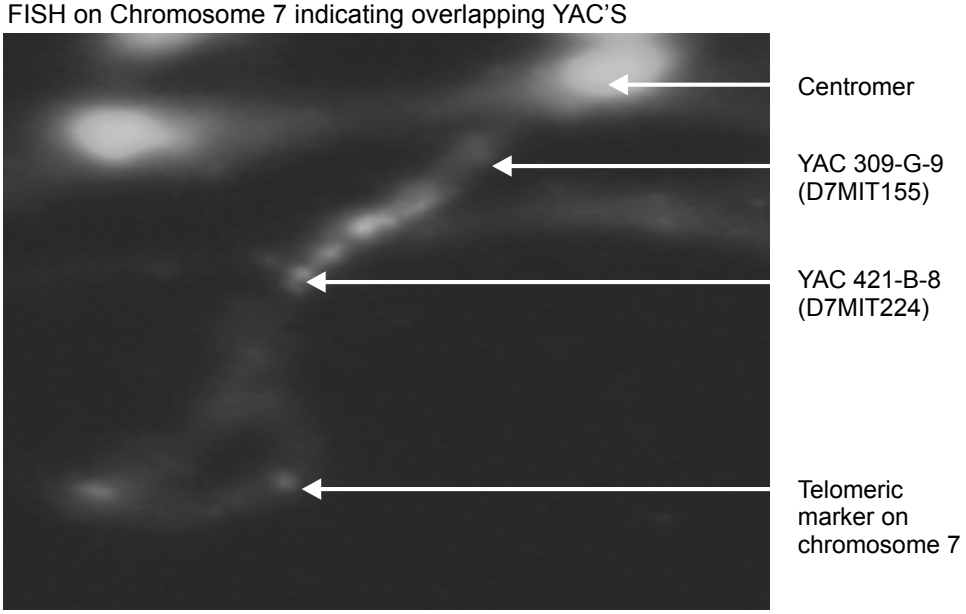

Figure 3. Fiber FISH analysis
FISH analysis was performed to localize the non-recombinant STS markers D7mit224 and D7mit155 to each other throughout the mouse genome. With red color the YAC clone containing D7mit155 and with green color the YAC clone containing D7mit224 is indicated. Furthermore is the color green used for localization of the telomeres, while blue shows the centromer.

Having identified four non recombinant markers in the genome (D7mit344, D7mit155, D7mit224 and D7mit308), we used these genome sequence data to define a region within the available databases, such as Celera http://www.celera.com, Ensembl http://www.ensembl.org, UCSC http://genome.ucsc.edu and NCBI http://www.nlm.nih.gov . This was still an area of 3.69 Mb according to Ensembl jun 2004. In the meantime, a recombination between Cast/Ei and C57Bl/6J in mouse 27R was identified by our collaborators. Phenotypically wildtype offspring were either heterozygous Cast/ei/C57Bl/6J for centromeric markers (D7mit266, and D7mit246)

and homozygote C57Bl/6J for telomeric markers or heterozygote C57Bl/6J, while the *clp/clp* offspring of this mouse was exclusively homozygote C57Bl/6J for the investigated STS markers. This result implicated that a recombination had occurred in mouse 27R between *clp* and D7mit155 (Fig 4).

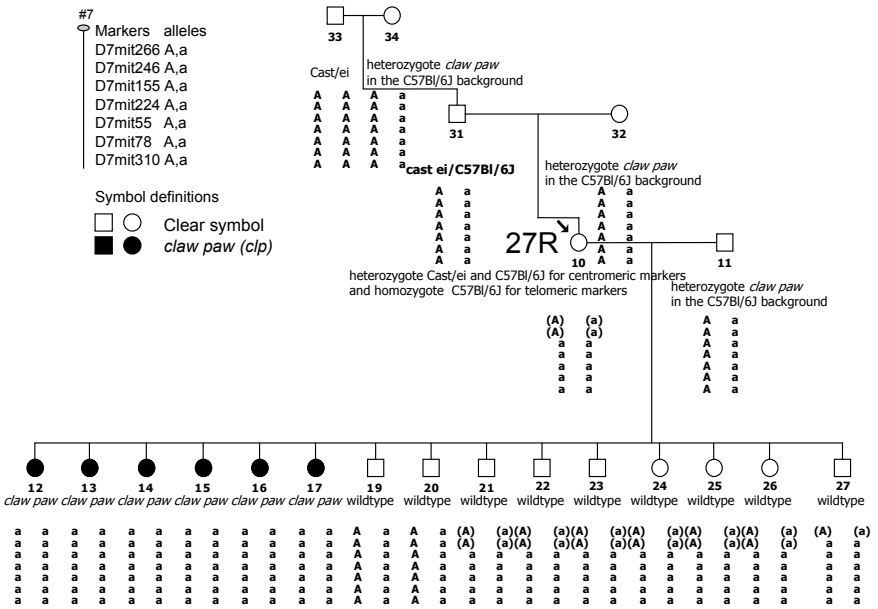

Figure 4. Crossing of Cast/Ei with heterozygote C57Bl/6J resulting in the offspring 27R Cast/Ei was crossed with a heterozygote *clp/clp* in the C57Bl/6J background. Offspring of these mice were heterozygote for Cast/Ei/C57Bl/6J background and claw paw. Subsequently these mice were crossed with a heterozygote claw paw in the C57Bl/6J background. All the offspring were tested for the selected STS markers shown in right corner. Among tested animals 27R had a striking genotype. This mouse was at the centromeric site heterozygote for Cast/Ei and C57Bl/6J background while at the telomeric site it was homozygote for C57Bl/6J background. This mouse was phenotypically wildtype. 27R was crossed with a heterozygote claw paw in the C57Bl/6J background and the offspring of these mice were as following: wildtype mice had either the same genotype as 27R or were homozygous C57Bl/6J while *clp/clp* mice were exclusively homozygous C57Bl/6J indicating that in 27R a recombination had occurred between *clp* and D7mit155. This mouse places *clp* on the centromeric site of D7mit155.

