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Role of Laminins in the Development of the Peripheral Nervous System

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ROLE OF LAMININS IN THE DEVELOPMENT OF THE PERIPHERAL NERVOUS SYSTEM

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To investigate the function of laminins in peripheral nerve development, the laminin γ1 gene was specifically disrupted in Schwann cells. Disruption of laminin γ1 gene expression resulted in depletion of all other laminin chains known to be expressed in Schwann cells. Schwann cells lacking laminins fail to differentiate to myelinating and non-myelinating Schwann cells and do not extend processes required for initiating axonal sorting and mediating axon-Schwann cell interactions. These cells also fail to down-regulate Oct-6 and they arrest at the premyelinating stage. Impaired axon-Schwann cell interactions prevent phosphorylation of β-neuregulin-1 receptors, which results in decreased cell proliferation. Postnatally, laminin-null Schwann cells exhibit reduced phosphatidylinositol 3-kinase activity and activation of caspase cascades, leading to apoptosis. Injection of a laminin peptide into mutant sciatic nerves partially restores PI 3-kinase activity and reduces apoptotic signals. In a Schwann cell/dorsal root ganglion co-culture system, disruption of laminins impairs podia formation as well as the elongation of Schwann cells. These results demonstrate that: 1) laminins initiate axonal sorting and mediate axon-Schwann cell interactions required for Schwann cell proliferation and differentiation; 2) laminins provide a PI 3-kinase/Akt-mediated Schwann cell survival signal.

I wish to dedicate my thesis to

my wife and my parents

for their constant support, devotion, and understanding throughout the

preparation of my thesis

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CHAPTER 1: INTRODUCTION

1.1. Development of Schwann cells

Myelination of axons is essential for proper function of the nervous system, predominantly by allowing for the fast conduction of action potentials. Myelination of the peripheral nervous system (PNS) is accomplished by Schwann cells (SCs), the major glial cells of the vertebrate PNS. During embryonic development, SC precursors are derived from the neural crest, which occurs at embryonic day (E) 14-15 in the rat and E12-13 in the mouse. The survival of SC precursors is dependent on axon-derived signals, comprised of the neuregulin family of growth factors. When immature SCs are generated from SC precursors (E15-17 for rat and E13-15 for mouse), they lose this axon dependence and support their own survival by establishing autocrine loops (Jessen and Mirsky, 2005). At this time, some SCs destined to myelinate will proliferate vigorously and differentiate into promyelinating SCs, from which individual cells extend their cytoplasmic processes into bundles of axons, progressively separate them into even smaller bundles, and finally establish a 1:1 relationship with each larger diameter axon, a process known as radial sorting (Webster, 1993). These cells will further differentiate and wrap axons concentrically with the extension of their membrane and produce myelin sheaths (myelinating SCs). In contrast, nonmyelinating SCs ensheath multiple small caliber axons (C fibers, <1 μm diameter) to form a Remak bundle and keep individual axons separated by

membrane extensions, but they do not form myelin sheaths (Jessen and Mirsky, 2005) (Fig. 1).

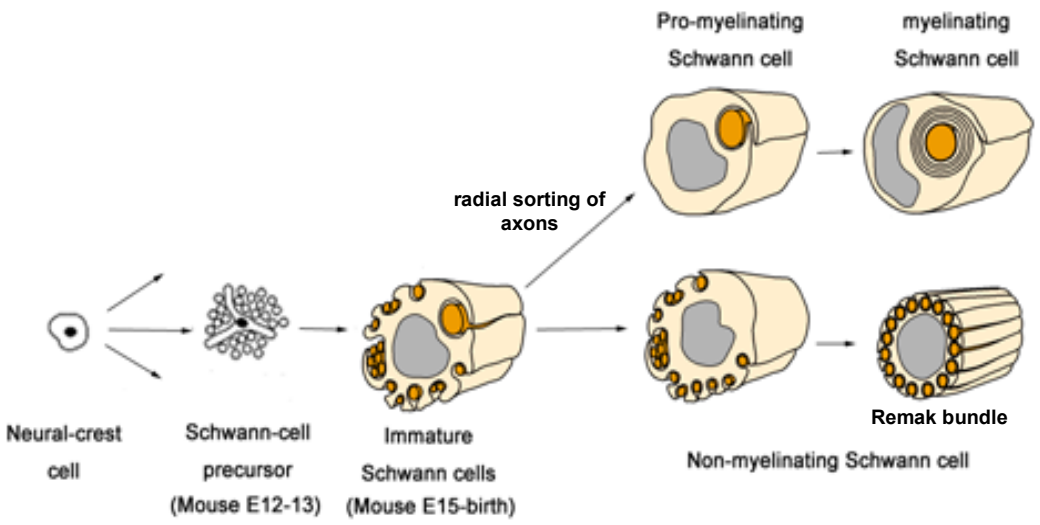


Figure 1. The mouse Schwann-cell lineage (modified from Jessen and Mirsky, 1999b; Basic Neurochemistry, 6th Ed, 1999, Fig 27-16).

1.2 Proliferation and survival of SCs

SC proliferation and survival are essential processes during PNS development and are crucially dependent on the regulation of at least two distinct signaling pathways, the neuregulin/ErbB and transforming growth factor-β (TGF-β) (Jessen and Mirsky, 2005). Neuregulin-1 (NRG1) consists of a family of proteins of more than 15 transmembrane and secreted isoforms that result from alternative splicing. They can be divided to three major types: I, II, and III. The NRG1 type III protein expressed in axons is the major isoform responsible for most of its effects on the SC development, mediated by the receptor tyrosine kinases, ErbB2 and ErbB3 (Nave and Salzer, 2006). During PNS development, NRG1 proteins are key regulators at multiple stages of the SC lineage. For example, they can act as a survival factor for SC precursors, as a major axon-derived mitogen for SC precursors and SCs, and as a regulator of myelin sheath thickness and ensheathment fate of SCs (Jessen and Mirsky, 2005; Michailov et al., 2004; Taveggia et al., 2005). The severe deficiency in SCs in neuregulin-1/ErbB deficient mice indicates that this growth factor plays an essential role in SC development (Meyer and Birchmeier, 1995; Riethmacher et al., 1997). SC precursors depend on axon-derived NRG-1 for survival through activation of both the mitogen-activated protein kinase and the phosphoinositide-3-kinase (PI 3-K) signaling pathways (Dong et al., 1999). When SCs generate from precursors, they acquire the ability to survive without axons by establishing autocrine survival loops that involve insulin-like growth factor (IGF-2), neurotrophin-3 (NT3), and platelet-derived growth factor-BB (PDGF-BB) (Meier et al., 1999). Laminins also

act synergistically with these autocrine survival signals to promote SC survival from short-term to long-term (Meier et al., 1999). In rat SC culture, NRG1 proteins induce cAMP response element binding protein (CREB) phosphorylation (Tabernero et al., 1998) and heregulin (a human homologue of NRG1) combined with forskolin (a compound which elevates cAMP levels and CREB phosphorylation) to synergistically promote SC proliferation (Rahmatullah et al., 1998). CREB is found in SC precursors and in mature SCs (Stewart, 1995), and its phosphorylation is significantly induced after axon-induced SC proliferation (Lee et al., 1999). NRG-1 mediated activation of the PI 3-kinase pathway are also crucial for the proliferative responses of SCs to axons (Maurel and Salzer, 2000).

TGF-β is required for the maintenance of the nonmyelinating, proliferating state of SCs by promoting proliferation and inhibiting myelination during development (Awatramani et al., 2002; D'Antonio et al., 2006; Einheber et al., 1995; Guenard et al., 1995; Ridley et al., 1989). The effects of the TGF-β signaling pathway on SC development and function are modulated by the proto-oncogene, Ski (Atanasoski et al., 2004). TGF-β blocks myelination by preventing Ski expression. Overexpression of Ski inhibits TGF-β-mediated SC proliferation and upregulates myelin genes. TGF-β has also been shown to induce SC embryonic apoptosis during development by activating c-Jun (Parkinson et al., 2001). In differentiating SCs, expression of Krox-20 (a myelin-associated transcription factor) turns off TGF-β signaling and renders the SCs resistant to apoptosis (Parkinson et al., 2004).

1.3 Differentiation and myelination of SCs

Several transcription factors participate in the onset of myelination, including Krox20 (also known as Egr-2), octamer-binding transcription factor 6 (Oct-6/Scip/Tst-1), brain 2 class III POU-domain protein (Brn2), Ski, and NGFI-A-binding proteins 1 and 2 (NAB1/2) (Atanasoski et al., 2004; Jaegle et al., 2003; Jaegle et al., 1996; Le et al., 2005; Topilko et al., 1994). Among these, Krox20 and Oct-6 are the two best characterized factors involved in a genetic hierarchy essential for the differentiation of myelinating SCs. Oct-6 expression in SCs is transient and peaks in the promyelinating stage but progressively down-regulates during postnatal development. Oct-6 function is required at promyelinating SCs for their timely differentiation into myelinating SCs (Bermingham et al., 1996; Jaegle et al., 1996; Jaegle and Meijer, 1998). In contrast, Krox-20 is expressed only in the myelin-producing SCs, and these cells continue to express detectable levels of Krox-20 protein throughout life (Topilko et al., 1994). Genetic and cell biological studies suggest that these transcription factors can interact with each other (Nagarajan et al., 2001; Zorick et al., 1996; Zorick et al., 1999). In promyelinating SCs, Oct-6 is strongly induced by an axonal contact-related signal and subsequently activates a set of genes, including Krox-20. High level expression of Krox-20 is required for the down-regulation of Oct-6 after the peak of myelination and for the activation of enzymes required for the synthesis of myelin lipids as well as for the activation of the major myelin genes, P_0 and myelin basic protein (MBP). Brn2 is expressed in a similar developmental profile as Oct-6, and its function largely overlaps with Oct-6 in regulating the transition

from promyelinating to myelinating SCs (Jaegle et al., 2003). Nab1/2 proteins form a complex with Krox-20 and are critical transcription modulators of Krox-20 in myelinating SCs (Le et al., 2005).

A series of studies revealed that neurotrophins play a critical role in regulating SC myelination (Chan et al., 2001; Cosgaya et al., 2002). The neurotrophin family includes NGF, BDNF, NT-3, and NT4/5 (Notterpek, 2003). Their signals are mediated through two types of receptors: the high affinity Trk receptor tyrosine kinases and the low affinity $p75^{NTR}$ (Chao, 2003). The $p75^{NTR}$ binds all four neurotrophins with similar affinity and acts as a co-receptor for Trk receptors. Three different types of Trk receptors are specific for a particular neurotrophin. Specifically, 1) TrkA binds NGF; 2) TrkB binds BDNF and NT4/5; and 3) TrkC interacts preferentially with NT3 (Chao, 2003). Neurotrophins play both positive and negative roles in the modulation of myelination. Using *in vitro* and *in vivo* model systems, BDNF was identified as a positive modulator while NT3 was found to be a negative modulator of peripheral nerve myelination (Chan et al., 2001). The $p75^{NTR}$ and TrkC receptors were identified as the major mediators of neurotrophin activity during the myelination process (Cosgaya et al., 2002). The myelin-promoting effect of BDNF is mediated by $p75^{NTR}$. On the other hand, NT3 mediates its myelin-inhibitory effect via TrkC. Recently, $p75^{NTR}$ has been demonstrated to associate with the polarity protein, Par-3, which recruits $p75^{NTR}$ asymmetrically to the axon-glial junction to establish radial SC polarity for myelination (Chan et al., 2006).

NRG1 proteins also act as key instructive signals for myelination through the activation of PI 3-K activity (Taveggia et al., 2005). The amount of NRG1 determines both the myelin thickness and the ensheathment fate of axons (Michailov et al., 2004; Taveggia et al., 2005). Low expression of NRG1 type III is required for nonmyelinating SCs to ensheath several small axons, whereas high levels of NRG1 type III is essential for myelinating SCs to myelinate large axons. Above the threshold to trigger myelination, the amount of myelin formed is proportional to the amount of NRG1 type III presented by the axon to the SC.

1.4 Laminins in SC development

Laminins are heterotrimeric glycoproteins composed of an α-, β-, and γ-chain. Five α-, four β- and three γ-chains have been identified, and 15 isoforms have been observed (Yin et al., 2003). Laminins play at least three overlapping roles in mammals (Miner and Yurchenco, 2004): 1) they compose a major structural element of the basement membrane (Tabernero et al., 1998; Timpl, 1996; Yurchenco et al., 2004); 2) they provide attachment sites for cells via cell surface proteins (e.g., dystroglycan) (Henry and Campbell, 1996); and 3) they act as ligands for receptors on cells (e.g., integrins), thereby initiating signals that influence cell behavior and survival (Schwartz, 2001). Laminins are present in many tissues including the central nervous system (Grimpe et al., 2002; Hagg et al., 1989; Indyk et al., 2003), the neuromuscular junction (Noakes et al., 1995; Patton et al., 1998; Patton et al., 2001; Sanes and Lichtman, 2001) and peripheral nerves (Doyu et al., 1993; Patton et al., 1997) (Fig. 2). The function of

laminins in the nervous systems is varied; they play a role in neurite outgrowth, axon pathfinding (Luckenbill-Edds, 1997), brain development (Colognato and Yurchenco, 2000; Liesi et al., 2001; Miner et al., 1998), pathology (Murtomaki et al., 1992), and PNS development (Chen and Strickland, 2003; Podratz et al., 2001; Yang et al., 2005; Yu et al., 2005).

There is substantial evidence that SCs require the formation of an organized basal lamina to properly ensheath and myelinate axons (Bunge, 1993). Laminins are major components of the basal laminae that appear to be especially important. *In vitro* studies using SC/neuronal co-culture have shown that laminin deposition is required for myelination (Fernandez-Valle et al., 1993; Fernandez-Valle et al., 1994; Podratz et al., 2001). The essential function of laminins in PNS development was discovered when a mutation in the α2 laminin gene was found to cause a peripheral neuropathy in both humans [Helbling-Leclerc, 1995 #455] and mice (Shorer et al., 1995; Sunada et al., 1995; Xu et al., 1994). Mutant mice have hypomelinated axons, where the naked axon bundles lack ensheathment and myelination, most obvious at the proximal region of the peripheral nerve (Bradley and Jenkison, 1973; Stirling, 1975). Also, the endoneurium basal lamina is disrupted, and nerve conduction velocity is reduced in these mutant mice (Rasminsky et al., 1978). Furthermore, there are changes in the nodes of Ranvier (for review see Feltri and Wrabetz, 2005). All these results suggest that laminin is critical for SC development, but the mechanistic details are not clear.

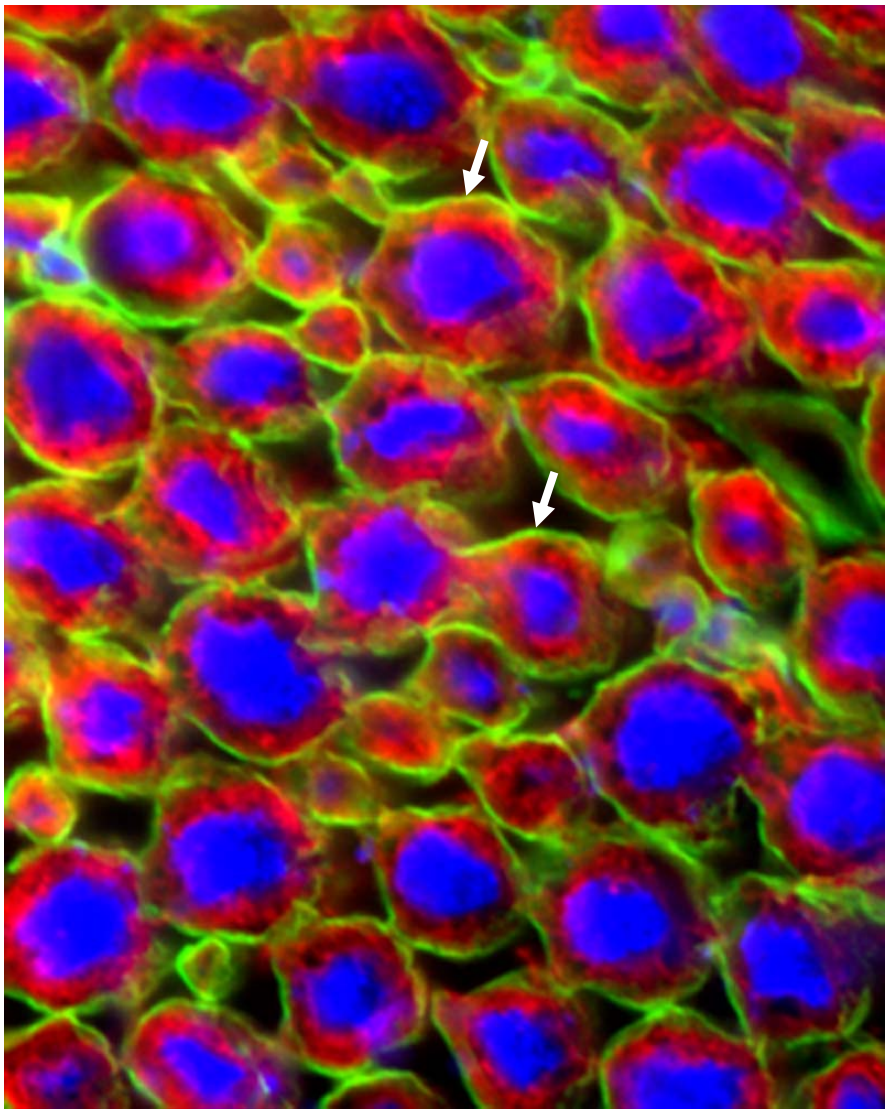


Figure 2. Triple immunofluorescent staining of mouse sciatic nerves.
Staining shows the normal relationship amongst laminins (green), myelin sheaths (red), and axons (blue) in the mouse peripheral nerve. The expression of laminin γ1 is abundant in the endoneurium outside Schwann cells (arrows).

1.5 Laminin receptors and cytoskeletal signaling in SC differentiation

The signaling effects of laminins are thought to be mediated primarily through integrins. Cell culture and *in vivo* studies have shown that interfering with β1 integrin function, a component of many laminin receptors, leads to inhibition of myelination (Feltri et al., 2002; Podratz et al., 2001; Relvas et al., 2001). *In vitro* work demonstrated that β1 integrin forms a multi-molecular complex with focal adhesion kinase (FAK), paxillin (an adaptor protein), and merlin/schwannomin when SCs begin to form the basal lamina and differentiate (Chen et al., 2000; Fernandez-Valle et al., 2002; Obremski et al., 1998). SCs lacking FAK are severely hypomyelinated with impaired axonal sorting (Grove et al., 2007). Phosphorylation of Schwannomin also leads to local reorganization of the SC cytoskeleton and the formation of a bioplor morphology characterized for differentiating SCs (Thaxton et al., 2007). Additionally, actin has been implicated in changes found in cell shape and in gene expression that is associated with SC differentiation (Fernandez-Valle et al., 1997). These studies suggest that laminins may influence SC differentiation by regulating these cytoskeletal signals.

Dystroglycan also functions as a laminin receptor (Previtali et al., 2001; Previtali et al., 2003). Mice lacking dystroglycan in SCs form myelin sheaths but the folding of myelin sheaths is abnormal (Saito et al., 2003). Mice deficient in a functional periaxin gene assemble compact myelin but the myelin sheath is unstable, which leads to a demyelinating phenotype (Gillespie et al., 2000). Dystroglycan forms a complex with dystrophin-related protein-2 and L-periaxin at the surface of myelin-forming SCs (Sherman et al., 2001). Specific disruption of

this complex in SCs results in a demyelinating neuropathy, suggesting that laminins may be linked to the SC cytoskeleton through this complex to stabilize the myelin sheath.

1.6 Disruption of laminin γ1 gene in SCs

Laminin γ1 is one of the most abundant chains as it is present in 10 of the 15 known isoforms (Grimpe et al., 2002), including all known laminin isoforms expressed in the PNS (laminin 2, 8, and 10) (Patton et al., 1997; Previtali et al., 2003). Given its widespread expression, global disruption of the *laminin γ1* gene causes lethality in mice at E5.5 (Smyth et al., 1999). Since SCs are first present at E12 in mice, this early lethality makes it impossible to analyze the effects of laminin on SC development and function in these mice (Jessen and Mirsky, 2005).

Therefore, in order to study the mechanism of laminin function in PNS development, mice were created that contain a *laminin γ1* gene with *loxP* recombination sites flanking the essential exon 2. The *laminin γ1* gene in these mice can be disrupted conditionally in various tissues by tissue-specific expression of Cre recombinase. Laminin γ1 was deleted from the nervous system using the calcium/calmodulin-dependent protein kinase II α promoter to drive Cre expression (referred to as *CaMKII/Cre:fLAMγ1* mice) (Chen and Strickland, 2003). The *laminin γ1* gene is disrupted in these mice during late embryonic stages in a subpopulation of SCs, causing a dysmyelinating phenotype. To specially disrupt laminins in SCs at their early development

stages, a SC-specific promoter (myelin protein zero, Mpz) is used to drive Cre expression. In transgenic mice, this promoter activates Cre expression specifically in SCs around E13.5-14.5 (Feltri et al., 1999) (Fig. 6). Since the laminin γ1 chain is a common component in all laminin isoforms expressed in SCs (Fig. 2), deleting laminin γ1 disrupts most if not all of the laminin isoforms in these cells. This particular study revealed novel functions of laminins in the PNS. SCs that lack laminins do not extend processes that are required for the radial sorting of axons. They have impaired interactions with axons, which leads to decreased proliferation and aberrant differentiation. These cells also have reduced PI 3-K signaling, which is needed to maintain viability, therefore leading to apoptosis.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mouse lines, genotyping and analysis of Cre-mediated *laminin γ1* gene recombination

Mice in which exon 2 of the *laminin γ1* gene was flanked by two loxP sites (*fLAMγ1*) were generated as described (Chen and Strickland, 2003). *mP$_0$TOT(Cre)* (*P$_0$Cre*) transgenic mice were described previously (Feltri et al., 1999). To obtain *P$_0$/Cre:fLAMγ1* mice, mice homozygous for the *fLAMγ1* allele were crossed with mice heterozygous for the *fLAMγ1* allele and hemizygous for the *P$_0$Cre* transgene. Genotypes of the resulting offspring were identified by PCR analyses of tail genomic DNA using the forward primer 5'-CTC AGA GCT GGC TTC TCA CAT-3' and reverse primer 5'-GAT TTT CAA AGA AGC AGA GTG TG-3'. The PCR conditions were as follows: 2 minutes at 94^{o}C; 35 consecutive cycles (94 ^{o}C for 45 seconds, 56 ^{o}C for 45 seconds, 72 ^{o}C for 3 minutes and 30 seconds), and 10 minutes at 72 ^{o}C. For detection of Cre-mediated *laminin γ1* gene recombination, genomic DNA was prepared from various tissues of *fLAMγ1/ fLAMγ1* (control) mice and *P$_0$/Cre:fLAMγ1* (mutant) mice and were analyzed by using the same primer pair and PCR conditions as described above. In some experiments, Cre recombinase activity was monitored using the LacZ/EGFP dual reporter mouse line (Novak et al., 2000).

2.2 Mouse Schwann cell/dorsal root ganglia (SC/DRG) neuronal co-cultures

DRG from E14 mice were isolated and dissociated as described (Kleitman et al., 1999). The dissociated explants (mostly neurons, with a few satellite SCs and fibroblasts) were dispersed and plated onto 25 mm collagen-coated coverslips and maintained in DMEM/F-12 (Invitrogen, Carlsbad, California) containing 5% FBS supplemented with 50 ng/ml NGF, 5 µg/ml insulin, 10 µg/ml mouse transferrin, 100 µM putrescine, 20 nM progesterone, and 30 nM sodium selenite (maintenance media) at a density of 25,000 cells/coverslip. The endogenous SCs were allowed to proliferate and populate axons for 10 days. SC/DRG neuronal co-cultures were infected with adenovirus expressing Cre recombinase (Microbix Biosystems, Toronto, Canada) or adenovirus expressing ß-galactosidase (Vector Biolabs, Philadelphia, PA) at a multiplicity of infection (MOI) of 20 for 2 days. Myelination was induced by the addition of fresh maintenance media containing 50 µg/ml ascorbic acid (myelinating promoting feed) with or without 25 µM exogenous mouse laminin (Invitrogen).

2.3 Immunohistochemistry and immunocytochemistry

Mice were euthanized at different ages by CO_2 asphyxiation. Sciatic nerves were removed from mice and embedded in Tissue-Tek OCT (Sakura, Torrance, CA), immediately frozen on dry-ice/methanol, and stored at −70°C. SC/DRG neuronal co-cultures were washed once with cold PBS and fixed in 4% paraformaldehyde. For immunostaining, 8 µm cryosections were prepared, fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 30 minutes, rinsed in

PBS, and then blocked in PBS containing 0.3% Triton X-100 and 5% normal goat serum. The primary antibodies used were diluted in PBS containing 0.3% Triton X-100, and 3% normal goat serum. Primary antibodies used were as follows: rat anti-laminin γ1 (Chemicon, Temecula, CA) (1:500), rat anti-laminin α1 (Chemicon) (1/500), rat anti-laminin β1 (Chemicon) (1/500), chicken anti-neurofilament H (Chemicon) (1/2000), rat anti-laminin α2 (Sigma, St. Louis, MO) (1/500), rabbit anti-laminin α4 (Santa Cruz Biotech, Santa Cruz, CA) (1/100), rabbit anti-laminin 1 (Sigma), rabbit anti-human MBP (Dako, Carpinteria, CA) (1/2000), rabbit anti-Oct6 (a gift from Dr. Meijer, Erasmus University, Rotterdam, The Netherlands) (1/200), rabbit anti-Krox-20 (Covance Research Products, Berkeley, CA) (1/50), rat anti-BrdU (Abcam, Cambridge, UK) (1/400), rabbit anti-S-100 (Sigma) (1/1000), rabbit anti-S100 (Swant, Bellinzona, Switzerland) (1/5000), rabbit anti-activated caspase-3 and -7 (Cell Signaling, Beverly, MA) (1/100). The samples were incubated with primary antibodies at 4^{o} C overnight. After rinsing with PBS, the samples were incubated with the appropriate Fluorescein-, Rhodamine Red-X-, Coumarin AMCA-, or Cy5- conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) (1/1000) for 1h at room temperature. After washing, the sections were mounted in Vectashield® mounting medium with or without DAPI (Vector Laboratories, Burlingame, CA), examined under an Axioskop 2 fluorescent microscope (Carl Zeiss USA, Thornwood, NY) equipped with appropriate filters and photographed with the AxioVision System (Carl Zeiss). For laser scanning confocal microscopy, cells were imaged with a Zeiss LSM 510 system (Carl Zeiss

Microscopy). The Argon 458 nm, 488 nm, and 514 nm and HeNe 543 nm, and 633 nm were switched on and set at 75% output intensity. The multitrack standard DAPI/FITC/rhodamine/Cy5 was selected. Pinhole diameter was maintained as 1 Airy Unit. A Plan-Apochromat 63X or 100X objective was used to collect 35 Z-stacks (0.2 μm per stack) for each sample. Images with maximum projection were obtained using LSM Image Examiner (Carl Zeiss).

2.4 Semithin and electron microscopic analyses

Mice at different ages were anesthesized, and the sciatic nerve was exposed. The nerve was immersed in 3% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.2. The nerve was then dissected, immersed in 3% glutaraldehyde in 0.1 M PB for 24 h, and postfixed in 2% osmium tetroxide solution. SC/DRG neuronal co-cultures were washed with cold PBS, fixed in 2.5% paraformaldehyde/glutaraldehyde, and postfixed in 2% osmium tetroxide solution. The nerves or cell layers were embedded in resin. Semi-thin sections were cut and stained with Richardson's staining to stain proteins. For electron microscopy (EM) analysis, ultra-thin sections were cut on a Reichert-Jung Ultracut E microtome and poststained with uranyl acetate and lead. Sections were examined and photographed on a JEOL100CXII at 80 kV.

2.5 BrdU incorporation assay

Female mice heterozygous for the *fLAMγ1* allele and hemizygous for the *mP$_0$TOT(Cre)* transgene were mated with male mice homozygous for the *fLAMγ1* allele. The time of detection of a vaginal plug was counted as 0.5 day post-coitum (dpc). Pregnant mice at 13, 15.5, 17.5, 19.5 dpc and P5 mice were injected intraperitoneally with 100 μg BrdU/g body weight. One hour later, the embryos of pregnant mice or the sciatic nerves of P5 mice were dissected and frozen in dry ice, and the upper bodies or tails were used for genotyping. Whole embryos (E13, E15.5, or E17.5) or sciatic nerve tangential cryosections (E17.5, E19.5, or P5) were prepared, fixed in cold methanol, denatured with 2N HCl for 20 min at 37 ^{o}C, and neutralized in 0.1M sodium borate, pH 8.5, for 10 min. Sections were co-incubated with rat anti-BrdU (Abcam) and rabbit anti-neurofilament H (Chemicon) antibodies. After staining with the appropriate secondary antibodies, the nuclei were counterstained with DAPI. Only cigar-shape nuclei inside the nerve tissues were counted, and double-labeled nuclei (both BrdU and DAPI) were determined. At each time point, six control and six mutant mice were analyzed. The differences in percentage of BrdU incorporated nuclei between control and mutant nerves were analyzed by two-tailed Student's t-Test.

2.6 TUNEL assay

TUNEL was performed using the *In Situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, Indiana) according to the manufacturer's instruction.

Whole embryo (E15.5) or sciatic nerve tangential cryosections (P0 to adult) were prepared, fixed in 4% PFA in PBS, pH 7.4, for 20 min, and permeabilized in 0.1% Triton X-100/0.1% sodium citrate on ice for 2 min. E15.5 embryo sections were stained with anti-neurofilament antibodies to identify nerves. P15 sciatic nerve sections were stained with anti-S100, anti-laminin γ1 or, anti-phosphoAkt antibodies, and nuclei were counterstained with DAPI. Double-labeled nuclei were determined. At each time point, six control and six mutant animals were analyzed. The differences in percentage of TUNEL positive nuclei in control and mutant sciatic nerves were analyzed by two-tailed Student's t-Test.

2.7 Western blot analysis

Control and mutant mice at different ages were anesthetized. The sciatic nerves were exposed, immersed in PBS containing the phosphatase inhibitor Cocktail I and II (Sigma), and dissected. The nerves (including perineurium) were homogenized in 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, phosphatase inhibitor Cocktail I and II and protease inhibitor Cocktail (Sigma). Extracts from co-cultures were obtained using the same lysis buffer. Protein concentrations were determined by the Lowry method (Bio-Rad, Hercules, CA). For immunoblotting of caspase-9, total proteins of sciatic nerves were extracted in CHAPS cell extract buffer (Cell Signaling) containing protease inhibitors. Proteins (15-20 μg) were fractionated on 4-15% SDS-PAGE, blotted onto PVDF membrane (Millipore, Billerica, MA), and probed with primary antibodies.

Antibodies against p-ErbB2, ErbB2, p-ErbB3, p-Akt, Akt, p-GSK-3β, GSK, and mouse caspase-9 were purchased from Cell Signaling and used at 1:1000 dilution. Other antibodies used were anti-Oct6 (Dr. Meijer), anti-ErbB3 (Abcam) (1:200), anti-MPZ (Dr. Archelos, Graz, Austria) and anti-β-actin (Sigma) (1:8000). After incubation with appropriate secondary antibodies (Amersham Biosciences, Piscataway, NJ), proteins were visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL).

2.8 Injections of laminin peptides

Injections were performed as previously described (Cosgaya et al., 2002). The laminin peptide EIKLLIS derived from the laminin α1 chain (conserved in laminin α2 chain) and a control peptide with a scrambled amino acid sequence ILEKSLI (Tashiro et al., 1999) were purchased from American Peptide Co. (Sunnyvale, CA). Laminin peptides (5 μg) were injected subcutaneously, starting from the caudal portion of the greater trochanter region and running parallel along the mutant sciatic nerves. The contralateral sciatic nerve served as the control and was injected with control peptides. Injections were performed on postnatal day 1 and reinjected two days later for control and mutant mouse pups (20 of each genotype). Nerves were extracted at postnatal day 5 (four days of total treatment), and 20 μg proteins were used for Western blot analysis.

2.9 Generation of recombinant adenoviruses

cDNAs encoding eGFP-tagged β-actin (Clontech, Mountain View, California) were subcloned using polymerase chain reaction (PCR)-mediated cloning into the *Sal*I and *Not*I sites of pShuttle-CMV (Stratagene, La Jolla, California). mCherry and mouse neurofilament light chain cDNAs (Invitrogen) were inserted into the *Bgl*II, *Sal*I, and *Not*I sites of pShuttle-CMV using PCR-mediated cloning, resulting in cDNAs encoding mCherry-tagged neurofilament light chain. All constructs were verified by automatic sequencing. Recombinant adenoviruses were produced using the AdEasy XL Adenoviral Vector System (Stratagene) according to the manufacturer's protocol.

2.10 Time-lapsed live cell imaging

SC/DRG neuronal co-cultures were plated in glass bottom dishes (Mattek, Ashland, Massachusetts) coated with collagens and infected with adenovirus expressing mCherry-tagged neurofilament light chain at the time of plating. After incubated for 10 days, co-cultures were infected with adenovirus expressing eGFP-tagged β-actin for 2 days and then switched to myelinating promoting feed. Only infected SCs expressing low levels of eGFP were chosen for imaging. Cells were imaged with a Carl Zeiss Axiovert 200M equipped with a 63×, 1.4-NA, Plan Apochromat objective. Imaging chamber was maintained at 37°C and 5% CO_2 with the aid of a microscope incubator system (Solent Scientific Limited, Segensworth, UK). A PerkinElmer Wallac UltraView confocal head with 488- and 568- nm excitation filter and Orca ER cooled CCD camera (Hamamatsu, Bridgewater, NJ) were used for high-resolution imaging. *Z*-stacks were collected

(1μm per *z*-stack, 7−8 z-stacks) every five mins during imaging. Images were processed and analyzed using MetaMorph (Molecular Devices, Downingtown Pennsylvania). Cells were usually imaged for 24 hours.

2.11 Statistical and imaging analysis

Images of immunostained sections were acquired by using an AxioVision System (Carl Zeiss) and processed with Photoshop (Adobe, San Jose, California). The electron micrographs and Western blot films were digitized using a scanner (Microtek, Carson, California). The signal intensity of the Western blot film was quantified by NIH Image. All statistical analyses were performed by Excel software (Microsoft, Redmond, Washington).

CHAPTER 3: RESULTS

3.1 Generation of mice lacking laminin γ1 specifically in SCs

To specifically disrupt the *laminin γ1* gene in SCs, mice were created to be homozygous for a floxed *laminin γ1* allele (*fLAMγ1*) (Chen and Strickland, 2003) and carry a P_0*Cre* transgene, *m*P_0*TOT(Cre)* (Fig. 3), which activates Cre-mediated recombination specifically in SCs between E13.5 and 14.5 (Feltri et al., 1999; Feltri et al., 2002). P_0*/Cre:fLAMγ1* mice (referred to as "mutant" mice hereafter) were born normally in accordance with the predicted Mendelian ratio. However, they showed tremor and progressive hind limb paralysis during early postnatal stages. By the end of the fourth week, they had complete hind limb paralysis (Fig. 4) and most did not live past two months due to emaciation.

To determine the specificity of Cre-mediated *laminin γ1* gene recombination, genomic DNA from various tissues was analyzed by PCR (Fig. 5A). Recombination only occurred in the mutant peripheral nerves (e.g., the sciatic nerve) and not in other tissues of mutant mice or in mice homozygous for a *fLAMγ1* allele but without the *cre* transgene (f/f, referred to as "control" mice hereafter). To confirm the elimination of laminin γ1 expression in mutant peripheral nerves, transverse sciatic nerve sections at postnatal day 0 (P0) were stained for laminin γ1. Laminin γ1 was present in control nerves, but was absent in mutant nerves in the endoneurium surrounding SCs; in mutant nerves, laminin γ1 was still present in blood vessels and the perineurium (outer region), where it is presumably produced by fibroblasts (Fig. 5B). Further analyses showed that

laminin γ1 gene was disrupted around E13.5 to 14.5 (Fig. 6), and the protein expression of most laminin chains known to be expressed in SCs was not detectable (α1, α2, α4, β1, γ1; Fig. 7). These results demonstrate that the *laminin γ1* gene was specifically disrupted in SCs around E13.5 to 14.5, resulting in the depletion of all laminins.

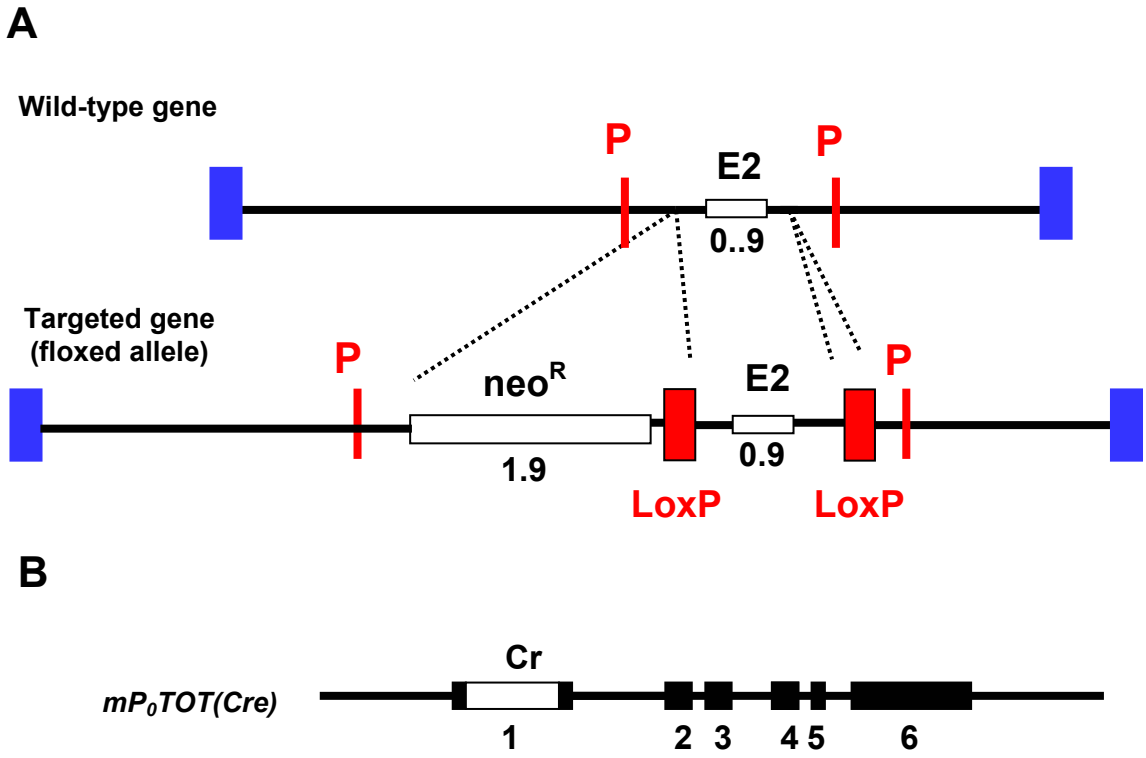


Figure 3. Generation of mice with Schwann cell-specific disruption of laminin γ1.
(A) Laminin γ1 targeting construct. A fragment containing exon 2 was used. The two loxP sites were inserted into exon 2 of the *laminin γ1* gene and is used to excise exon 2. P1 and P2 denote the primer sites used to genotype and monitor recombination. (B) *P0-Cre* transgene, *mP0TOT(Cre)*, was created by inserting the *cre* gene into exon 1 of the *mpz* gene.

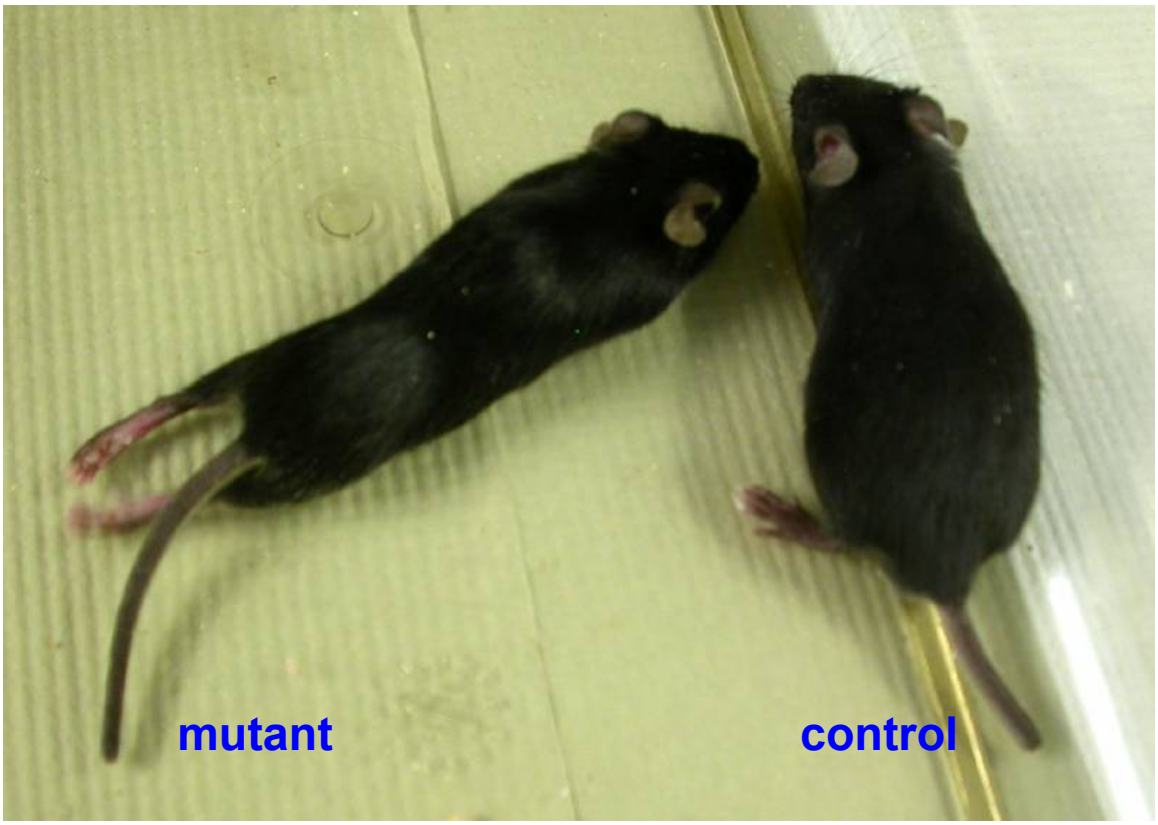


Figure 4. Mutant mice show motor defects.
The control mouse on the right side is homozygous for a *floxed laminin γ1* allele (*fLAM γ1*). The mutant mouse on the left is homozygous for a *floxed laminin γ1* allele and carries a P_0*Cre* transgene (P_0*/Cre:fLAM γ1*, mutant). The mutant mouse is smaller than control mice and has complete hind limb paralysis.