Thus these data demonstrate that *clp* is localized on the centromeric site of D7mit155. This recombination in that specific mouse reduced the area of 3.69 Mb to approximately 737 kb (736724 basepairs) consisting of 41 defined and predicted genes. Furthermore, *clp* is localized in between D7mit344 (centromeric) and D7mit155 (telomeric). Only the STS marker D7mit343 remained non recombinant from the 4 non-recombinant STS markers (see Fig 5 next page). This region is compared with the syntenic region, localized on chromosome 19, in the human genome database. The genes are similar in the human syntenic region, only the order is reversed. In an area of approximately 821 kb (821036 bp) 130 defined and predicted genes/proteins are localized.

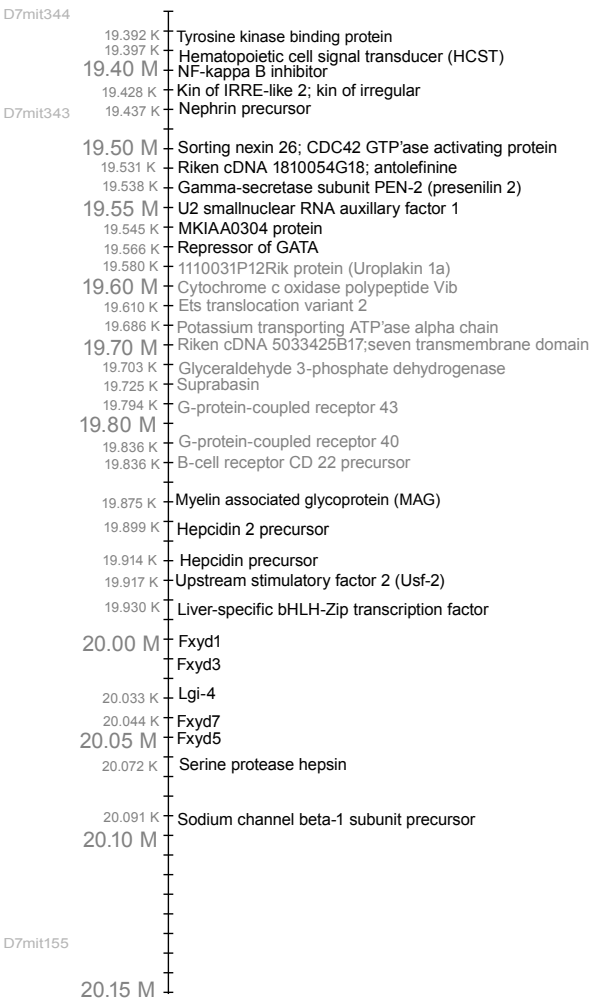

Figure 5. The *clp* area as defined by linkage analysis.
Via linkage analysis a certain region is defined. Additional data of transcripts, genes and proteins within this area are derived from the mouse genome databases: Celera, NCBI, UCSC and Ensembl.

From this collection of candidate genes a small number of genes were selected. These genes encode proteins expressed in the peripheral nervous system and which are involved in generation of action potentials (*Scn1b* [26]), or in maintenance of membrane potential *Fxyd3* [27] *and Fxyd7* [28]). The selected genes are at a distance of 127 kb from each other within the *clp* area on chromosome 7. Each exon of these selected genes, with the flanking splicing regions, were amplified from *clp/clp* genomic DNA. No mutation was found in either the protein coding part of the gene or in the splice junction sequences.

Additionally, Scn1b, Fxyd3 and *Mag* (myelin associated glycoprotein) were tested for the levels of mRNA expression. We selected *Mag* for RT-PCR rather than Fxyd7, as Fxyd7 had a weak expression already in control nerve. *Mag*, localized on chromosome 7 in the vicinity of *clp*, a minor constituent of CNS and PNS myelin, was already ruled out as a candidate gene for *clp* via sequencing cDNA [29]. Furthermore, the *Mag* knock-out mouse had a phenotype distinct from *clp* [30, 31]. The reason why we preferred analysing *Mag* was based on a study where expression of *Mag* preceded sodium channel clustering [32]. Therefore, we were interested in the expression of *Mag* during development of the peripheral nervous system in *clp/clp* where sodium channel clustering had not yet occurred [3]. By RT-PCR a reduction of mRNA expression of voltage gated sodium channel (*Scn1b*) and chloride channel or chloride channel regulator (*fxyd3* = *mat 8*) and *Mag* was observed at postnatal day 8-11 *clp/clp* nerves (Fig 6).

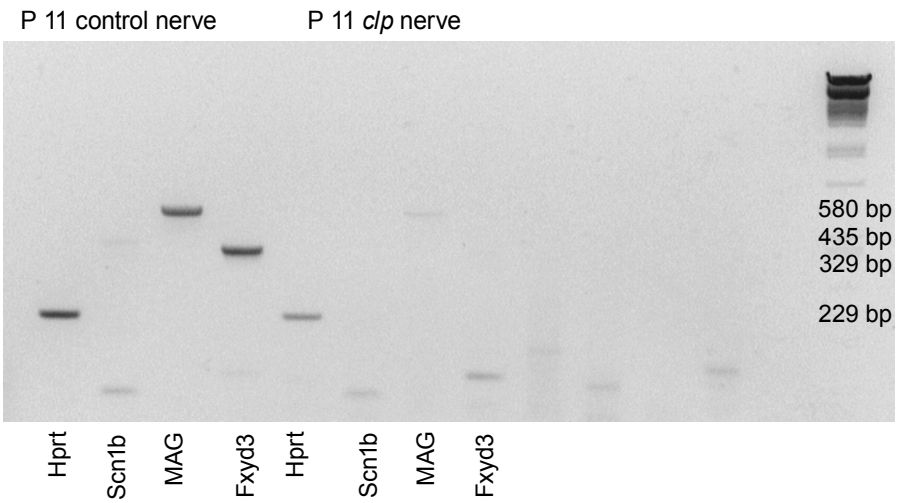

Figure 6. Ethidium bromide stained acrylamide gel with final cDNA product of genes shown below. Quantitative RT-PCR has elucidated that expression of Scn1b, Fxyd3 and MAG are reduced if compared with HPRT.

No reduction of control gene expression (hypoxanthine phosphoribosyl-transferase =HPRT) was observed in the same experiments. To see whether reduction in mRNA level of Scn1b, Fxyd3 and *Mag* was a general phenomenon in *clp/clp* mice or restricted to the peripheral nerves, other tissues were included. Heart and testis (Scn1b), muscle (Fxyd3) and spinal cord (*Mag*) were selected. We did not found a similar reduction as observed for peripheral nerves (not shown) suggesting that the downregulation of these genes is Schwann cell specific.

Discussion

In the present study we sought to further define the position of the *clp* gene through increasing the resolution of the genetic map and to identify the genetic defect underlying the claw paw phenotype. We have demonstrated that the *clp* gene is between the markers D7mit155 and D7mit344, spanning an area of approximately 737 kb. We selected three candidate genes in this area for their expression in the peripheral nervous system, adhesive function combined with generation of action potentials (*Scn1b*) [26] and maintenance of membrane potential (*Fxyd3* [27] *and Fxyd7* [28]). No mutations were found in these genes. However, we observed reduced mRNA levels in *clp/clp* nerves for the genes encoding *MAG, Fxyd3* and *Scn1b*. All these genes are present at or near the *clp* area, and while Fxyd3 and Scn1b were *clp* candidate genes, *Mag* was not.

Although our data of reduced mRNA levels for several of these genes have to be confirmed by either northern blot experiments or in situ hybridisation, a recent report indicate that it is very likely that *clp* affects more genes at the mRNA level. RNA in situ hybridisation analysis suggest that the genes encoding P2, a myelin component and member of the fatty acid binding protein, *dendrin* and the *LIM-domain protein cysteine rich protein-2 (CRP-2)* are all expressed at reduced levels in the sciatic nerve of *clp/clp* animals [33].

The fact that the reduced genes within the defined *clp* area are at a distant of 308 kb from each other implicates that there is an underlying mechanism which regulates gene expression in this area. We speculate that this mechanism depends on *clp*. Alternatively, the reduced expression of these genes reflect the delayed sorting and myelination observed in *clp/clp* nerves. A developmental profile of gene expression of all these genes in wildtype and *clp/clp* animals need to be obtained to resolve this issue.

An example of a similar global effect on gene expression levels of a selected group of genes is provided by *quaking. Quaking* is a mouse mutant with generalized hypomyelination throughout the central and peripheral nervous system [13]. *Quaking* is associated with reduced expression of several myelin genes including *Mag* [34], proteolipid protein (PLP), Myelin basic protein (MBP). The *Quaking I* (*QKI*) protein belongs to the family of STAR proteins (signal transduction activator of RNA metabolism) and the reduction of many myelin proteins at the mRNA level seems to be associated with impairment of mRNA metabolism. A recent investigation addressed the question whether *QKI* interacts with mRNAs of myelin proteins to regulate homeostasis, or whether posttranscriptional misregulation interrupts normal myelination [35, 36]. Indeed, destabilization and mislocalization of at least MBP mRNA is reported [36]. Furthermore, a series of N-ethyl-N-nitrosurea (ENU)-induced quaking alleles established that *quaking* is essential in embryonic development where

it was required for blood vessel formation [37]. A similar mechanism as observed in *quaking* could be the underlying mechanism in *clp* where instead of the central nervous system the peripheral nervous system is affected.
The limb abnormality observed in *clp/clp* is strikingly similar to KIF1b beta mouse mutant with a disruption in axonal transport implicating that *clp/clp* could have a neuronal component.
Indeed a neuronal component is not excluded in our results. However, progressive axonal degeneration would have ultrastructurally resulted in degeneration/regeneration phenotype, which was not observed [1].

The yet undefined *clp* gene is localized in the 737 kb region on chromosome 7 defined in this study. Mutation analysis of each of the 41 genes in this area would be the most direct way to identify the *clp* gene. Even when a mutation is found which disrupts the open reading frame of one of the genes additional experiments need to be done to confirm that this mutation is causing the phenotype as no additional *clp* alleles are known. The most obvious one would be a genetic complementation experiment. However, if the genetic defect underlying *clp* is a mutation in a regulatory region the absence of additional *clp* alleles severely limits our chances of identifying the mutation. In this case the mapping will have to be further refined analysing much larger numbers of meiotic chromosomes, to be able to sequence directly the minimal critical region of claw paw and C57Bl/6J DNA. The *clp* mutation is identified, and further details are given in chapter 6.