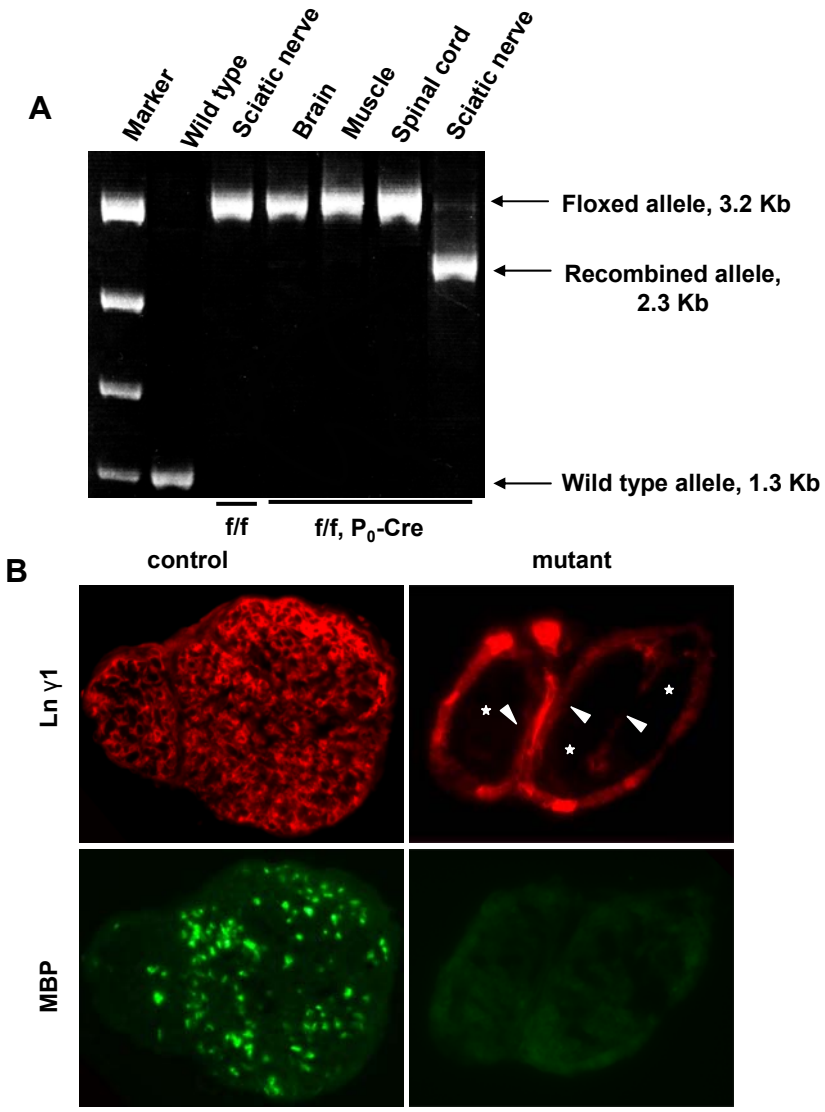


Figure 5. Schwann cells lacking laminin γ1 expression exhibit severe hypomyelination.
(A) PCR analysis of genomic DNA from various tissues of wild type, homozygous *fLAM γ1* mice (*f/f*; control), and *P0/Cre:fLAM γ1* mice (*f/f, P0-Cre*; mutant). The primers used amplified the wild type (1.3 kb), unrecombined (3.2 kb), and recombined (2.3 kb) *fLAM γ1* alleles. (B) Transverse sections of control and mutant sciatic nerves at postnatal day (P)0 were double stained for laminin γ1 (red) and MBP (green). In the mutant nerve, laminin γ1 expression was absent in the endoneurium (asterisks) and only remained in the perineurium (arrowheads). MBP expression was not detected.

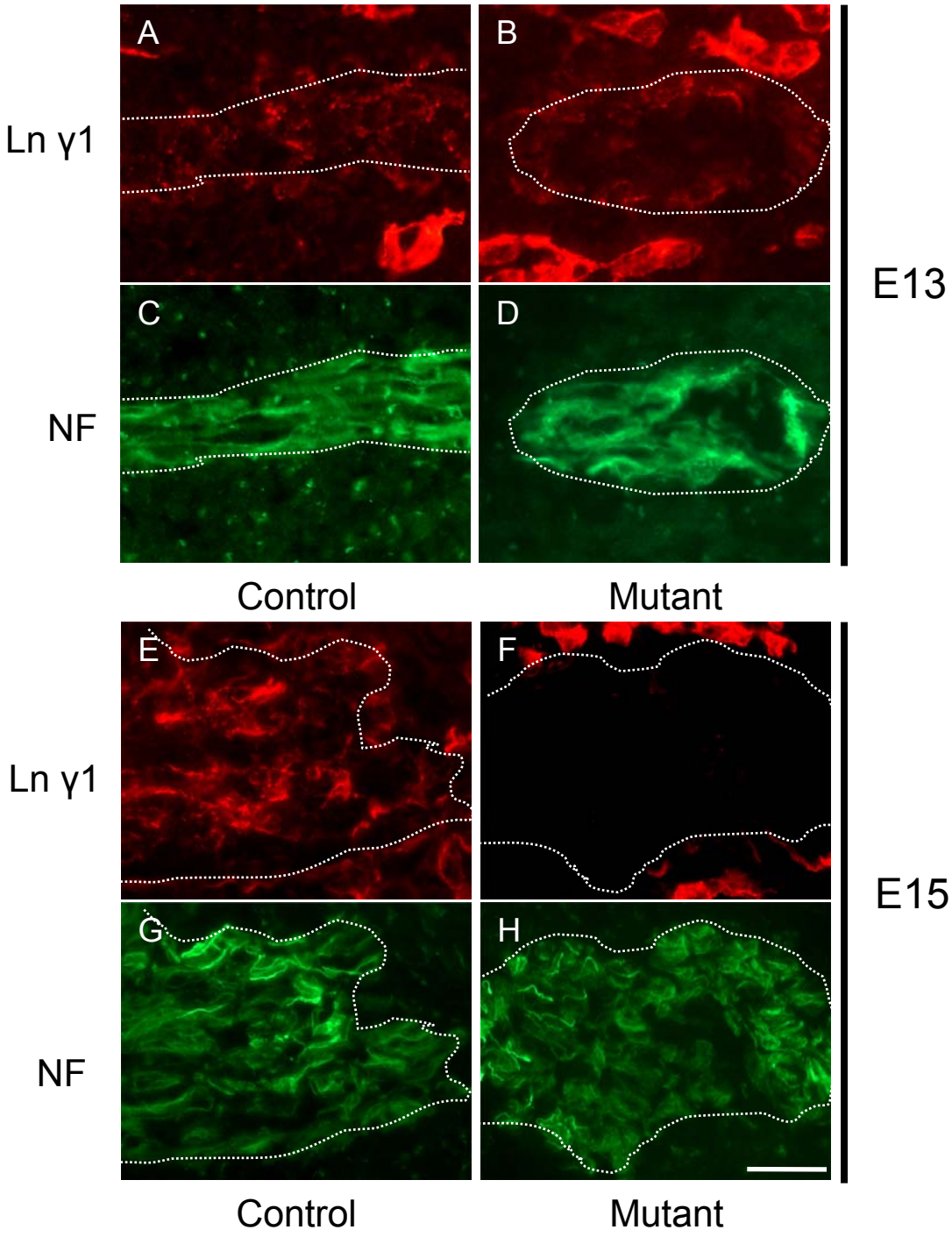


Figure 6. mP0TOT(Cre) activates Cre-mediated *laminin γ1* gene disruption specifically in the PNS between embryonic day (E) 13.5 and 14.5.
Co-immunostaining of laminin γ1 (A, B, E, and F; red) and neurofilament (C, D, G, and H; green) of whole embryo sections from control (A , C, E, and G) and mutant (B, D, F, and H) mice at E13 (A-D) and E15 (E-H) revealed that the expression of laminin γ1 was similar in control and mutant nerves at E13 but greatly reduced in mutant peripheral nerves at E15.

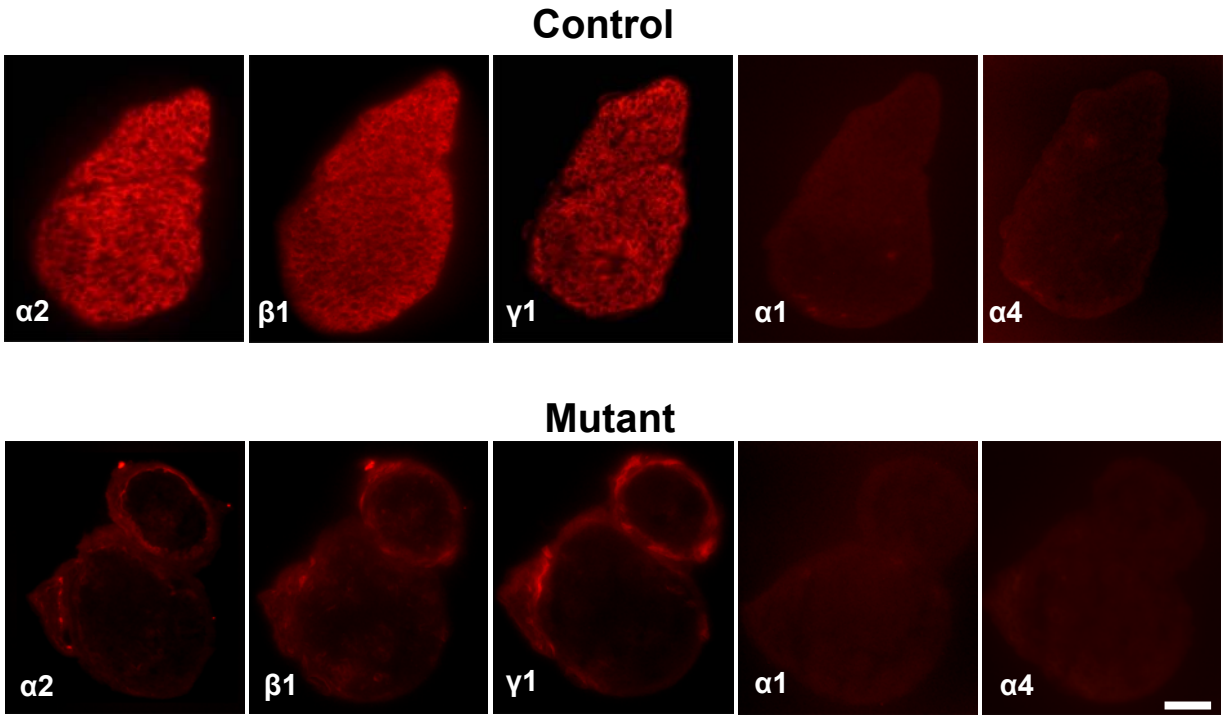


Figure 7. Disruption of *laminin γ1* gene expression resulted in depletion of all other laminin chains known to be expressed in Schwann cells.
Adjacent transverse sections of control and mutant sciatic nerves at P0 were stained for different laminin subunits, including α1, α2, α4, β1 and γ1. In the mutant nerves, disruption of *laminin γ1* gene expression resulted in concurrent depletion of other laminin chains. Note that the laminin α1 chain, which is expressed in the perineurium in mature nerves, and the laminin α4 chain, which is expressed at low level in adult nerves, are both nearly undetectable at the P0 stage. Bar=25 μm.

3.2 Mutant SCs are severely hypomyelinated and fail to extend processes to initiate radial sorting of axons

The onset of myelination was compared between mutant and control sciatic nerves at P0 by staining transverse sections for myelin basic protein (MBP), a myelin marker. In control nerves, MBP was expressed normally, whereas no MBP expression could be detected in mutant nerves (Fig. 5B). At late postnatal stages (P28), myelination was complete in control nerves (Fig. 8A). In contrast, mutant sciatic nerves showed large unsorted axonal bundles and only a few SCs with normal myelin sheaths (arrows in Fig. 8A). In mice lacking β1 integrin in SCs, perineurial cells are abnormally located along microfasciculations in the center of the nerve (Feltri et al., 2002). In agreement with this observation, laminin γ1 expression was not detectable in the endoneurium surrounding mutant SCs before P5 (Fig. 5B and Fig. 7) but appeared at later stages in the endoneurium of some centrally located SCs in mutant sciatic nerves (P28, Fig. 8*A*). Therefore, these centrally located SCs with normal myelin sheaths in mutant nerves might obtain laminin from nearby perineurial cells or escape recombination of the *laminin γ1* gene and thus undergo normal differentiation**.** To address this question, mutant mice were crossed with an EGFP reporter mouse line Z/EG (lacZ/EGFP) (Novak et al., 2000). Mice that were obtained were homozygous for the floxed laminin γ1 allele and were also hemizygous for both P_0-Cre transgene and the Z/EG reporter transgene (Fig. 9). In mice carrying the reporter gene, upon Cre mediated recombination, EGFP was expressed. The sciatic nerves from these mice were analyzed at P28; the few SCs that had

laminin γ1 surrounding them expressed the reporter gene EGFP. This result indicated that these SCs have undergone Cre-mediated laminin γ1 gene recombination since the reporter gene was expressed, but they have obtained laminin γ1 from other cells (see Fig. 9). However, it is also possible that some SCs might escape Cre-mediated laminin γ1 gene recombination or undergo incomplete (monoallelic) recombination.

The morphology of control and mutant SCs was compared at P1 and P28 by EM. In control sciatic nerves at P1, most SCs had extended processes to segregate axons and some axons had formed a 1:1 relationship with individual SCs (Fig. 8B). In contrast, mutant SCs at P1 did not extend processes (arrows in Fig. 8B) and left axons unsorted. Ultrastructural analysis of mutant sciatic nerves at late postnatal stages showed that SCs closely associated with unsorted axonal bundles (SC in Fig. 8C) lacked a continuous basal lamina (compare fuzzy materials indicated by arrows and denuded areas indicated by arrowheads in Fig. 8D), and did not extend their cytoplasmic processes between axons, thus failing to segregate, interact, and myelinate axons.

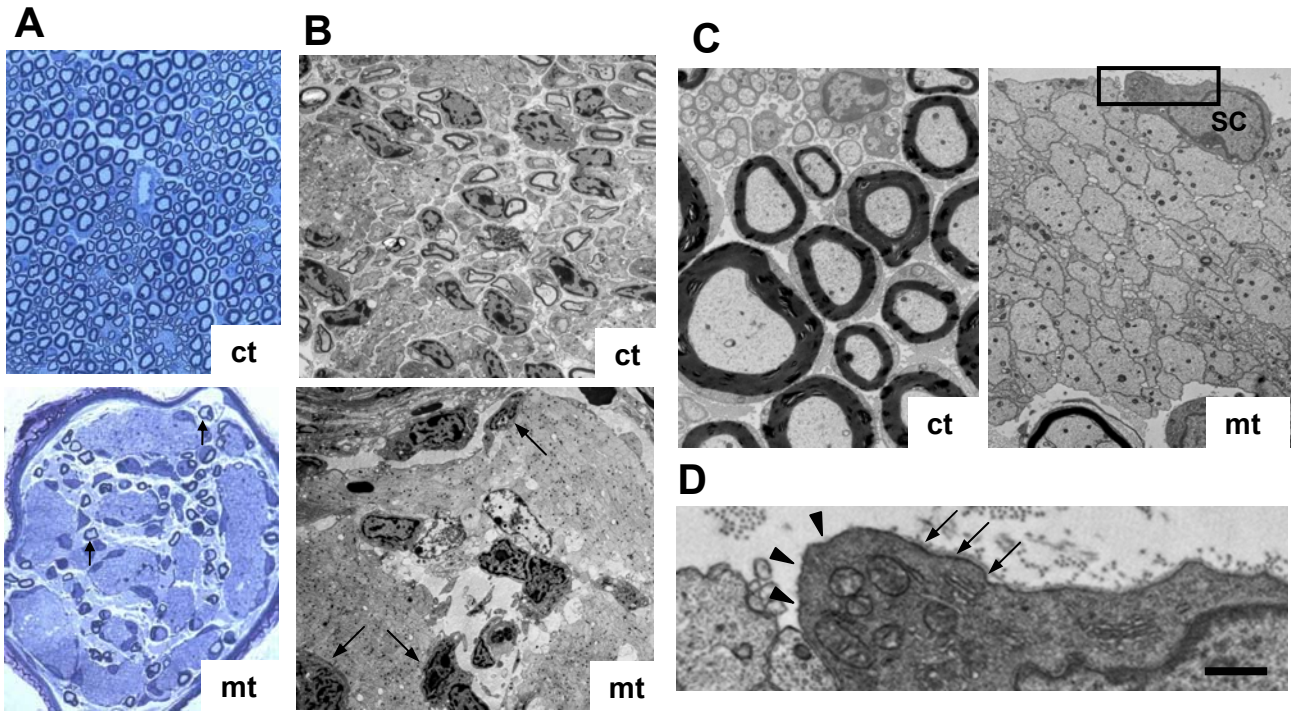


Figure 8. Mutant sciatic nerves show severe hypomyelination and impaired axonal sorting.
(A) Transverse semi-thin sections from P28 control (ct) and mutant (mt) sciatic nerves show that mutant nerves have large unsorted axonal bundles and few Schwann cells with myelin sheaths near the perineurium (arrows). (B) Electron micrographs of P1 control (ct) and mutant (mt) sciatic nerves show that mutant Schwann cells (arrows) do not extend cytoplasmic processes and leave axons unsorted. (C) Electron micrographs of P28 control (ct) and mutant (mt) sciatic nerves show that mutant nerves have large bundles of unsorted axons with some Schwann cells located outside. (D) Higher magnification of the boxed region in (C) shows that the mutant Schwann cell closely associated with unsorted axonal bundles lacks a continuous basal lamina (compare fuzzy materials indicated by arrows and denuded areas indicated by arrowheads) and does not extend processes between axons. Bar= (A) 18.5 μm; (B) 5.6 μm; (C) 1.7 μm; (D) 0.3 μm.

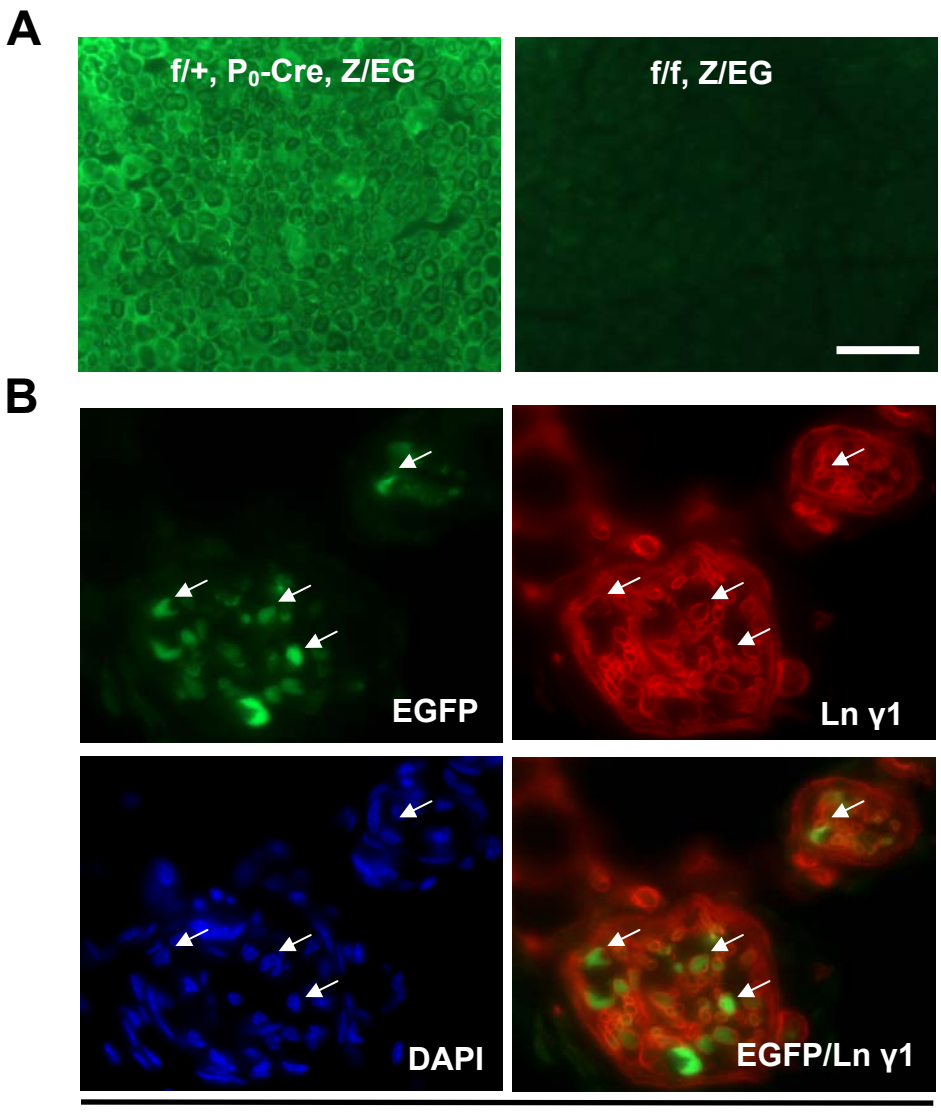


Figure 9. Schwann cells with laminin γ1 surrounding them in mutant sciatic nerves express Cre.