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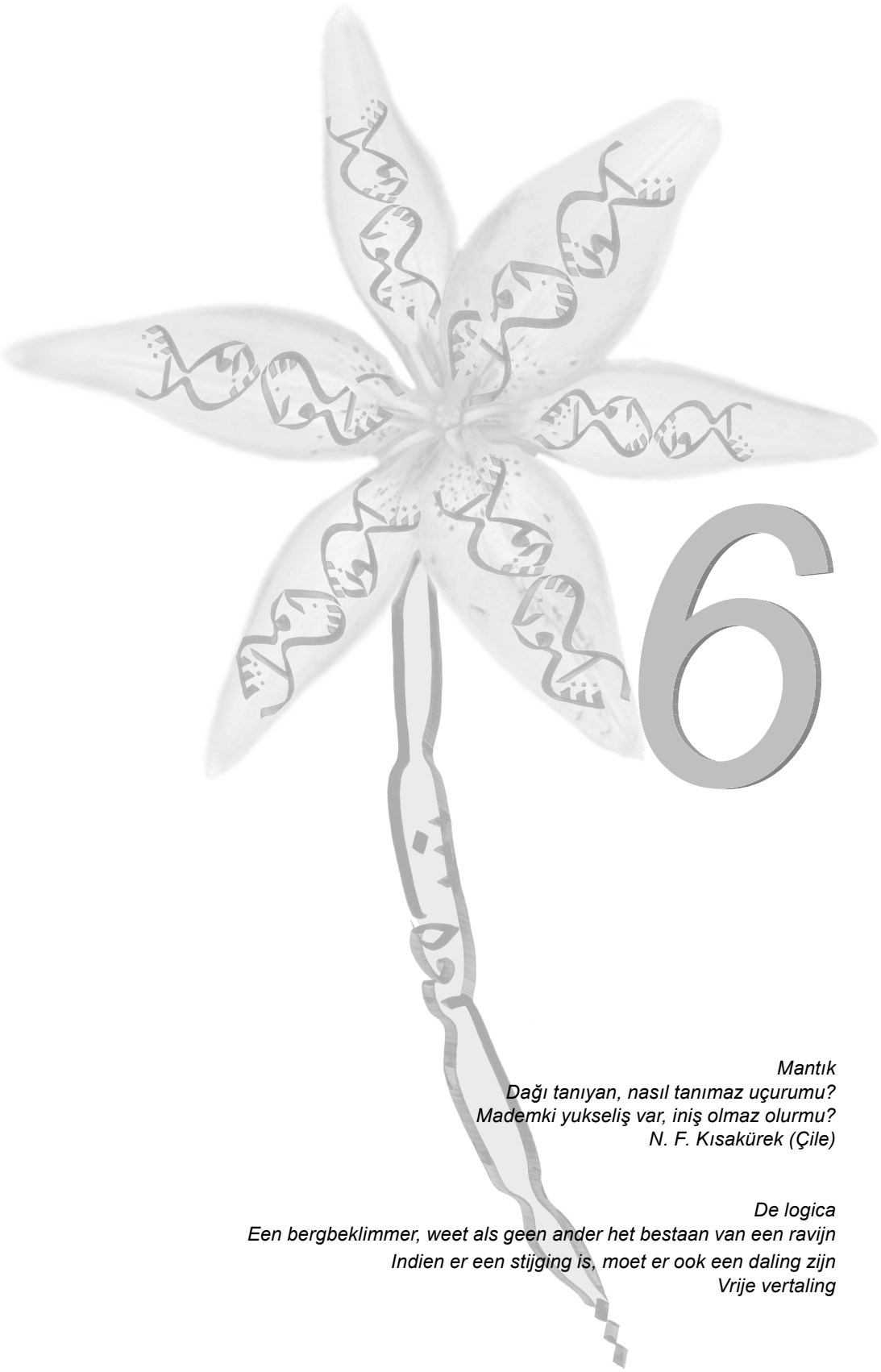

6

Mantık
Dağı tanıyan, nasıl tanımaz uçurumu?
Mademki yukseliş var, iniş olmaz olurmu?
N. F. Kısakürek (Çile)

De logica
Een bergbeklimmer, weet als geen ander het bestaan van een ravijn
Indien er een stijging is, moet er ook een daling zijn
Vrije vertaling

Chapter 6

Concluding remarks and future perspectives

Chapter 6

Concluding remarks and future perspectives

Postnatally the autosomal recessive mouse mutant *clp/clp* [1] can be selected based on the limb abnormalities, already present in utero [2]. Additionally, *clp/clp* mice are characterized by congenital hypomyelination of the peripheral nervous system.

It is generally accepted that axonal contact-associated signals initiate the myelination process through coordinate activation of sets of genes. Myelin, a lipid-rich insulation layer around axons, is the endpoint of Schwann cell differentiation. Although each Schwann cell has the potency to form myelin, yet unknown axonal signals determine whether myelination occurs [3-6]. The genetic cascade that is activated by these axonal contact associated signals include the transcription factors Oct-6 and Krox-20. Myelination is delayed in both Oct-6 [7-10] and Krox-20 [11] mouse mutants. Whereas the absence of the former results in a transient arrest of Schwann cell differentiation, the latter is essential during terminal differentiation. Hence Schwann cells of Oct-6 null mice eventually form normal myelin, whereas Krox-20 null mutants remain devoid of myelin for the timepoints studied. Extensive analysis of these transcription factors revealed a genetical hierarchy within the myelination program where Oct-6 expression induces Krox-20 gene expression directly [12]. We have investigated the interaction between these transcription factors and the *clp* gene (chapter 3 and 4). Analysis of abnormal myelination in men and mice has resulted in the identification of several mutations in genes encoding proteins either required for myelin synthesis or axonal function. Thus dys-/demyelination does not necessarily mean that the Schwann cell is the defective cell type. In the past, impaired myelination in mice has been studied through nerve grafting experiments, which allows the study of regenerating axons in contact with genetically distinct Schwann cells. Analysis of remyelination (considered a reiteration of development) in the grafted nerve segment, indicates whether impaired myelination is due to an axonal or Schwann cell defect, or both [13-15].

To examine the role of the Schwann cell in dysmyelination as observed in *clp* mutants we studied Schwann cells during development and regeneration, as described in chapters 3 and 4. In these studies we provide evidence that *clp/clp* Schwann cells express Oct-6 normally in development, but are transiently inhibited to express successive genes involved in myelination. Although initially this would indicate that *clp* is a target gene of Oct-6, other findings are restricted to *clp/clp* Schwann cells such as delay in laminin and periaxin expression, suggesting a yet unidentified parallel role of *clp* in myelination process. Besides delayed expression of laminin, other proteins such as periaxin, myelin associated glycoprotein, P-zero are considered to be involved in cellular polarization.

It seems as if *clp/clp* Schwann cells are transiently blocked during cell polarization (chapter 4).
In order to be able to understand the hypomyelination observed in *clp/clp* peripheral nerves and its relation to limb posture abnormalities the gene has to be identified. Previous studies had already identified the position of *clp* in the mouse genome on chromosome 7 [1]. In order to find the *clp* gene, a positional cloning strategy was developed as described in chapter 5. A selection of anonymous markers surrounding *clp* region both on telomeric and centromeric site was made. Subsequently linkage analysis resulted in refining the *clp* area. Additionally, Fxyd3, Scn1b (genes involved in generation of membrane potential) and *Mag* (a myelin gene) mRNA expression were reduced in developing *clp/clp* nerves. Finally, while this thesis was written, our collaborators identified the *clp* gene. *Clp* turned out to be an insertion of 224 basepairs, 5 nucleotides upstream of exon 4 of the Lgi-4 gene.

Lgi proteins

Lgi-1 (leucine rich glioma inactivated-1), was cloned in a translocation breakpoint of a glioblastoma cell line [16]. Subsequently in silico mapping identified Lgi-2, Lgi-3 and Lgi-4 [16]. It was logical to assume a role for Lgi-1 in tumorigenesis, as the gene is strongly downregulated in malignant gliomas. Surprisingly, mutations in Lgi-1 were found to cause lateral temporal lobe epilepsy in several families with the disease. This was all the more surprising as genetic forms of epilepsy were invariably associated with mutations in genes encoding ion channels [17]. Therefore, it was assumed that Lgi-1 interacts with ion channels.

LGI-proteins are constitutively secreted and functionally related to other protein families such as tyrosine kinase receptors and SLIT, which are involved in development and maintenance of the nervous system, in particular axon guidance [18].
Several domains can be recognized in Lgi proteins:
1) a putative signal peptide
2) 3 LRR domains
3) 4 EAR domains
4) a putative N-linked GlcNac part

LRR repeat proteins

Cells of multicellular organisms are surrounded by extracellular matrix. The latter plays an essential role in many processes such as development, cell proliferation, differentiation, migration and adhesion. Among the extracellular matrix molecules there is a group characterized by its leucine-rich region (LRR) which dominates the structure of the core protein [19].

LRR proteins have been found intercalated into the cell membrane as observed in tyrosine kinase family of neurotrophin receptors, attached to the cell membrane via a GPI-linkage and in the cytoplasm of the cell. This wide distribution of LRR proteins implicate diverse biological functions of LRR proteins [19]

EAR domain

Mutations in epilepsy-associated-repeat (EAR) have been identified in families with epilepsy [20]. For example mutations in the third EAR domain has been identified in idiopathic partial epilepsy syndrome. Additionally, this class of domains are thought to be involved in interactions between proteins [21].

A potential N-linked GlcNac part

Protein modifications through covalent linkage of sugars, phosphates, acetyl groups etcetera, is a common mechanism to regulate the function of a protein. These covalent modifications involve specific amino acid residues flanked by a loosely defined consensus sequence. For example, asparagines flanked by a serine or threonine is a potential site for glycosylation. N-glycans (linkage of N-acetylglucosamine with L-asparagine) are involved in receptor sorting, ligand binding, dimerization of receptors and signal transduction [22]. Such a site is present in Lgi-4 and might be important for its function. However, whether it is used as a glycosylation site remains to be determined.

Lgi-4 and claw paw phenotype

Do we understand the claw paw phenotype after the identification of the mutation in the Lgi-4 gene? Why would a mutation in a gene homologous to a tumor suppressor gene result in hypomyelination and arthrogryposis? It is too early to even begin to answer this question as virtually nothing is known about these proteins. Even their subcellular localization is unknown. All assigned functions to these proteins are guesses based on the domain structure of the proteins. A preliminary report has suggested that Lgi4 and the other Lgi proteins are secreted proteins. However, expression of a myc-tagged Lgi4 gene in COS cells suggest a vesicular localization of Lgi4 (unpublished observations of Martine Jaegle and Dies Meijer).