Mice heterozygous for the *fLAMγ1* allele and hemizygous for the *P_0Cre* transgene were crossed with mice homozygous for the *fLAMγ1* allele and hemizygous for a Cre reporter transgene, *Z/EG* (*lacZ/EGFP*), to obtain *P_0/Cre:fLAM γ1//Z/EG* mice (*f/f, P_0-Cre, Z/EG*) mice, *P_0/Cre:fLAM γ1/+//Z/EG* mice (*f/+, P_0-Cre, Z/EG*, positive control), and *fLAM γ1//Z/EG* mice (*f/f, Z/EG*, negative control). This Z/EG Cre reporter transgene activates expression of EGFP upon Cre-mediated gene recombination. (A) Transverse sciatic nerve sections from *P_0/Cre:fLAM γ1/+//Z/EG* mice and *fLAM γ1*//Z/EG mice at P28 served as positive (*f/+, P_0-Cre, Z/EG*) and negative controls (*f/f, Z/EG*) for Cre-mediated EGFP expression. (B) Transverse sections of sciatic nerves from *P_0/Cre:fLAM γ1//Z/EG* mice at P28 were stained for laminin γ1 (red) and counterstained with DAPI (blue). EGFP expression was directly visualized under fluorescent microscopy. Some Schwann cells in the sciatic nerve of the mutant mice that also carry the reporter gene have laminin γ1 surrounding them and express EGFP by Cre-mediated recombination (arrows). This result indicates that these Schwann cells expressed Cre but may have obtained laminin γ1 from other cellular sources. Bar= 2 μm.

3.3 Mutant SCs show aberrant differentiation and arrest in premyelinating stages

Upon the deposition of the basal lamina, the essential step for SC differentiation is the up-regulation of the transcription factor, Oct-6 (Bermingham et al., 1996; Jaegle et al., 1996). Oct-6 function is required in promyelinating SCs for timely differentiation into myelinating SCs (Jaegle et al., 1996; Jaegle and Meijer, 1998). Krox-20 is another transcription factor essential for SC myelination, which is expressed continuously and specifically in myelin-producing SCs (Topilko et al., 1994). Oct-6 and Krox-20 cross-regulate each other and act in the same genetic cascade (Nagarajan et al., 2001; Zorick et al., 1999). Oct-6 is required for timely expression of Krox-20. High level expression of Krox-20, in turn, is required for the down-regulation of Oct-6 after the peak of myelination and for the activation of the major myelin genes. Oct-6 and Krox-20 expression were examined in mutant peripheral nerves at different developmental stages to detect whether their expression was altered by laminin deficiency. In both control and mutant peripheral nerves, Oct-6 was observed at a low level around E15 (Fig. 10A), became readily detectable at E17.5, and peaked around P3, even in the absence of laminin γ1 (Fig. 11A). During postnatal development, most control SCs gradually down-regulated Oct-6 (Fig. 11B, control panel and Fig. 11D); however, mutant SCs were unable to down-regulate Oct-6, indicating that these SCs were arrested at the premyelinating stage (Fig. 11, B,D). Krox-20 was detected at very low levels around E17.5 in both control and mutant nerves (Fig. 10B). At E19.5, normal Krox-20 expression was observed in the absence of laminin γ1 (Fig. 11C).

In contrast, during postnatal development, when control SCs progressively up-regulated Krox-20, only a few SCs in mutant nerves expressed high levels of Krox-20 (Fig. 11C), indicating that up-regulation of Krox-20 is impeded in mutant SCs.

Taken together, these results suggest that laminins are not required for the induction of Oct-6 and Krox-20, but are critical for the maintenance of Krox-20 expression and ensuing down-regulation of Oct-6, and are therefore necessary for differentiation of myelinating SCs. The maintenance of Krox-20 expression requires continuous axonal signals (Jessen and Mirsky, 2002). Therefore, the failure of mutant SCs to maintain Krox-20 expression also suggests that these cells have impaired axon-SC interactions.

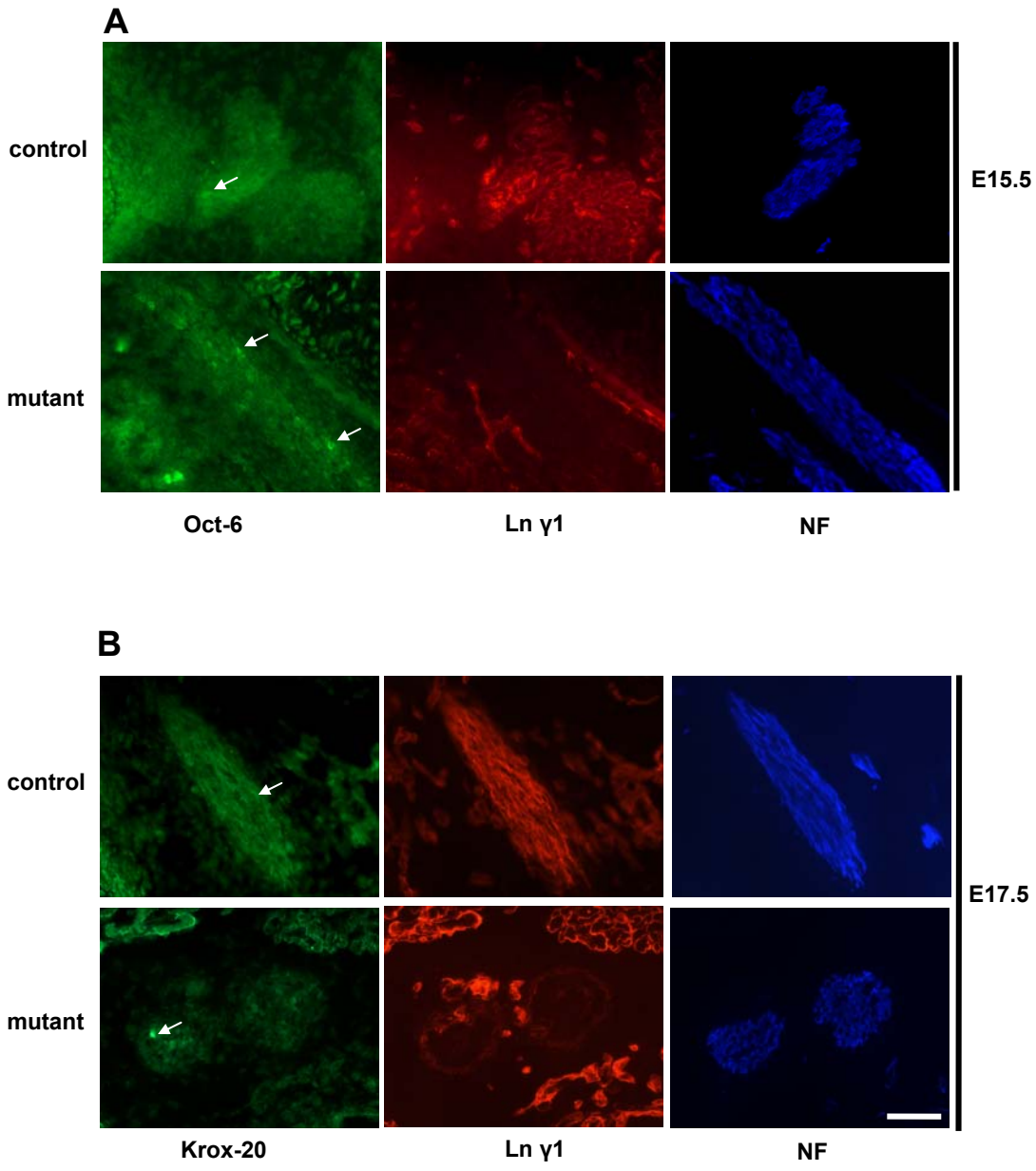
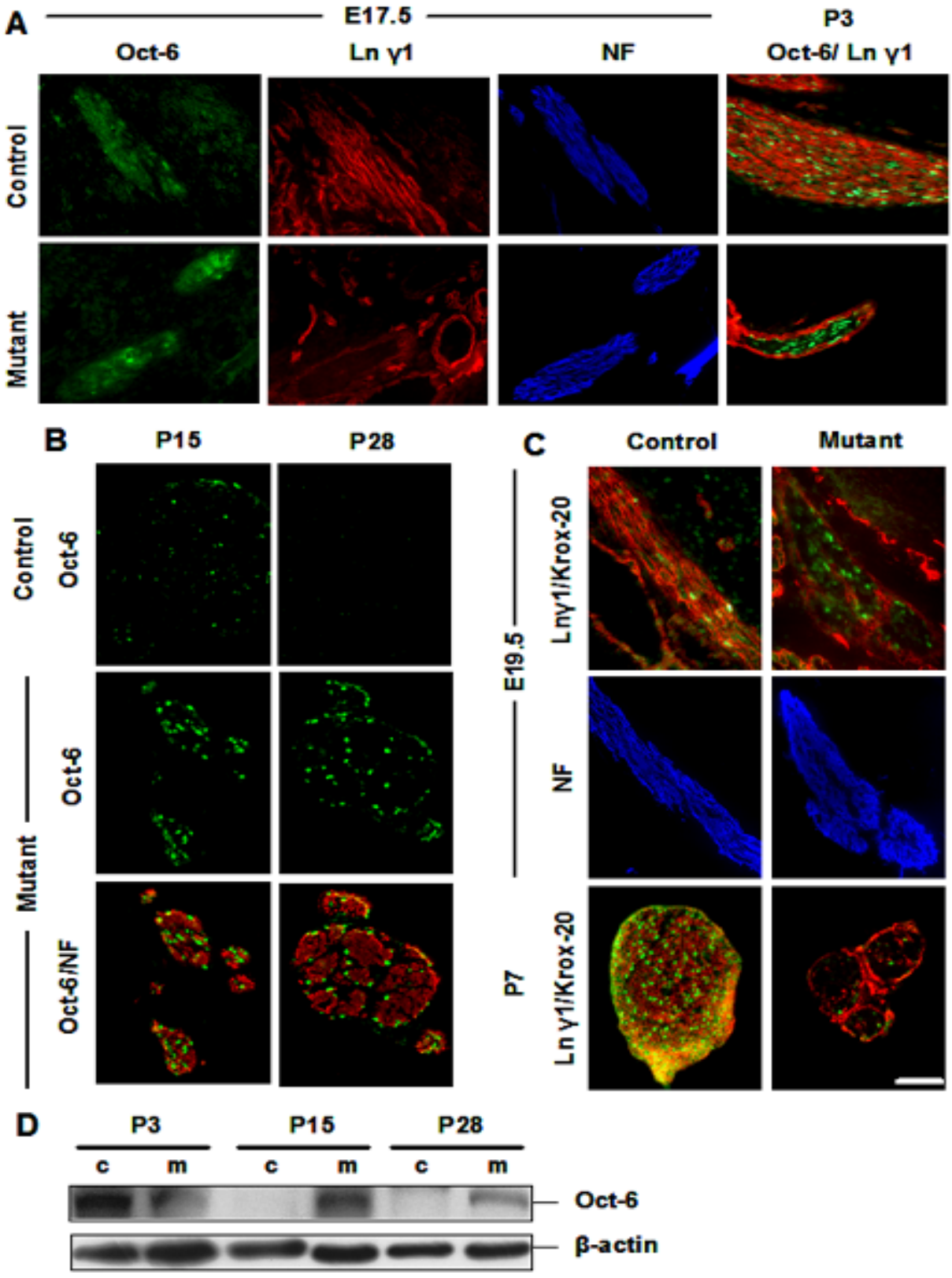


Figure 10. Expression of Oct-6 and Krox-20 in embryonic mouse peripheral nerves.
(A) Whole embryo sections at E15.5 were stained for Oct-6 (green), laminin γ1 (red), and neurofilament (blue). Oct-6 expresses at low levels in both control and mutant nerves at this time point (arrows). (B) Whole embryo sections at E17.5 were stained for Krox-20 (green), laminin γ1 (red), and neurofilament (blue). Low levels of Krox-20 expression can be detected in both control and mutant nerves at this stage (arrows). Bar= 50 μm.

Figure 11. Mutant Schwann cells fail to up-regulate Krox-20 and down-regulate Oct-6.
(A) Whole embryo sections at E17.5 and longitudinal sciatic nerve sections at P3 were stained for Oct-6 (green) and laminin γ1 (Ln γ1; red), and these images were merged. E17.5 embryo sections were stained for neurofilament (blue) to identify nerves. These images indicate that initiation of Oct-6 does not require laminin γ1. (B) Transverse control and mutant sciatic nerve sections at P15 and P28 were stained for Oct-6 (green) and neurofilament (red), and the images from mutant nerves were merged. During postnatal development, Oct-6 fails to down-regulate in mutant Schwann cells. (C) Whole embryo sections at E19.5 and transverse sciatic nerve sections at P5 were stained for Krox-20 (green) and laminin γ1 (red), and the images of Krox-20/ laminin γ1 were merged. E19.5 embryo sections were stained for neurofilament (blue) to identify nerves. Although initiation of Krox-20 does not require laminin γ1, high level expression of Krox-20 in Schwann cells is impaired during the postnatal development. Bar= 50 μm. (D) Oct-6 expression in control and mutant sciatic nerves at P3, P15, and P28 (n=15 per genotype per age) was assessed by Western blotting with antibodies against Oct-6. β-Actin was the loading control. Mutant Schwann cells were unable to down-regulate Oct-6 and showed aberrant consistent expression of Oct-6. (c: control; m: mutant).


A
E17.5
P3
Oct-6
Ln γ1
NF
Oct-6/ Ln γ1
Control
Mutant
B
P15
P28
Control
Oct-6
Mutant
Oct-6
Oct-6/NF
C
Control
Mutant
E19.5
Lnγ1/Krox-20
NF
P7
Ln γ1/Krox-20
D
P3
P15
P28
c
m
c
m
c
m
Oct-6
β-actin

3.4 Mutant sciatic nerves contain fewer SCs

By gross observation, mutant sciatic nerves were smaller and thinner than controls (Figs. 12A and B), and this could be due to reduced total cell number inside mutant nerves. Transverse sections from similar distal parts of mutant and control nerves were stained with DAPI at various ages to measure the total number of nuclei (Figs. 12C, D, and E). The cell number in mutant sciatic nerves compared to control was diminished to 43% at P0, 28% at P5, and 33% at P28 (Fig. 12E).

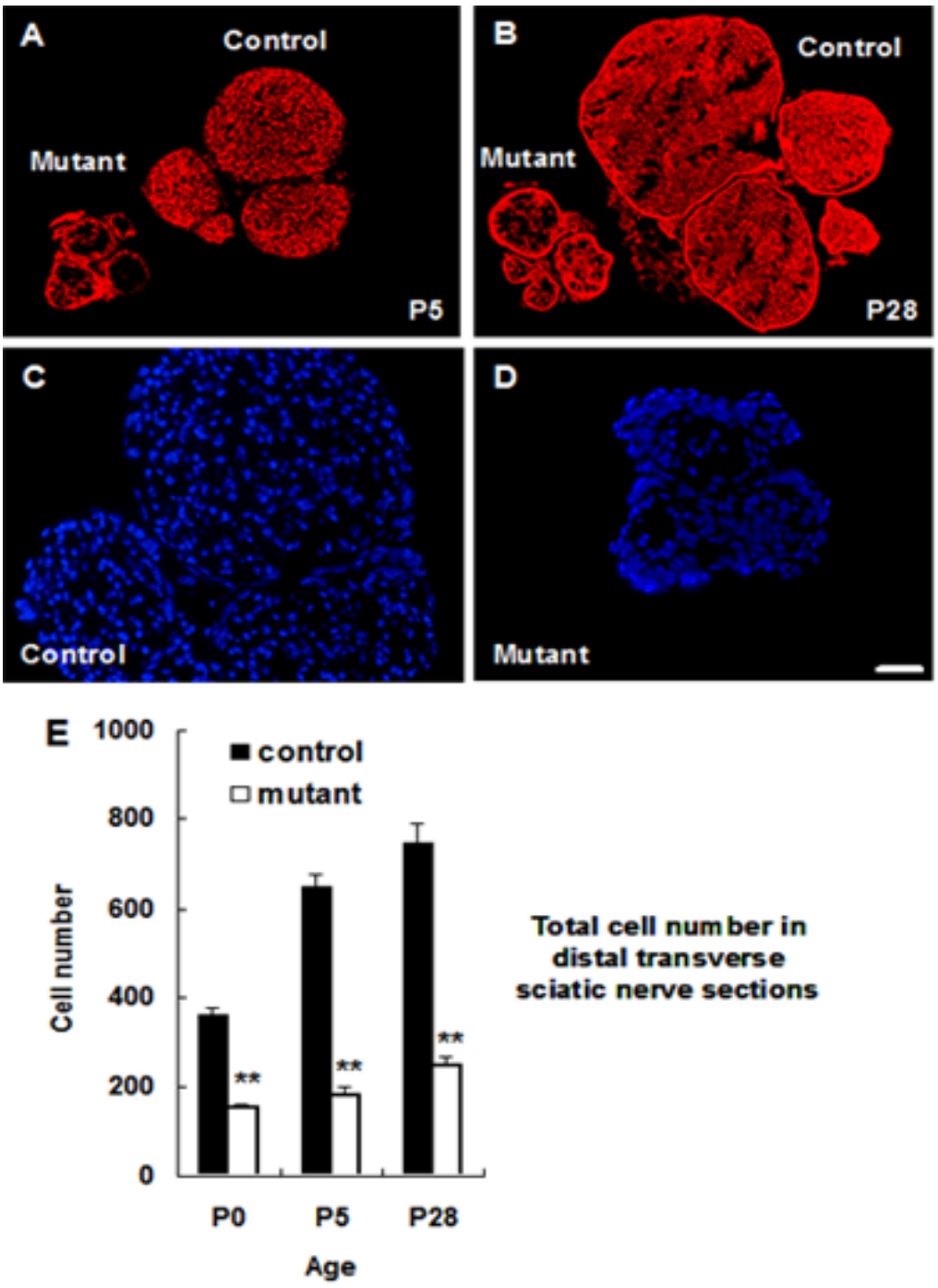


Figure 12. The mutant sciatic nerves are smaller and show decreased cell number.

Transverse sections of nerves at P5 (A, C and D) and P28 (B) were stained with DAPI (C and D, blue) to visualize individual cells and laminin γ1 (A and B, red) to show the size of nerves. At P5, the mutant nerve was smaller than control nerve (A), and the difference of size was greater at P28 (B). (E) Transverse sections of the distal part of control and mutant sciatic nerves at P0, P5, and P28 were stained for DAPI, and the total cell number was measured in four nerves in each group (mean±SEM). Bar: (A and B)= 100 μm; (C and D)= 50 μm.

3.5 SC proliferation is severely reduced in mutant peripheral nerves

During late embryonic and perinatal stages, SCs proliferate vigorously to rearrange, sort, and ensheath axons (Stewart et al., 1993). Reduced SC proliferation could cause the decreased total cell number observed in mutant nerves. To determine whether SCs proliferate normally, BrdU-incorporating nuclei were compared between control and mutant nerves at various ages. During the peak of SC proliferation (E17.5 to 19.5) (Stewart et al., 1993), control nerves showed extensive cell proliferation, whereas mutant nerves had few proliferating cells (Fig. 13A). Statistical analyses showed that the percentage of BrdU-incorporating nuclei was similar before *laminin γ1* gene disruption (E13) but was significantly decreased in mutant nerves as compared to controls at E15.5 (20.8% vs. 30.6%), E17.5 (12.7% vs. 29.2%), E19.5/P0 (11.8% vs. 29.6%), and P5 (3.8% vs. 5.6%) (Fig. 13B), indicating that mutant SC proliferation is reduced.

3.6 Laminin mediated axon-SC interactions are critical for SC proliferation

Since axons are a major source of SC mitogens (Morrissey et al., 1995; Wood and Bunge, 1975), impaired axon-SC interaction could result in reduced SC proliferation. β-neuregulin-1 (NRG-1) is a major axon-derived SC mitogen that can interact with and stimulate the phosphorylation of receptor tyrosine kinases ErbB2 and ErbB3 on SCs (Morrissey et al., 1995). The phosphorylation levels of ErbB2 and ErbB3 were measured in sciatic nerve extracts during early postnatal stages (Fig. 14). Although ErbB2 and ErbB3 receptor levels were not significantly changed, the phosphorylation of both proteins was greatly reduced,

suggesting that the inability of mutant SCs to be exposed to axonal mitogens is a major cause of decreased proliferation. This result indicates that laminin-mediated axon-SC interactions are essential for proliferation.

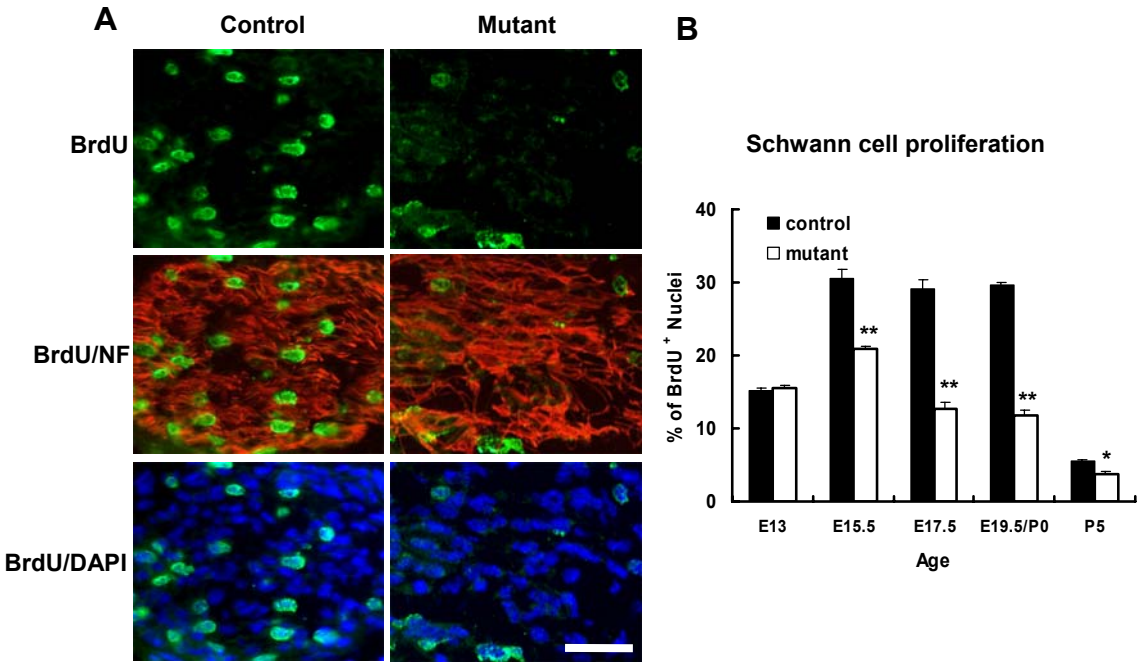


Figure 13. Laminin γ1-null Schwann cells show reduced Schwann cell proliferation.
(A) Longitudinal sections of control and mutant sciatic nerves at E19.5 were triple-stained for BrdU (green), neurofilament (red), and DAPI (blue) after a 1-h pulse of BrdU. The images of BrdU/neurofilament and BrdU/DAPI were merged. Mutant Schwann cells show reduced nuclei BrdU incorporation as compared with control Schwann cells. Bar= 60 μm. (B) Plot of the percentage (mean ± SEM) of BrdU-positive nuclei at various ages. The percentages of BrdU-incorporated nuclei are significantly reduced in mutant nerves (open bar) at E15.5, E17.5, E19.5/P0, and P5 as compared to control nerves (closed bar) (n=6 per genotype per age; * P<0.01, ** P<0.001).

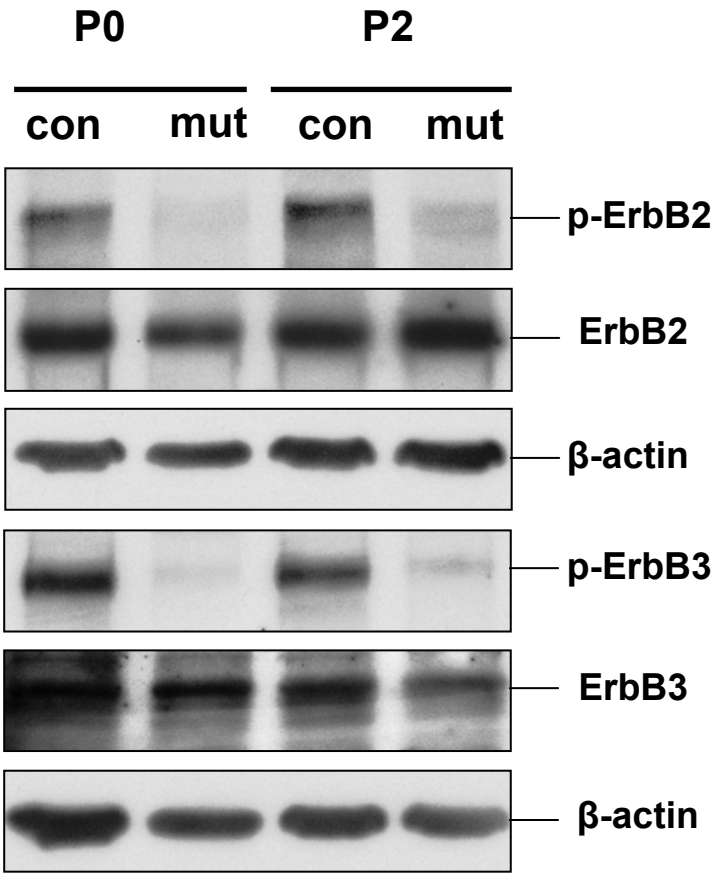


Figure 14. The response of mutant Schwann cells to the mitogenic effect of NRG-1 is impaired.
The response of Schwann cells to NRG-1 at P0 and P2 was assessed on immunoblots with antibodies recognizing phosphorylated ErbB2 (p-ErbB2) and ErbB3 (p-ErbB3). β-Actin was used as the loading control. Mutant Schwann cells show severe reduction in response to axonal mitogens. (c: control; m: mutant).

3.7 SCs lacking laminin expression undergo apoptosis at postnatal stages

ECM molecules, such as laminins, are often essential for maintenance of cell viability (Chen and Strickland, 1997; Meredith et al., 1993). Disruption of laminin γ1 may affect SC survival. Terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) was performed to determine the extent of SC apoptosis at various ages (Fig. 15**)**. There was little apoptosis at E15.5 and E17.5 (Fig. 15B), and the percentages of apoptotic cells were similar in control and mutant nerves. However, in early postnatal stages, the percentage of dying cells in mutant nerves progressively increased, peaked at P15 (Fig. 15A), and gradually declined as the nerves matured (Fig. 15B). In contrast, apoptotic cells were seldom found inside control nerves during these stages. TUNEL-positive cells in mutant nerves were significantly higher than in controls at P0 (1.8% vs. 0.5%), P5 (3.3% vs. 0.1%), P15 (7.1% vs. 0.04%), and P28 (3.0% vs. 0.2%), but not at E15.5 (1.1% vs. 0.6%) and adult (0.5% vs. 0.2%) (Fig. 15B).

Mesenchymal cells are recruited to peripheral nerves to generate the perineurium at E15-17 (Parmantier et al., 1999). To determine whether the apoptotic cells in the sciatic nerve were SCs and lack laminin expression, mutant nerve sections were stained at P15 for laminin γ1, TUNEL, and a SC marker. Cytoplasmic S100 staining showed that the apoptotic cells in mutant nerves were SCs (Fig. 16A) which had lost laminin expression (Fig. 16B).

Since axonal survival signals are only important in early (before P6) but not in later postnatal developmental stages (Grinspan et al., 1996), it is unlikely that late postnatal apoptosis (P15 to P28, Fig. 15 B) is due to a lack of proper

SC/axon relationship. This delayed apoptosis may indicate that laminin is required for long term survival of SCs, which has been previously suggested (Meier et al., 1999). Consistent with this observation, the *CaMKII/Cre:fLAMγ1* mice, in which laminin disruption in SCs occurs later and is incomplete (around E17.5 to E19.5, Chen and Strickland, 2003) as compared to that in *P_0/Cre: fLAMγ1* mice (cre expression around E13.5 -14.5; Fig. 6) showed a later onset of SC apoptosis (Fig. 17). SC apoptosis continued through adulthood in *CaMKII/Cre:fLAMγ1* mice (Fig. 17), but not in *P_0/Cre: fLAMγ1* mice (Fig. 15B); possibly because most SCs die before *P_0/Cre: fLAMγ1* mice reach adulthood, and fewer SCs remain within the sciatic nerve. Therefore, significant increases in apoptosis cannot be detected in these mouse lines.

Taken together, the results coupled with previous studies indicate that loss of laminin could be a direct cause of apoptosis. However, one cannot rule out the possibility that impaired erbB signaling in early postnatal development (Fig. 14) may contribute to part of increased apoptosis, since the axon-derived neuregulin signal still regulates SC survival to some extent in early postnatal stages (Grinspan et al., 1996).

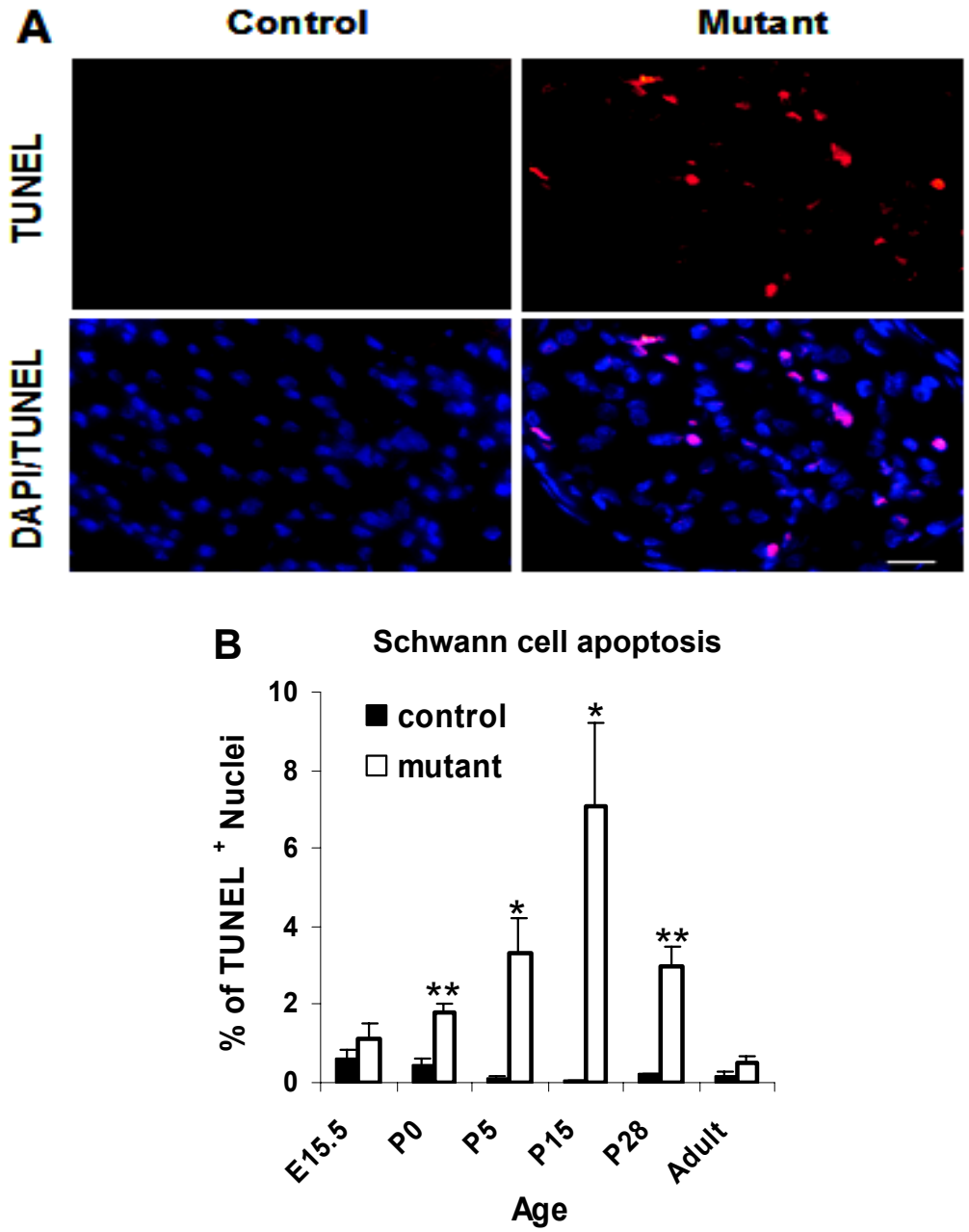


Figure 15. Mutant peripheral nerves show an increased percentage of cell death at postnatal stages.
(A) Representative transverse sciatic nerve sections of control and mutant mice at P15 were stained with TUNEL (red) and counterstained with DAPI (blue), and the images were merged. Apoptotic cells can be detected in mutant but not control nerves. (B) Plot of the percentage of TUNEL-positive nuclei (mean±SEM) at various ages. The percentages of TUNEL-positive nuclei are higher in mutant (open bar) than in control nerves (close bar) at P0, P5, P15, and P28 (n=6 per genotype per age; * P<0.01, ** P<0.001). Bar= 20 μm.

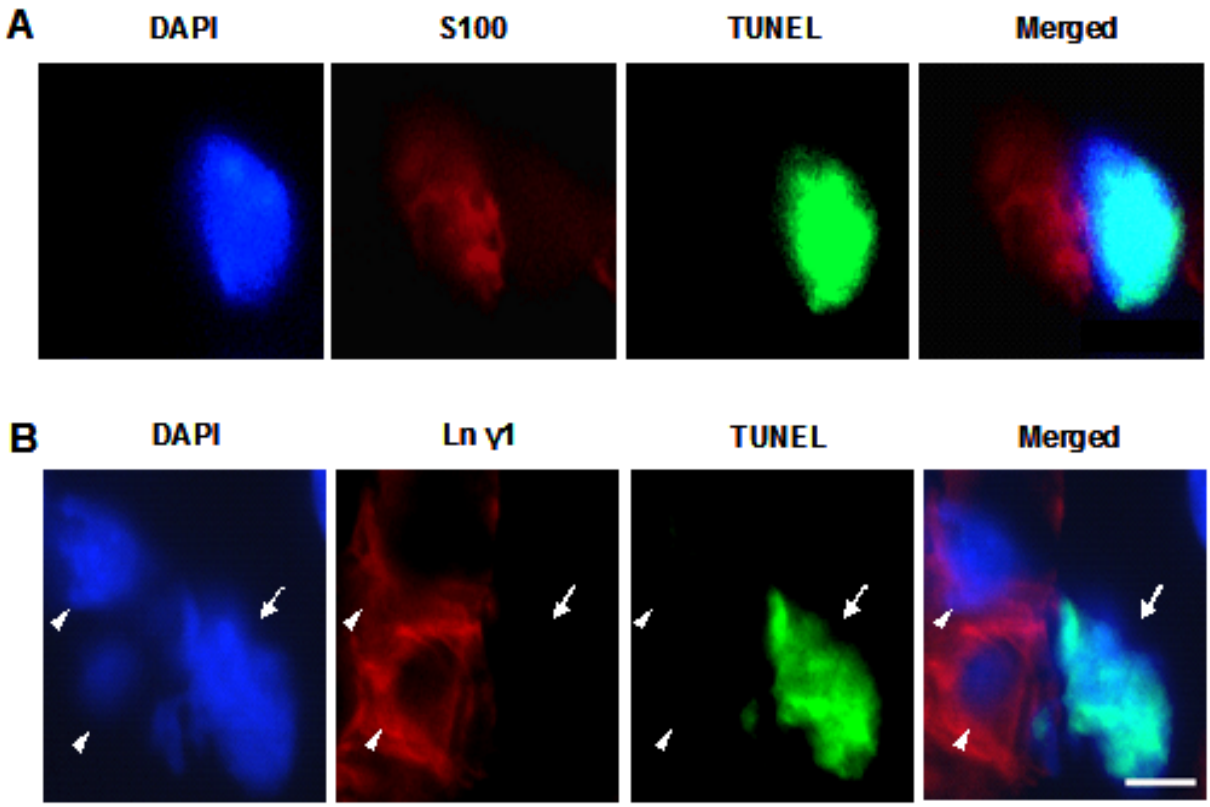


Figure 16. Apoptotic cells in mutant peripheral nerves are Schwann cells that lack laminins.
(A) P15 mutant sciatic nerve sections were stained with DAPI (blue), S-100 (red), and TUNEL (green), and the images were merged. The staining indicates that cells with TUNEL-positive nuclei are Schwann cells. (B) P15 mutant sciatic nerve sections were stained with DAPI (blue), laminin γ1 (red), and TUNEL (green), and the images were merged. The staining shows TUNEL-positive cells do not express laminins (arrows), whereas the TUNEL-negative cells express laminins (arrowheads). Bar= 8 μm.

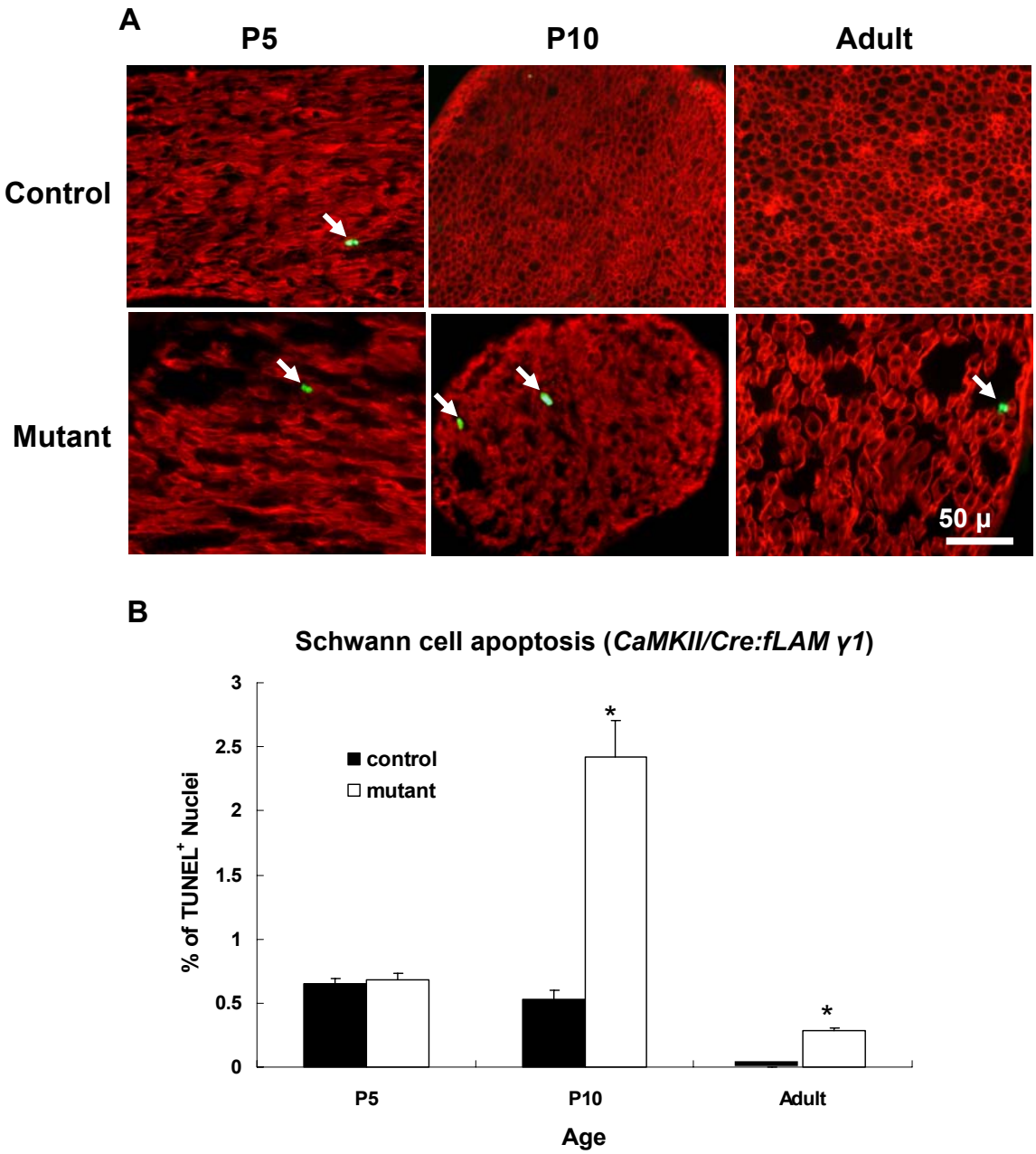


Figure 17. Increased Schwann cells apoptosis in later developmental and adult stages in *CaMKII/Cre:fLAM γ1* mutant mice.
(A) Representative longitudinal sciatic nerve sections of control and mutant mice at P5, and transverse sections of control and mutant mice at P10 and adult were stained with TUNEL (green) and counterstained with laminin γ1 (red). The images were merged. Apoptotic cells can be detected in mutant sciatic nerves at all stages (arrows) and in control nerves at P5. Apoptotic cells were barely detectable in control nerves at P10 and adult. (B) Plot of the percentage of TUNEL-positive nuclei (mean±SEM) at various ages. The percentages of TUNEL-positive nuclei were higher in mutant (open bar) than in control nerves (closed bars) at P10 and adult (n=6 per genotype per age; ** P<0.001), but there was no significant difference at P5.

3.8 Mutant SCs show reduced PI 3-kinase activities

The PI 3-kinase/Akt pathway plays a critical role in controlling the balance between cell survival and apoptosis (Burgering and Coffer, 1995). Several *in vitro* studies have shown that this pathway is important for SC viability (Cheng et al., 2000; Maurel and Salzer, 2000). The decreased SC survival (Fig. 15) led us to examine whether PI 3-kinase/Akt signaling is reduced in mutant SCs. PI 3-kinase activity was assessed by measuring the phosphorylation level of Akt/PKB (p-Akt) protein in sciatic nerve extracts at various ages (Fig. 18A). Akt phosphorylation progressively increased in control nerves during postnatal development. However, in mutant sciatic nerves, Akt phosphorylation decreased from P0 to P28 as compared to control, with a maximum decrease around P15. We stained nerve sections for both S100 and p-Akt and confirmed that the majority of PI 3-kinase activity was from SCs (Fig. 18B).