Lgi-4 has a leucine rich region, which is implicated in both axonal guidance and protein-protein interactions. Additionally, LRR proteins have been found at several places within the cell, attached or intercalated to the cell membrane and within the cytoplasm. Therefore it might be that impaired Lgi-4 expression is disturbing axonal guidance and protein-protein interactions at various places within the cell or between two celltypes.

Lgi-4 was cloned and a susceptibility allele (polymorphism) within this gene was identified for childhood absence epilepsy [23].

Furthermore, predominantly the epilepsy associated repeat (EAR) domain is mutated in epilepsy. This domain is associated with ligand recognition of the receptor and protein protein interactions. Its absence may influence protein interactions and signaling via the receptor.

Silencing of gene expression is regulated in several manners in the human genome. One known post-transcriptional silencing mechanism is the formation of antisense RNA. Antisense RNA can be formed when two distinct genes overlap at the DNA level, and are transcribed from opposing DNA strands. When both genes are expressed in the same cell-type at the same time, it is possible that the two RNA's bind to each other and influence further processing and translation. This might result either in absence or reduced protein levels of the respective genes. Lgi-4 and Fxyd3 are genes that overlap at the 3' end, have a reverse orientation and indeed are expressed in the same tissues at the same time. Potentially it is possible that RNA from these genes bind to each other and interfere with gene expression [24].

Future perspectives

With the identification of the *clp* gene we can finally begin to investigate the underlying cellular defects in *clp/clp* mice through detailed study of the function of the Lgi-4 protein and possibly provide an explanation for the remarkable link between congenital hypomyelination and limb contractures observed in *clp/clp* mice. First, it is important to generate antibodies for Lgi-4 to determine the localization of this protein and to start to study its function. Additionally, it is preferred to generate a knock-out with the identified mutation to see whether claw paw phenotype is observed.

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Summary

Based on anatomical and functional grounds, the nervous system is usually described to consist of two components: the central (brain and spinal cord) and peripheral nervous system (motor and sensory nerves, sensory ganglia, sympathetic and parasympathetic ganglia and nerves and the enteric nervous system). A large proportion of nerve fibers in both systems is covered with a lipid rich membranous layer (myelin), which allows fast impulse propagation at low energy costs. Myelin is formed in the central nervous system and peripheral nervous system by oligodendrocytes and Schwann cells respectively. Work described in this thesis focuses on the genetics and cell biology of myelin formation (myelination) in the peripheral nervous system.

The formation of the myelin sheath represents the last stage of Schwann cell differentiation, a process that starts with the generation of Schwann cell precursors from the neural crest. The proliferation, migration and differentiation of Schwann cell precursors into myelinating Schwann cells requires the differential recruitment of genetic information contained within the chromosomes of the cell. A number of nuclear proteins that play an important role in this differential recruitment of genetic information have been studied in detail over the last decade. In particular, it has been demonstrated that the transcription factors Oct-6 and Krox-20 are involved in the promyelin to myelinating transition and myelination respectively. In the absence of Oct-6, Schwann cells transiently arrest at the promyelin stage of cell differentiation, while in the absence of Krox-20 Schwann cells fail to initiate myelin formation. In addition to these engineered mouse mutants, natural mouse mutants have been characterized in which peripheral nerve myelination is affected. One such natural mouse mutant is the claw paw (*cp/clp)* mouse. *Clp/clp* mice are characterized by non-fixed limb abnormalities at birth (in clinical practice referred to as arthrogryposis; arthron = “joint” and gryposis = “bent”) and congenital hypomyelination of the PNS with delayed onset of myelination. We choose to study this mouse mutant in detail for a number of reasons. First, the similarities in genetics and peripheral nerve phenotype between *clp/clp* and Oct-6 mutants suggest a possible interaction between these genes. Second, the combination of limb abnormalities and congenital hypomyelination suggest a common neurogenic origin and third, the *clp/clp* mouse serves as a mouse model for the human syndrome of arthrogryposis multiplex congenita with congenital hypomyelination.

1) To shed light on these and related issues, we addressed two key questions in this thesis: what cell type is affected by the *clp* mutation and how does this mutation affect Oct-6 and Krox-20 in Schwann cells?
2) what is the underlying genetic defect of the claw paw phenotype?

In chapter 2 I describe the experimental approach chosen to answer these questions. In chapters 3 and 4 experiments are described that demonstrate that the *clp* gene is cell-autonomously required in Schwann cells and possibly in neurons. Furthermore,

we show that the *clp* defect affects radial sorting of nerve bundles suggesting an impairment of axon-Schwann cell interactions and cellular polarization. In chapter 5 I describe our positional cloning efforts that resulted in the definition of a minimal critical region in which the *clp* gene resides. Chapter 6 contains a general discussion of the work and suggestions for future directions.

Samenvatting

Gebaseerd op functionele en anatomische gronden wordt het zenuwstelsel onderverdeeld in twee componenten: centraal (brein en ruggemerg) en perifere zenuwstelsel (motore en sensore neuronen en ganglia, sympatische en parasympathische ganglia en zenuwen). Het merendeel van de zenuwen in beide systemen worden omgeven door een vetrijke isolatielaag welke myeline wordt genoemd. Myeline wordt in het centraal en perifeer zenuwstelsel gevormd door respectievelijk oligodendrocyten en Schwann cellen. De myeline laag rondom axonen is van cruciaal belang voor het functioneren van het zenuwstelsel. Afbraak van myeline leidt tot ernstige neurologische problemen zoals in multiple sclerose en de erfelijke perifere neuropathiën. In dit proefschrift wordt werk beschreven dat tot doel had een aantal genetische en celbiologische aspecten van myelinisatie in het perifere zenuwstelsel op te helderen.