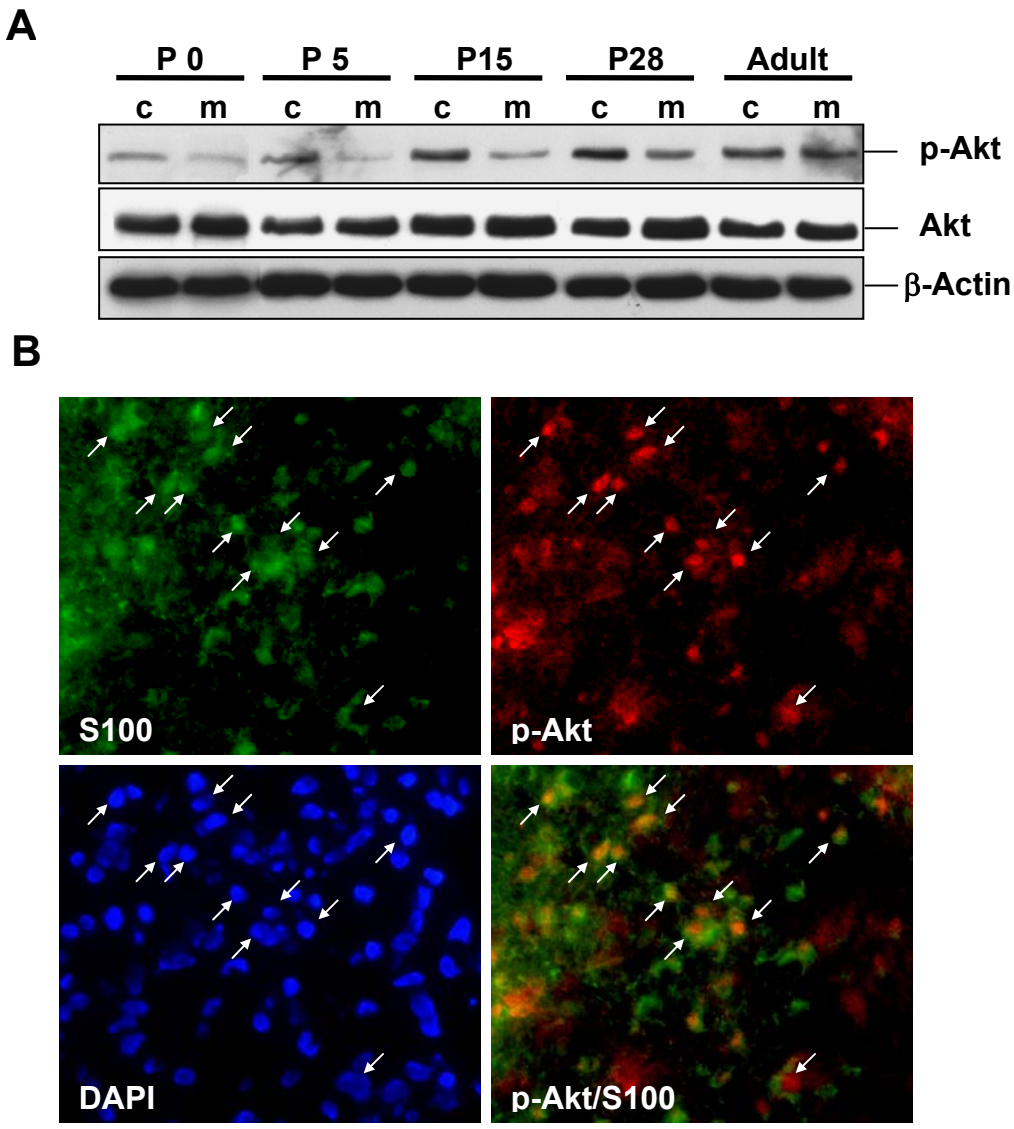


Figure 18. Mutant Schwann cells show impaired PI 3-kinase activity. (A) PI 3-kinase activity of control and mutant sciatic nerves at P0, P5, P15, P28, and adult (n=15 per genotype per age) were assessed on immunoblots with antibodies against phosphorylated Akt (p-Akt), the downstream effector of PI 3-kinase. Mutant sciatic nerves compared to control nerves show reduced PI 3-kinase activity at P0, P5, P15, and P28 (c: control; m: mutant). (B) Transverse mouse sciatic nerve sections at P15 were triple-stained with S100 (green), phospho-Akt (red), and counterstained with DAPI (blue). The images of S100 and p-Akt were merged. These images show that the majority of PI 3-kinase activity was from Schwann cells.

3.9 Mutant SCs show elevated apoptosis/caspase signaling

The reduced PI 3-kinase activity in mutant SCs could indicates that the loss of laminins impaired SCs' ability to receive the axon-derived myelinating signal since the myelin-promoting effect of axon-derived NRG1 type III is mediated by PI 3-kinase/Akt activities (Taveggia et al., 2005). Additionally, the reduced PI 3-kinase/Akt activity may also reflect that mutant SCs have reduced survival signaling. To investigate whether the reduced PI 3-kinase activity activates the downstream cell death signals in SCs, the anti-apoptotic effect of Akt was examined.

Upon activation, Akt phosphorylates and inactivates several downstream targets, including glycogen synthase kinase 3 (GSK-3) and caspase-9, to execute its anti-apoptotic effect. GSK-3 promotes apoptosis and, its activity can be inhibited by Akt-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β (Cross et al., 1995). To determine the Akt-mediated anti-apoptotic effect, the endogenous levels of phospho-GSK-3β were examined in sciatic nerve extracts at P5 and P15, the peak of apoptosis in mutant nerves. In mutant sciatic nerves, GSK-3β protein was comparable to controls but its phosphorylation level was reduced at both ages (Fig. 19A).

Caspase-9 is inhibited by Akt-mediated phosphorylation (Cardone et al., 1998) and is the key initiator of the intrinsic apoptotic pathway (Budihardjo et al., 1999). Upon apoptotic stimulation, procaspase-9 (49 kDa in mice) is processed into a large active subunit (37 or 39 kDa) by self-cleavage. Cleaved caspase-9 activates other effector caspases, including caspase-3 and -7 and initiates a

caspase cascade, leading to programmed cell death. P5 and P15 sciatic nerves were examined for the large active fragment of caspase-9 by Western blotting (Fig. 19B) and the activation of downstream effector, caspase-3 and -7 (Fig. 19C) by immunostaining. At these ages, activated caspase-9, -3, and -7 could be detected in mutant nerves but not in controls (Fig. 19, B and C). These results further suggest that PI 3-kinase/Akt-mediated anti-apoptotic effects are impaired in mutant SCs, resulting in elevated GSK-3 activity and the initiation of a caspase cascade to promote apoptosis.

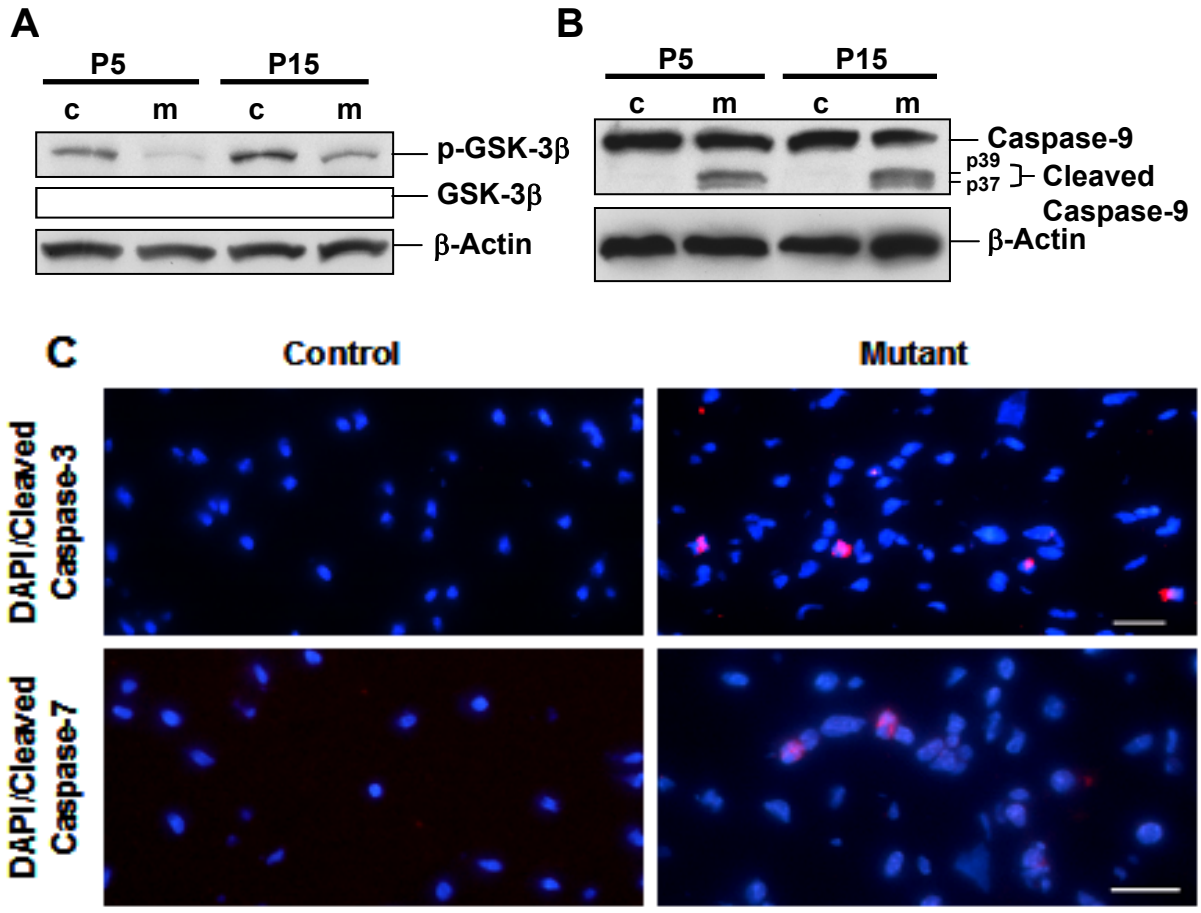


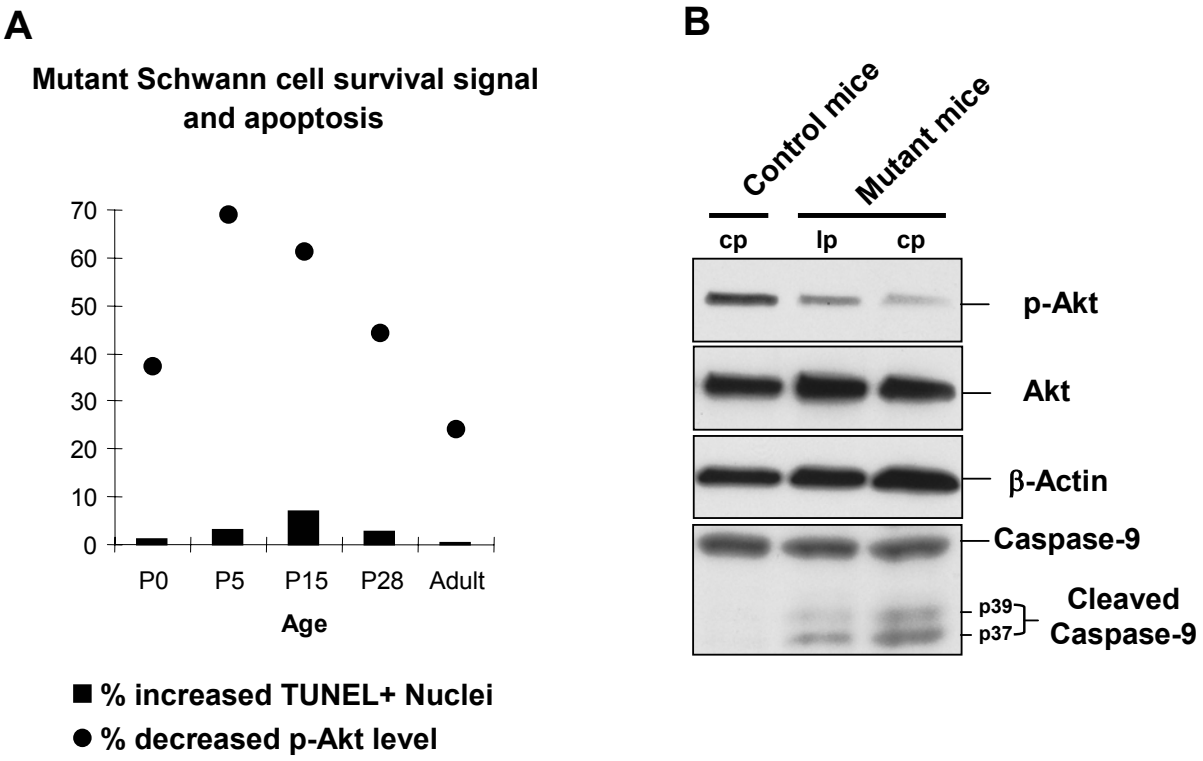
Figure 19. Mutant Schwann cells exhibit elevated apoptosis/caspase signaling.
(A) Akt kinase activity of control and mutant sciatic nerves at P5 and P15 were assessed on immunoblots with antibodies recognizing phospho-GSK-3β (p-GSK-3β). Mutant Schwann cells have reduced Akt kinase activities at P5 and P15 as judged by decreased phosphorylation of GSK-3β (c: control; m: mutant). (B) Endogenous level of activated (cleaved) caspase-9 in control and mutant sciatic nerves at P5 and P15 was assessed by immunoblots with antibodies recognizing both full length (49kDa) and the large fragment of mouse caspase-9 following cleavage at Asp353 (37 kDa) and Asp368 (39 kDa). The mutant sciatic nerves show activation of caspase-9 at both time points (c: control; m: mutant). (C) Longitudinal sciatic nerve sections of control and mutant mice at P15 were stained with activated capase-3 and -7 antibodies (red) and counterstained with DAPI (blue), and the images were merged. Mutant sciatic nerves show increased activated caspase-3 and -7. Bar= 20 μm.

3.10 Laminin-induced PI 3-kinase/Akt activation is required for SC survival

The increased apoptosis and decreased PI 3-kinase activities in mutant sciatic nerves are temporally correlated (Fig. 20A). This suggests that the disruption of laminins in SCs may cause impaired PI 3-kinase/Akt signaling, thus leading to apoptosis. To further confirm that laminins activate PI3-kinase/Akt signals to promote SC survival, a rescue experiment was performed by injecting a laminin peptide into mouse sciatic nerves. It has been shown that a short peptide derived from laminins containing the sequence EIKLLIS, which may represent the integrin-activating site of laminins, can activate PI 3-kinase/Akt-mediated survival signaling in neuronal cell culture (Gary and Mattson, 2001; Tashiro et al., 1999).

Laminin peptide was injected into one sciatic nerve of mutant mice, while the contralateral nerve was injected with a scrambled control peptide ILEKSLI (Tashiro et al., 1999). Injection of laminin peptide but not the control peptide along the mutant sciatic nerves partially restored Akt phosphorylation (increase 75%; normalized with total Akt) and also partially suppressed the activation of caspase-9 (decrease 50%) (Figs. 20 B and C). The control peptide was not toxic since injection into control nerves did not induce apoptosis. Together, these experiments indicate that the disruption of laminins in SCs results in impaired PI 3-kinase/Akt signaling, leading to SC apoptosis.

Figure 20. Restoration of PI 3-kinase activity in mutant sciatic nerves suppresses caspase-mediated death signaling.
(A) The levels of phospho-Akt in mutant sciatic nerves from Fig. 18(A) were quantified (normalized to total Akt level) and compared with control nerves to obtain the percentage of decreased p-Akt level. The percentage of increased TUNEL-positive nuclei in mutant sciatic nerves were obtained by subtracting the average percentages of TUNEL-positive nuclei in control from mutant nerves in Fig. 15(B). A plot of the percent increase in TUNEL-positive nuclei (bar) and decreased p-Akt level (circle) in mutant sciatic nerve against the ages of mice shows that reduced PI 3-kinase activities correlated temporally with increased Schwann cell death. (B) Control sciatic nerve or laminin peptide- (lp) and control peptide- (cp) injected mutant sciatic nerve extracts were immunoblotted with antibodies recognizing Akt or phosphorylated Akt. Caspase signaling was evaluated on the same blot using antibodies against caspase-9. β-actin served as a loading control . As compared to control nerves, the PI 3-kinase activities were partially restored in mutant sciatic nerves injected with the laminin peptide, resulting in the reduction of activated caspase-9 level. (C) Plot of the signal intensity (mean±SEM) of phospho-Akt (p-Akt) and cleaved caspase-9 from quantitative analysis of Western blots from (B). The signal intensity of p-Akt and cleaved caspase-9 in laminin peptide-injected mutant sciatic nerves increased 75% and decreased 50 %, respectively, as compared to contralateral control nerves (n=20 mice per genotype per lysate, three independent experiments; * $P<0.05$, ** $P<0.01$).


A
Mutant Schwann cell survival signal and apoptosis
70
60
50
40
30
20
10
0
P0
P5
P15
P28
Adult
Age
% increased TUNEL+ Nuclei
% decreased p-Akt level
B
Control mice
Mutant mice
cp
lp
cp
p-Akt
Akt
β-Actin
Caspase-9
p39
p37
Cleaved Caspase-9
C
80
60
40
20
0
Signal Intensity
control
mutant/Ln peptide
mutant/con peptide
*
**
p-Akt
cleaved caspase-9

3.11 Exogenous laminins rescue the defects of ensheathment and myelination of mutant SCs in SCs/dorsal root ganglion (SC/DRG) neuronal co-cultures

SC proliferation plays a vital role in establishment of solitary relationships between myelinating SCs and axons during the onset of radial sorting (Martin and Webster, 1973; Stewart et al., 1993), and laminins promote this perinatal SC proliferation (Fig. 13B) (Yang et al., 2005). The severe impairment of radial sorting in laminin-deficient nerves could largely result from the decrease in perinatal SC proliferation, leaving the question whether laminins have direct roles in regulating SC cytoskeleton to initiate ensheathment and myelination of axons. Moreover, the severe reduction of SC number in *P_0/Cre: fLAMγ1* mice also preclude us from using these mice to further study the direct effect of laminins on SCs. To investigate whether laminins regulate SC process formation during ensheathment and myelination, SC/DRG neuronal co-cultures were used. The ensheathment and myelination of this co-culture system is induced by addition of ascorbic acid to stimulate deposition of basal lamina. SC/DRG neuronal co-cultures from mutant mice show incomplete recombination of *laminin γ1* gene (Fig. 21A), presumably due to the unrecombined *laminin γ1* gene present in neurons and fibroblasts. These non-SC autonomous laminins partially rescue the mutant phenotype (Fig. 21B). To circumvent this problem, SC/DRG neuronal co-cultures from *floxed laminin γ1* mice were infected with an adenovirus expressing Cre recombinase (Ad-Cre) to completely disrupt the *laminin γ1* gene (Fig. 21A).

In co-cultures infected with a control adenovirus (Ad-LacZ), laminins were present (Fig. 22A) and myelination was first detectable two days after ascorbic acid addition. After eight days in myelinating promoting feed (maintenance media with ascorbic acid), extensive myelinated fibers were observed (Fig. 22A). Ultrastructural analysis of these control cultures showed normal ensheathment of axons (EM in Fig. 22A). In contrast, laminins were completely absent from Ad-Cre-infected co-cultures (Fig. 22A), and these mutant co-cultures did not myelinate (Fig. 22A), even after two weeks of ascorbic acid exposure (Fig. 22B). Ultrastructural analysis of these control cultures revealed that most SCs did not form processes to properly ensheath axons (EM in Fig. 22A).

To investigate if exogenous laminins could rescue the defects in mutant cultures lacking endogenous laminins, soluble laminins were added to the media (Fig. 22A). Addition of exogenous laminin restored myelination (Fig. 22A) as well as the defect of ensheathment of SCs (EM in Fig. 22A).