De vorming van myeline is het laatste stadium van een differentiatie proces van de Schwann cel dat begint met de emigratie van dorsale neuro-epitheliale cellen uit de neuraalbuis. Deze migrerende populatie cellen wordt de neurale lijst genoemd. In een vroeg stadium van de ontwikkeling associëren neurale lijst cellen met de uitgroeiende axonen. Deze zogenaamde Schwann cel voorlopers prolifereren en differentiëren uiteindelijk tot myeline vormende Schwann cellen. Celdifferentiatie vereist de gereguleerde expressie van een set genen die belangrijk zijn voor de specifieke vorm en functie van de cel. De expressie van deze genen wordt gereguleerd door een klasse van DNA bindende eiwitten welke transcriptiefactoren worden genoemd. Een aantal transcriptiefactoren die een belangrijk bijdrage leveren aan Schwann cel differentiatie zijn de laatste decennia gedetailleerd bestudeerd. Uit dit werk bleek dat twee transcriptiefactoren, namelijk Oct-6 en Krox-20, van belang zijn voor respectievelijk de overgang van het promyeline naar myeline stadium en myelinisatie. Terwijl in afwezigheid van Oct-6 myelinisatie tijdelijk geblokkeerd is in het promyeline stadium, wordt er totaal geen myeline gevormd in afwezigheid van Krox-20.

Naast deze muize mutanten die door genetische modificatie ontstaan zijn, zijn er ook spontane mutanten bekend die een gestoorde myelinisatie vertonen. Een voorbeeld is de muis mutant *claw paw* (*clp*). *Clp/clp* muizen worden gekarakteriseerd door pootafwijkingen (in de kliniek wordt dit beschreven als arthrogryposis; arthron=joint and gryposis=gebogen) en congenitale hypomyelinisatie gebaseerd op vertraagde myelinisatie. Deze muis werd om een aantal redenen uitgekozen voor nader onderzoek. Ten eerste; de genetische en neuropathologische overeenkomsten tussen *clp/clp* en *Oct-6* mutanten suggereren een interactie tussen deze genen. Ten tweede; de combinatie van arthrogryposis en congenitale hypomyelinisatie suggereert een gemeenschappelijk neuronale oorzaak van het fenotype.

Ten derde; de *clp/clp* muis kan als model gebruikt worden om de relatie tussen arthrogryposis en congenitale hypomyelinisatie beter te begrijpen.

Om inzicht te verkrijgen in deze en andere gerelateerde vraagstukken, hebben we onszelf twee vragen gesteld:

1) welk celtype is defect door de *clp* mutatie en hoe worden de transcriptiefactoren Oct-6 en Krox-20 hierdoor beïnvloed?
2) Wat is de genetische oorzaak die resulteert in dit fenotype?

In hoofdstuk 2 beschrijf ik de gekozen experimentele benadering om op deze vragen een antwoord te geven.
In hoofdstuk 3 en 4 worden experimenten beschreven die aantonen dat het *clp* gen functioneert in Schwann cellen en mogelijk in neuronen. Verder tonen we aan dat het *clp* defect resulteert in een vertraagde axonale rangschikking door Schwann cellen, mogelijk als gevolg van verstoorde axon-Schwann cel interactie en cellulaire polarisatie.
In hoofdstuk 5 beschrijf ik de afbakening van een kritische regio waarin het *clp* gen moet liggen, door middel van 'linkage analysis'.
Hoofdstuk 6 omvat een discussie van het werk en suggesties voor verder onderzoek.

Dankwoord

Het schrijven van het dankwoord is geen gemakkelijke opgave. In principe zijn er in de afgelopen jaren heel wat mensen geweest die bedankt moeten worden, echter vaak ben je genoodzaakt om een selectie te maken. Soms vergeet je bepaalde individuen te noemen, die wel veel voor je hebben betekend. Vandaar bij deze, iedereen die vindt dat hij of zij direct dan wel indirect betrokken is geweest bij het tot stand komen van dit geheel, bedankt.

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Mehmet bey, benim düşüncelerimi gercekleştirdiğiniz için size minnettarım.

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Appendix

Exons of selected genes	DNA sequence	Annealings temperature	Amplified product size
fxyd3exon1 sense	TTAAgggAggAATggAgTggg	61	310
fxyd3exon1 anti sense	AgCAgAggCTCAgggAggAA	61	310
fxyd3exon2 sense	TggTgAgCAACAggTgAgACA	61	419
fxyd3exon2 anti sense	gAgAgAgAgTCTgCCCgTTgA	61	419
fxyd3exon3-4 sense	AACCTCCATggATCCggCT	61	401
fxyd3exon3-4 anti sense	ACTgAgCAggACgggAAgAA	61	401
fxyd3exon 5-6 sense	AAgggCCTgggTTTgCTTT	58	444
fxyd3exon 5-6 anti sense	TCgggATgATTTgCCACA	58	444
fxyd3exon 7-8 sense	ACAgTgggAAACCAgCCAAg	65	582
fxyd3exon 7-8 anti sense	gCCTCACTCCAAgCTCCACA	65	582
Scn1bexon1 sense	gCCTCggAATTggTgTATgg	55	1374
Scn1b exon1 anti sense	gATCCTTCTgTgCCgTCCA	55	1374
Scn1b exon2 sense	AgTgACATCCTCTTTCCCAgg	54	679
Scn1b exon2 anti sense	ATgCACTCAgACAAgCCAATC	54	679
Scn1b exon3 sense	gggAACAggTTgggAATgg	61	413
Scn1b exon3 anti sense	TgTTAgCTggCCTCTgTgTgg	61	413
Scn1b exon4 sense	ACTTgCCCgAgCTCACACA	61	401
Scn1b exon4 anti sense	TTCgAgTTgCggCTTTgC	61	401
Scn1b exon5 sense	ggCAgAgTTACAgCTTgTCgg	55	367
Scn1b exon5 anti sense	AAgAgTgTgTCCCTgAgCCCT	55	367
Scn1b exon 6 sense	AgggCTCAgggACACACTCTT	61	614
Scn1b exon 6 antisense	AAgAgAggAggCCgAAgAgg	61	614
fxyd7 exon 1 sense	TCAgTgCCTCTTgTgCgAAA	55	396
fxyd7 exon 1 anti sense	TCgTCTTCCTTTCCgTATggg	55	396
fxyd7 exon 2 sense	TTggTCCATCTTgCTCCTCAA	55	298
fxyd7 exon 2 anti sense	ggTTCACAgCCTTCATCAggg	55	298
fxyd7 exon 3 sense	ggAggTgAgAATCCAgAAggg	55	399
fxyd7 exon 3 anti sense	gCAggCAgAggACCTTCAAg	55	399
fxyd7 exon 4-5 sense	TTgAgggTTTCCTgCTggTC	55	437
fxyd7 exon 4-5 anti sense	TCCgAggCTTgCATTTCTTT	55	437
fxyd7 exon 6 sense	gCAgCCATCCTTggACTCTg	65	510
fxyd7 exon 6 anti sense	gACAgTCAACTCAgCCTCCCA	65	510

Stellingen

1. *De wederzijds afhankelijke functie en differentiatie van verschillende celtypen binnen een weefsel bemoeilijkt de identificatie van het celtype dat is aangedaan door een genetisch defect.*
   Dit proefschrift

2. *Verstoorde cellulaire communicatie tussen neuron en glia cel leidt tot vertraging in differentiatie.*
   Dit proefschrift

3. *Polarisatie is een van de eerste stappen in het cellulaire differentiatieproces.*
   Dit proefschrift

4. *Positioneel kloneren is iets uit de oude doos: rechtstreeks sequencen van het genoom is de snelste weg naar identificatie van genetische afwijkingen.*
   Dit proefschrift

5. *Om vanuit het fenotype het defecte gen te voorspellen binnen een kritische regio blijft een hachelijke onderneming.*
   Dit proefschrift

6. *De term 'Junk' DNA blijkt achteraf een ongelukkig gekozen term.*
   S. Brenner *Nature's Gift to Science* Nobel lecture 2002

7. *The world is full of willing people; some willing to work, the rest willing to let them.*
   R. Frost

8. *The noblest pleasure is the joy of understanding.*
   L. da Vinci

9. *Education makes a people easy to lead, but difficult to drive; easy to govern but impossible to enslave.*
   Baron Henry Peter Brougham

10. *We must not believe the many who say that only free people ought to be educated, but we should rather believe the philosophers who say that only the educated are free.*
    Epictetus

11. *Ondanks dat vandaag de dag onafhankelijkheid wordt nagestreefd, zijn we massaal afhankelijk van de computer.*

Curriculum vitae

Personalia:

Naam:	Darbaş
Voornaam:	Aysel
Adres:	Banierstraat 1
Postcode:	4841 VE
Plaats:	Prinsenbeek
Geboorteplaats:	Elazığ (Turkije)
Geboortedatum:	17-12-1970
Burgerlijke staat:	Ongehuwd
Geslacht:	Vrouw

Opleiding & diploma's:

1983-1987	Virgo Maria Mavo
1987-1989	Mencia de Mendoza Lyceum (HAVO)
1989-1991	Mencia de Mendoza Lyceum (VWO)
1991-1995	Erasmus Universiteit Rotterdam
1997-1997	Co-schappen
Maart 1998	Artsenbul

Onderzoekservaring:

1992-1993	Onderzoek bij de afdeling neuroanatomie naar structtuur en verbindingen van het cerebellum en de vestibulaire kernen bij het konijn. (Begeleider: professor dr. J. Voogd)
1995- 1996	Afstudeeronderzoek op de afdeling neuroimmunologie: Gekeken is of dat antistoffen tegen neurale eiwitten in sera van GBS patiënten voorkomen en/of de aanwezigheid van antistoffen tegen specifieke eiwitten geassocieerd is met bepaalde klinische manifestaties en het klinisch beloop van de ziekte. (Begeleider: drs. B. Jacobs)

1996	Onderzoek op de afdeling klinische neurofysiologie: Er werd gekeken naar de zenuwgeleiding bij proefpersonen. Deze gegevens zijn vergeleken met de waarden die gevonden worden bij mensen met de ziekte GBS, HMSN en CIDP. Hierover is een artikel verschenen in The journal of neurology, neurosurgery and psychiatry. (Begeleider: dr.J. Meulstee)
1998 –2002	Promotieonderzoek op de afdeling Celbiologie en Genetica met als titel The mouse mutant Claw paw: cellular and genetic aspects onder supervisie van Dr. Ir. D.N. Meijer en Prof. Dr. F.G.Grosveld te Rotterdam aan de Erasmus Universiteit.
2002-2004	Arts-assistent in de klinische genetica (counseling op de oncogenetica)
2005	arts assistent interne geneeskunde