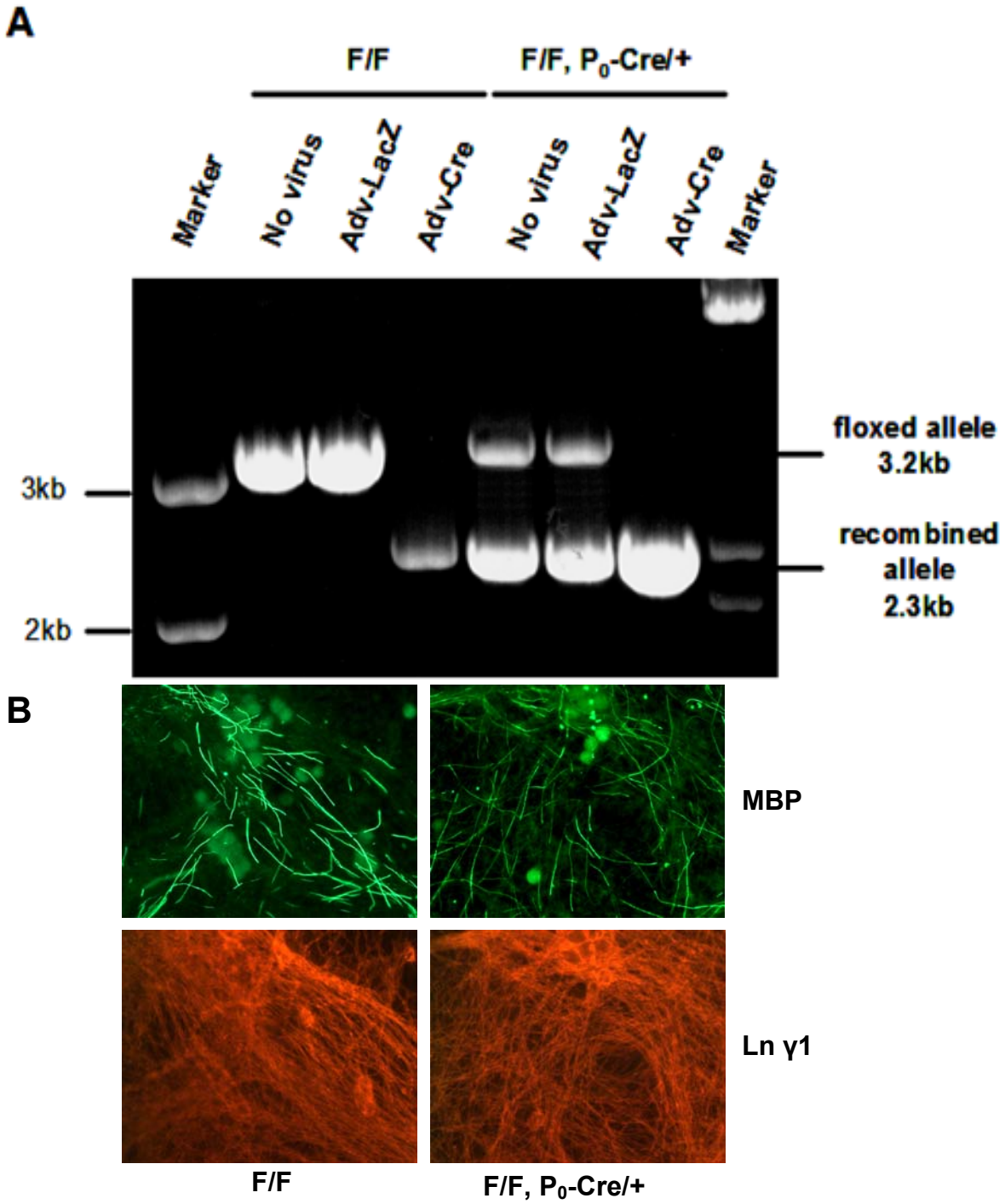
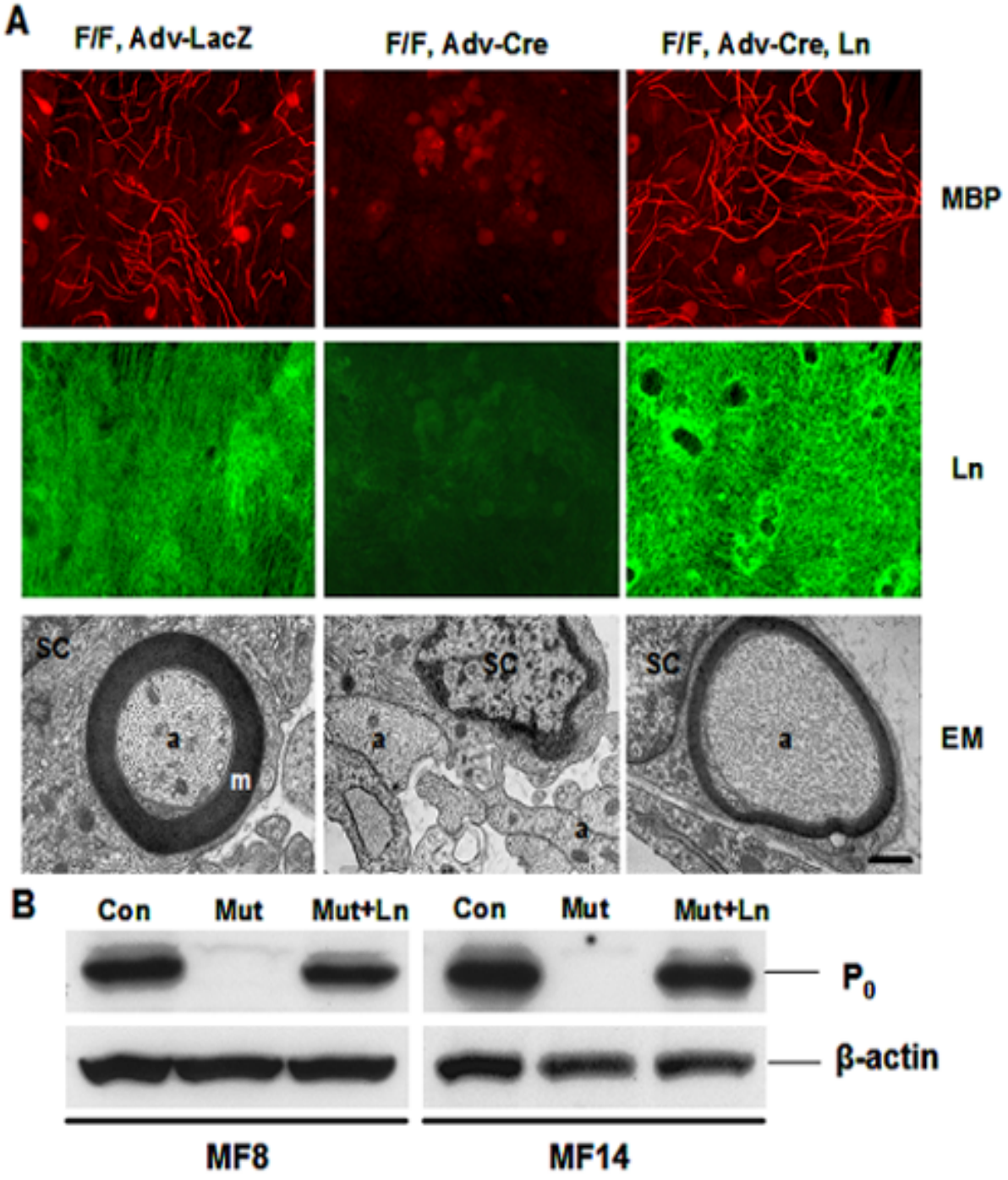


Figure 21. SC/DRG neuronal co-cultures from *P0/Cre:fLAM γ1* mice show incomplete recombination of laminin γ1 and are partially myelinated.
(A) PCR analysis of genomic DNA of co-cultures from homozygous *fLAM γ1* mice (*F/F*) and *P0/Cre:fLAM γ1* mice (*F/F, P0-Cre*) infected with adenoviruses expressing LacZ or Cre. The primers used amplified the unrecombined (3.2 kb) and recombined (2.3 kb) *fLAM γ1* alleles. (B) Myelination of mouse SC/DRG neuronal co-cultures eight days after addition of ascorbic acid were detected by immunostaining for MBP (green) and laminin γ1 (red) . Control co-cultures show extensive myelination. Mutant co-cultures have incomplete disruption of laminin γ1 and show some myelination.

Figure 22. Schwann cells lacking laminins show defects in ensheathment and myelination.
(A) Myelination of mouse SC/DRG neuronal co-cultures infected with Ad-LacZ or Ad-Cre eight days after addition of ascorbic acid or exogenous laminins was detected by immunostaining for MBP (red) and laminins (green) or analyzed by electron microscopy (EM). Co-cultures infected with a control virus show extensive myelination. In contrast, co-cultures infected with Ad-Cre (mutant) show complete disruption of laminins and have no myelination. Extensive myelination was restored in mutant co-cultures treated with exogenous laminin. Electron micrographs of SC/DRG neuronal co-cultures eight days in myelinating promoting feed show that mutant Schwann cells have no processes to enwrap axons and fail to myelinate axons. Bar= 1 μm. (B) The expression of myelin protein zero in control and mutant co-cultures eight days or 14 days after addition of ascorbic acid was assessed by Western bloting with antibodies against P_0. β-Actin served as the loading control. No myelination was dectected in mutant co-cultures after two weeks in myelinating promoting feed. (con: control; mut: mutant).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F/F, Adv-LacZ
F/F, Adv-Cre
F/F, Adv-Cre, Ln
MBP
Ln
EM
SC
a
m
B
Con
Mut
Mut+Ln
Con
Mut
Mut+Ln
P0
β-actin
MF8
MF14

3.12 Mutant SCs can not form bipolar morphology and elongate

A hallmark of SC differentiation and myelination is to form a bipolar morphology and to elongate in order to precisely match the extension of nerve growth (Court et al., 2004). To determine if SC morphology was altered upon disruption of laminins, SCs were identified by staining co-cultures with S100, and morphology was visualized using confocal microscopy. Upon eight days in myelinating promoting feed, most SCs in control co-cultures form an internodal myelin segment along axons with the canonical bipolar extension morphology (Fig 23A). In contrast, SCs lacking laminins did not differentiate properly and fail to establish cell polarity (Fig. 23B). These cells showed either no filapodia extension (mutant 2 in Fig. 23B) or extended membrane in multiple directions (mutant 3 in Fig. 23B). A few mutant SCs differentiated asymmetrically and form a unipolar morphology with a short myelin segment (mutant 1 in Fig. 23B). These SCs may have their laminins disrupted after the initiation of myelination. Addition of exogenous laminin restored the normal morphology of differentiated SCs (Fig. 23C). Statistical analysis revealed that the length of mutant SCs is significantly decreased as compared to control (Fig. 23D). Thus, laminins are required for SCs to form a bipolar morphology and the subsequent elongation during myelination.

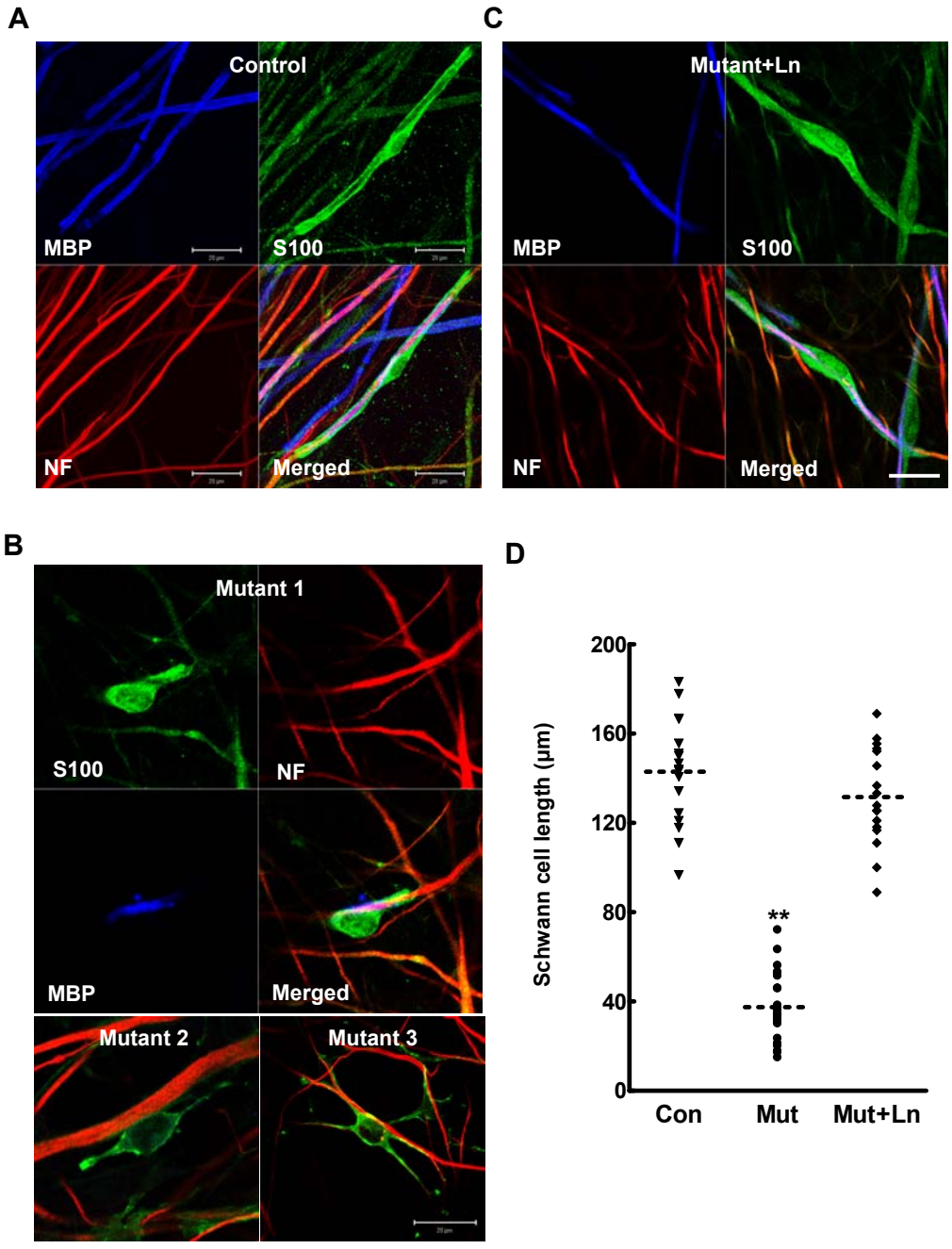


Figure 23. Schwann cells lacking laminins show defects in formation of bipolar morphology and elongation.
Control (A), mutant (B), and mutant co-cultures with exogenous laminins (C) eight days after addition of ascorbic acid were stained for the MBP (blue), s100 (green), and neurofilment (red) and were imaged by confocal microscopy. The images were then merged. Mutant Schwann cells show defects in formation of bipolar morpholgy. Bar= 20 µm. (D) Comparison of Schwann cell length in co-cultures after eight days in myelinating promoting feeds (3 fields per co-culture, 6 co-cultures in control and mutant+Ln and 8 co-cultures in mutant, ** $P<0.001$ compared with control, student's t test). Broken lines represent the average.

3.13 Mutant SCs show less formation of lamellipodia and filopodia upon the onset of myelination

Mutant SCs lack processes necessary to form a close relationship with axons (EM in Fig. 22A). Therefore, the regulation of SC process formation during myelination was examined by performing time-lapsed live cell imaging in SC/DRG neuronal co-cultures. To specifically label neurites with red fluorescence and SCs with green fluorescence, co-cultures were infected with two different recombinant adenoviruses at different time points. At the time of plating, the dissociated explants contain mostly neurons with only a few satellite SCs and were infected with adenovirus expressing mCherry-tagged neurofilament light chain. Red fluorescence appeared in the soma and proximal neurites within one day after infection, and by eight days it had extended into the distal neurites (Fig. 24A). Two days before imaging (10 days after plating), the co-cultures were completely packed by endogenous SCs and were infected with another adenovirus expressing eGFP-tagged β-actin. This resulted in co-cultures with axons of red fluorescence and SCs of green fluorescence, respectively (Fig. 24A). Infecting co-cultures with these adenoviruses did not impede SC differentiation or myelination (Fig. 24B).

Labeled control and mutant co-cultures were imaged for 16 hours with a spinning disk confocal microscope to acquire high-resolution 3D images. Imaging of control co-cultures showed SCs form extensive dynamic cytoplasmic processes to attach neurites (Fig. 25A, see video 1 for details). In contrast, mutant SCs show less process formation with some SCs undergo cell death (Fig.

25A, see video 2 for details), consistent with our previous observations that laminins are also required for SC survival (Fig. 15). We measured the number of processes formed per hour and the length of formed processes and found both parameters were significantly decreased in mutant SCs (Figs. 25 B and C).

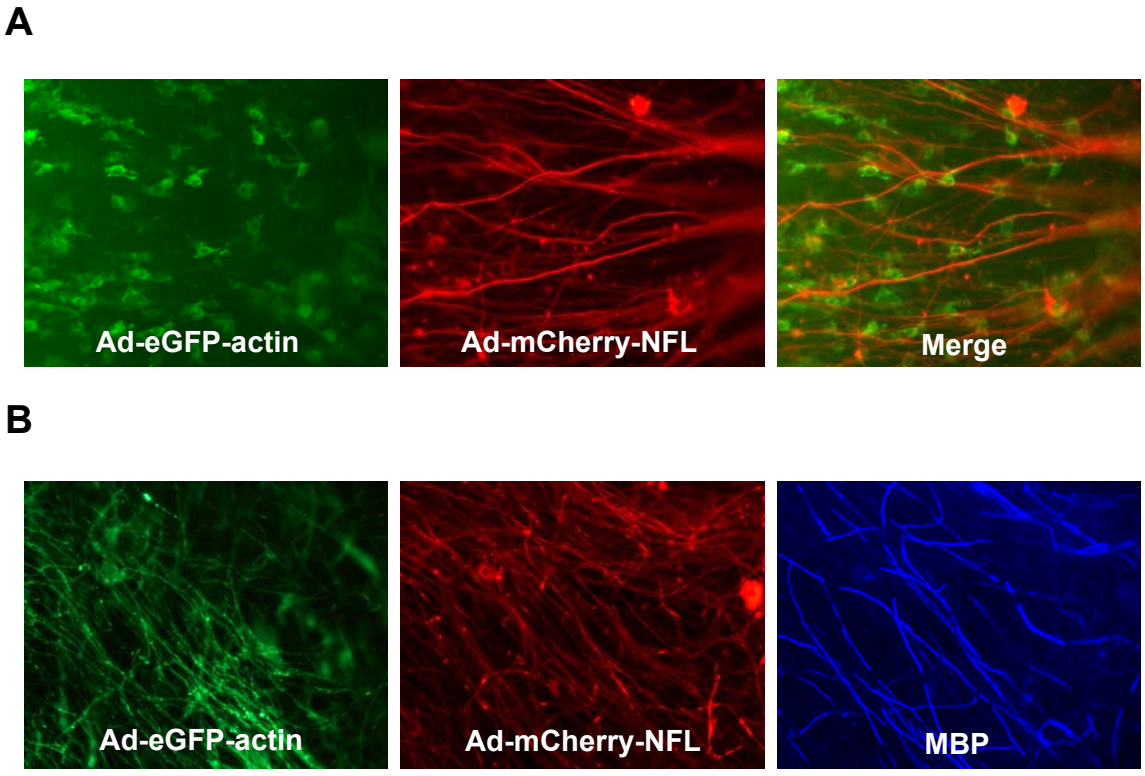


Figure 24. Mouse SC/DRG neuronal co-cultures were infected with Ad-eGFP-actin and Ad-mCherry-NFL and used for time-lapsed imaging. (A) SC/DRG neuronal co-cultures were infected sequentially with Ad-mCherry-NFL at day 0 and Ad-eGFP-actin at day 10 in maintenance media and images were taken and merged two days after last infection. (B) Co-cultures were infected with Ad-mCherry-NFL and Ad-eGFP-actin, incubated for eight days in myelinating promoting feed and stained for MBP (blue). Infection of co-cultures with Ad-mCherry-NFL and Ad-eGFP-actin does not inhibit myelination.

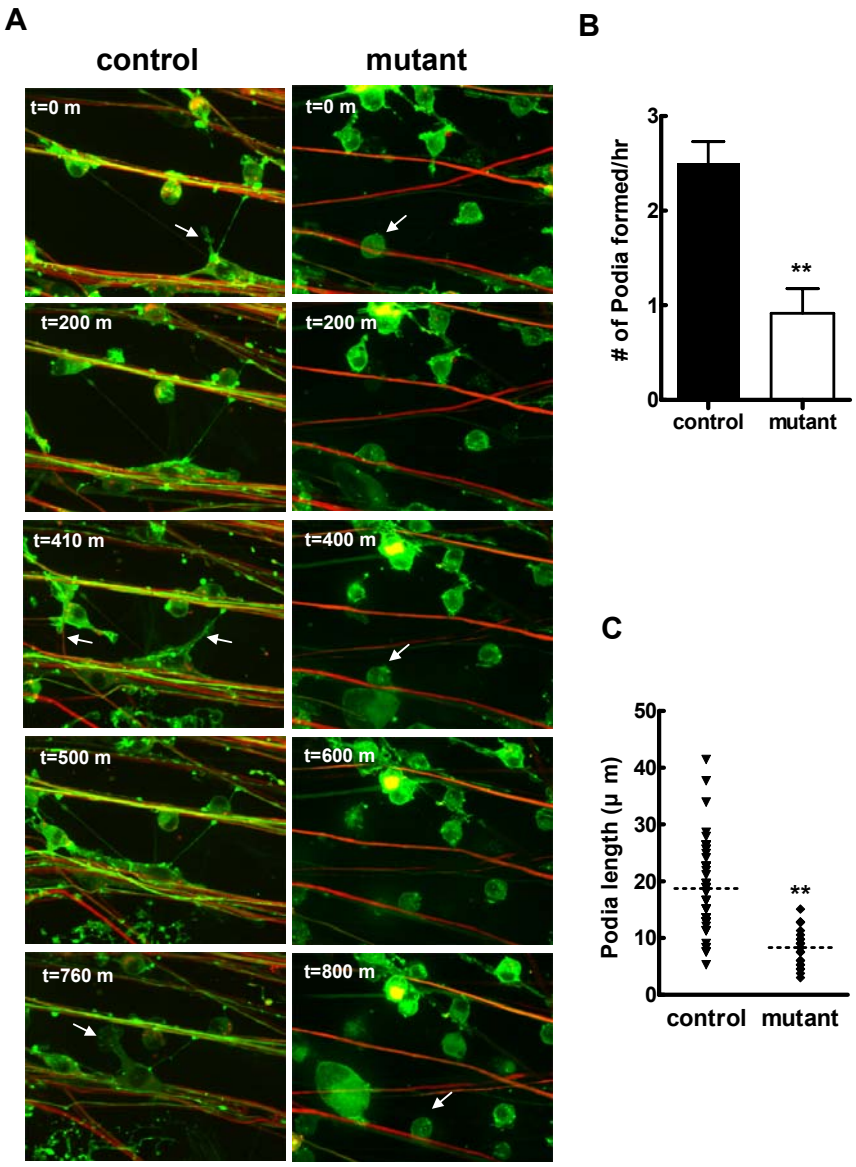


Figure 25. Formation of lamellipodia and filopodia is decreased in mutant Schwann cells.

(A) SC/DRG neuronal co-cultures were labeled with Ad-mCherry-NFL and Ad-eGFP-actin and then imaged for 16 hours using a spinning disk confocal microscope. *Z*-stack were taken every five mins. Each panel is a maximum projection of a *z*-stack. Control Schwann cells form extensive podia (arrows), while mutant Schwann cells form fewer and shorter podia. (B) Quantification of podia formation per hour over a 12-hour period reveals mutant Schwann cells have less podia formation (**: $p<0.001$, student's t-test). (C) Quantification of the length of formed podia over a 12-hour period reveals mutant Schwann cells form shorter podia (**: $p<0.001$, student's t-test). Broken lines represent the average.

3.14 Disruption of laminins impedes non-myelinating SC differentiation

At P28, the differentiation of both myelinating and non-myelinating SCs was completed in control nerves. The non-myelinating SCs form several processes to enwrap multiple small caliber axons (Remak bundles in Figs. 26 A and C). However, at the same age, the mutant nerves contain only large unsorted axonal bundles and few myelinating SCs without Remak bundles (Figs. 26 B and D). To determine whether nonmyelinating SCs differentiate properly in peripheral nerves lacking laminins, sciatic nerve sections were stained with L1 and N-CAM, two cell adhesion molecules that serve as markers of differentiated nonmyelinating SCs (Martini and Schachner, 1986; Martini and Schachner, 1988). L1 and N-CAM are normally expressed by immature and all promyelinating SCs, but are down-regulated in myelinating SCs when myelination starts and restricted to nonmyelinated axons and nonmyelinating SCs (Martini and Schachner, 1986). In both control and mutant embryonic nerves, L1 and N-CAM staining appeared normal in growing axons and immature SCs (Figs. 27 A, D, I, and L). During postnatal stages, L1 and N-CAM were gradually confined to nonmyelinating SCs and nonmyelinated axons in control nerves (Figs. 27 A-C, G, H, and I-K); however, their expression progressively diminished in all areas of mutant sciatic nerves in early postnatal stages (Figs. 27 B and E), then completely disappeared around P15 (Figs. 27 C, F, J, and M) and could not be detected thereafter (Figs. 27 G, H, K, and N).

At this developmental stage, evidence of apoptosis of L1 and N-CAM positive cells could not be found in mutant sciatic nerves. These results suggest

that the disappearance of L1 and N-CAM positive cells results from a lack of differentiation of non-myelinating SCs and not apoptosis.

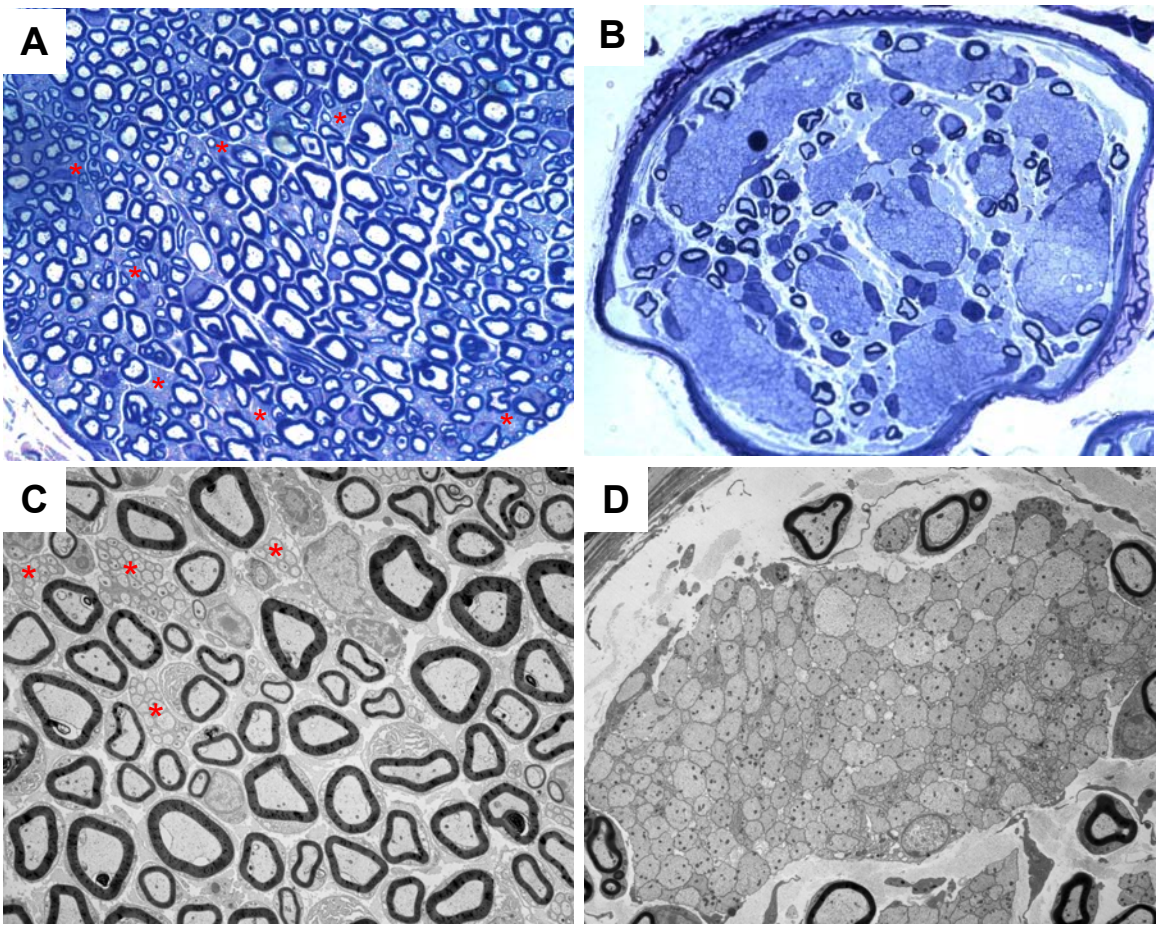
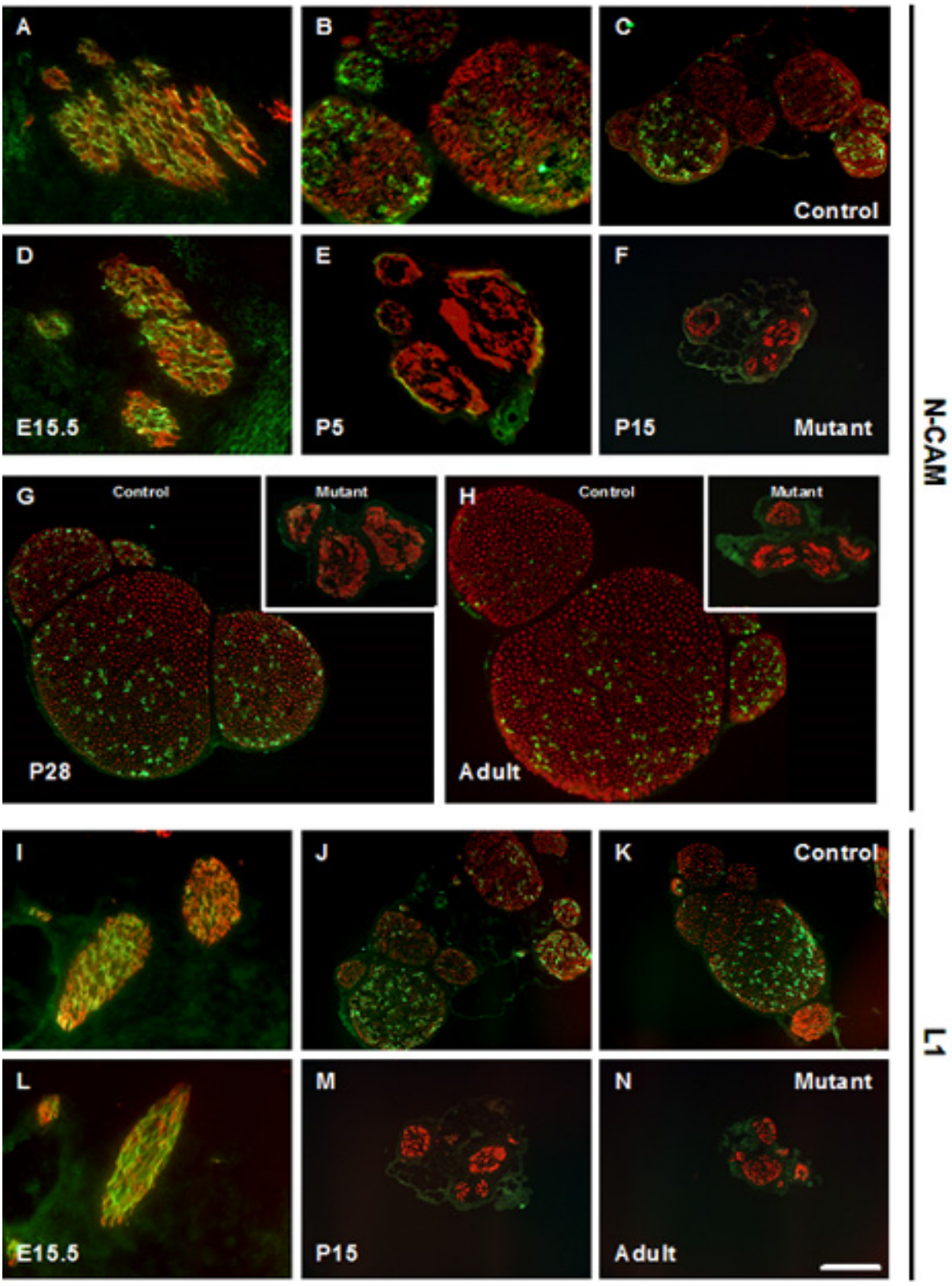


Figure 26. P28 mutant sciatic nerves do not have non-myelinating Schwann cells.
(A-D) Transverse semi-thin (A and B) and ultrathin (C and D) sections from P28 sciatic nerves show that control nerves (A and C) have non-myelinating Schwann cells and form several Remak bundles (asterisks) while mutant nerves only contain unsorted axonal bundles and few Schwann cells with myelin sheaths (B and D).

Figure 27. Non-myelinating Schwann cells in mutant peripheral nerves do not differentiate.
Whole embryo sections at E15.5 (A, D, I, and L) and transverse sciatic nerve sections at P5 (B and E), P15 (C, F, J, and M), P28 (G), and adult (H, K and N) were stained for neurofilament (red) and N-CAM (green, A-H) or L1 (green, I-N) to detect the differentiation of non-myelinating Schwann cells. In mutant sciatic nerves, the non-myelinating Schwann cells did not differentiate and failed to express N-CAM (E-H)and L1 (M and N) at postnatal stages. Bar: (A, B, D, E, I, and L)= 39.5 μm; (C, F, J, K, M and, N)= 79 μm; (G and H)= 50 μm.


A
B
C
Control
D
E15.5
E
P5
F
P15
Mutant
N-CAM
G
Control
Mutant
P28
H
Control
Mutant
Adult
I
J
K
Control
L
E15.5
M
P15
N
Mutant
Adult
L1

CHAPTER 4: DISCUSSION

Using the Cre/LoxP system, *laminin γ1* gene was specifically disrupted in SCs during their early developmental stages. Disruption of *laminin γ1* gene resulted in complete depletion of all laminin isoforms in SCs. These mice have revealed novel mechanistic insights into laminin function in SC development. First, laminin is essential for differentiation of SCs; mutant SCs fail to down-regulate Oct-6 and myelinate, and are arrested in the premyelinating stage. Second, laminins regulate the bipolar extension and the formation of lamellipodia and filopodia of SCs, which are two essential steps for axon-SC interaction, axonal sorting, ensheatment, and myelination. Impaired axon-SC interaction prevents exposure of the cells to axon-derived signals and causes severe reduction of proliferation and aberrant differentiation. Third, laminins maintain SC long-term survival through regulation of a survival signal. Fourth, laminins are required for differentiation of non-myelinating SCs as well.

4.1 The primary function of laminins in PNS development is to initiate radial sorting of axons and mediate axon-SC interaction.

Using time-lapsed live cell imaging, it was demonstrated that laminins regulate the formation of podia in SCs (Fig. 25). This laminin-mediated podium formation is essential for axon defasciculation as well as interaction between SCs and axons, as the disruption of laminins in SCs results in severe impairment of axonal sorting (Fig. 8) and inappropriate relationship of SCs and axons (Figs. 22A and

23B). In mutant nerves, SCs show less proliferation, and fail to down-regulate Oct-6 and up-regulate Krox-20 at later developmental stages. All these steps critically depend on axon-derived signals (Jessen and Mirsky, 2005; Murphy et al., 1996; Scherer et al., 1994). Thus, many defects in P_0/Cre:fLAMγ1 mice, including the aberrant differentiation and decreased proliferation, appear to be a secondary consequence of impaired axon-SC interaction. In support of this view, mutant SCs have reduced response to NRG-1 at early postnatal stages (Fig. 14) and decreased PI 3-Kinase activities during the course of myelination (Fig. 18A). Since the activation of PI 3-kinase pathway by axon-derived NRG-1 type III is a hallmark of SC myelination (Maurel and Salzer, 2000; Ogata et al., 2004; Taveggia et al., 2005), part of the decreased PI 3-kinase activities may simply reflect the failure of mutant SCs to expose to axonal membrane-bound NRG-1 type III.

4.2 The role of laminins in SC proliferation

Many phenotypes of mice lacking laminin γ1 in SCs (Fig. 8) are similar to those observed in mice lacking β1 integrin in SCs (Feltri et al., 2002), indicating that β1 integrin plays a major role in mediating laminin signaling. However, proliferation is reduced in SCs lacking laminins (Fig. 13) but is not significantly affected in β1 integrin-null SCs. There are three likely possibilities for why laminin γ1 affects proliferation: 1, laminin may act as a direct SC mitogen since this effect has been observed *in vitro* (Baron-Van Evercooren et al., 1986; Macica et al., 2006; McGarvey et al., 1984; Yang et al., 2005); in this case, laminin γ1 would employ

receptors other than β1 integrin; 2, the laminin basal lamina could act as a scaffold to attract and bind growth factors and influence SC proliferation; 3, the proliferation effect might be secondary to axonal sorting and provision of axon-derived growth factors (Fig. 14). These three possibilities are not mutually exclusive and can coexist. The third possibility is favored based on our results (Fig. 14) and the following observations. In early postnatal stages (P1), SCs lacking β1 integrin send abnormally shaped, thick cytoplasmic processes to ensheath groups of axons. The formation of unsorted axonal bundles in later stages partly results from the retraction of processes (Feltri et al., 2002), at which time SCs may have the opportunity to encounter to axon-derived mitogens. However, SCs lacking laminin γ1 are unable to extend cytoplasmic processes (Figs. 8C, 22A and 25) and fail to interact with axonal mitogens.

In the presence of basal lamina, β1 integrin forms a complex with focal adhesion kinase (FAK) and paxillin (Chen et al., 2000). Schwannomin (merlin) is then recruited to the plasma membrane through its interaction with paxllin and forms a complex with β1 integrin and the erbB2 receptor (Fernandez-Valle et al., 2002; Obremski et al., 1998). NRG-1 also induces the association of FAK with the erbB2/erbB3 receptor (Vartanian et al., 2000). If any process of formation of this multimolecular complex is disrupted, many deleterious events may occur. For example, inactivation of schwannomin causes abnormal SC proliferation in Schwannomas found in Neurofibromatosis type 2 patients (Rouleau et al., 1993). In addition, disruption of FAK results in similar phenotypes to our mutant mice, characterized as impaired radial sorting of axons and decreased SC proliferation

(Grove et al., 2007). All these studies, coupled with our finding that laminins regulate the response of SCs to NRG-1, indicate that laminins cooperate with NRG-1 to coordinate SC proliferation through the formation of a multimolecular complex.

4.3 The role of laminins in SC survival

At E12-E13 in mice, the survival of SC precursors depends on axon-derived β-neuregulin 1/Erb B pathway (Dong et al., 1995; Riethmacher et al., 1997). After this stage, SCs establish an autocrine loop but also require laminins for long-term survival (Meier et al., 1999). Since P0/Cre-mediated laminin γ1 disruption occurs between E13.5 to E14.5 (Fig. 6) and since there was no significant cell death around this stage (Fig. 15), it is unlikely that the neuregulin 1/Erb B signaling pathway is affected in mutant nerves at this time point.

The PI 3-kinase activity in mutant SCs was severely reduced (Fig. 18A). This reduced PI 3-kinase activity may be a consequence of the impaired differentiation of mutant SCs (see above discussion). However, disruption of laminins may also contribute to the reduction of this survival signaling pathway and results in apoptosis based on the following observations: At P0/P1, both control and mutant SCs are at similar differentiating stages (premyelinating stage), but the mutant SCs had reduced PI 3-kinase activity and increased apoptosis (Figs. 15B and 18A). Additionally, mutant SCs infused with laminin peptides showed partial restoration of PI 3-kinase activity and reduced apoptosis (Fig. 20).

We observed reduced Krox-20 expression and increased SC death in mutant sciatic nerves (Figs. 11 and 15). Since Krox-20 can suppress c-Jun-mediated TGFβ-induced SC apoptosis (Parkinson et al., 2004), increased SC apoptosis could result from the failure of Krox-20 to inhibit c-Jun activation. However, the phosphorylation of c-Jun at postnatal stages between control and mutant sciatic nerves was similar (data not shown), suggesting that the TGFβ pathway did not play a major role in the increased apoptosis of mutant SCs.

4.4 Other mouse models with similar defects

The defects in the PNS observed in mice lacking laminins (Fig. 8) are more severe than those found in *dystrophic* mice (*dy2J/dy2J* or *dy3K/dy3K*), which have a mutation or complete deficiency in their *laminin α2* gene, resulting in a lack of laminin-2 (α2 β1 γ1) expression (Nakagawa et al., 2001; Xu et al., 1994). However, in the laminin α2 mutant mice, laminin-1 and laminin-8 are up-regulated, which can partially compensate for the loss of laminin-2 (Pagenstecher et al., 1998; Patton et al., 1997; Previtali et al., 2003; Yang et al., 2005). Since laminin-8, laminin-1, and laminin-2 all contain the γ1 chain, compensation in the laminin γ1-depleted mice is not possible and results in a more severe phenotype. Consistent with this observation, combined deficiency of laminin 2/8 (*dy2J/α4null* mice) caused more severe defects than those in dystrophic mice (Yang et al., 2005), and the severity is similar to the mutant mice presented here. Yang et al. also provided evidence that laminins are important for SC proliferation. As with

dy2J/α4null and *Lnα2/α4-DKO* mice, the mutant mice discussed herein also show severe defects in SC proliferation (Fig. 13).

4.5 Laminin receptors in SC development

SCs express several potential laminin receptors, including α6β1, α6β4 integrins, and dystroglycan (Previtali et al., 2003). α6β1 integrins is thought to be the major laminin receptor in SCs. The β1 integrin-null SCs can ensheath axons prenatally and myelinate axons after birth with some delay (Feltri et al., 2002), which is in contrast to laminin γ1-null SCs that do not exhibit these processes. The postnatal myelination difference may be due to compensation of β1 integrin by another laminin receptor, for example α6β4 or dystroglycan, both of which are expressed in postnatal SCs (Previtali et al., 2003). The prenatal ensheathment difference is more difficult to explain, since only α6β1 integrin is observed before birth (Previtali et al., 2003). This observation suggests that an unidentified laminin receptor expressed in embryonic SCs is involved during the ensheathment of axons.

In contrast to β1 integrin-deficient SCs, SC-specific ablation of dystroglycan results in abnormal folding of myelin sheaths and a reduction of sodium channels at the nodes of Ranvier, but it does not severely affect radial sorting of axons (Saito et al., 2003). During PNS development, L-periaxin-null mice show abnormal folding of myelin sheaths and late-onset of demyelination similar to dystroglycan-null mice (Gillespie et al., 2000). L-periaxin is required for the formation of the dystroglycan-dystrophin-related protein-2 (DG-DRP2)

complex and is involved in the linkage between the ECM and the SC cytoskeleton (Sherman et al., 2001). Additionally, periaxin-null mice exhibit disruption of the Cajal bands, a cellular structure of SCs with a nutritive function, resulting in impaired SC elongation during nerve growth (Court et al., 2004). Our findings that disruption of laminins in SCs causes impairment of axonal sorting (Fig. 8) as well as elongation of SCs (Fig. 23) suggest that laminins may employ distinct sets of receptors in different stages of myelination (Fig. 28). During the initiation of myelination, the action of laminins in SCs is mediated by β1 integrin. The multimolecular complex formation of β1 integrin/FAK/paxllin/schwannomin upon the deposition of laminins then regulates Rac1/Cdc42 activities, which are required for the formation of lamellipodia and filopodia. This multimolecular complex also coordinates with the NRG-1/ErbB signaling pathway to regulate SC proliferation. Both podia formation and SC proliferation are required for radial sorting and ensheathment of axons during the initial stages of myelination. At later stages of myelination, laminins may exert their functions by switching to another set of receptors, namely dystroglycan. The formation of DG-DRP2-periaxin complex coordinates the length of myelinating SCs with growing axons and maintains the stability of myelin sheaths. A definitive assessment of this "dual-receptor" model requires further analyses which as follows: 1) The integrity of β1 integrin-containing or dytroglycan-containing multi-molecular complexes in SCs lacking laminins will be examined by co-immunoprecipitation and immunoblotting; 2) The activities of Rac1/Cdc42 will be assessed in SCs lacking laminins using Rac1/Cdc42 activation assay (Taylor and Shalloway, 1996); 3)

Two channel time-lapsed imaging will be performed in co-cultures that lack β1 integrin or that are exposed to β1 integrin functional blocking antibodies. The podia formation of SCs in these co-cultures will be analyzed; 4) Two channel time-lapsed imaging will be performed in co-cultures that lack dystroglycan or contain dystroglycan functional blocking antibodies (Ervasti and Campbell, 1993). The elongation rate of myelinating SCs in these co-cultures will be measured.

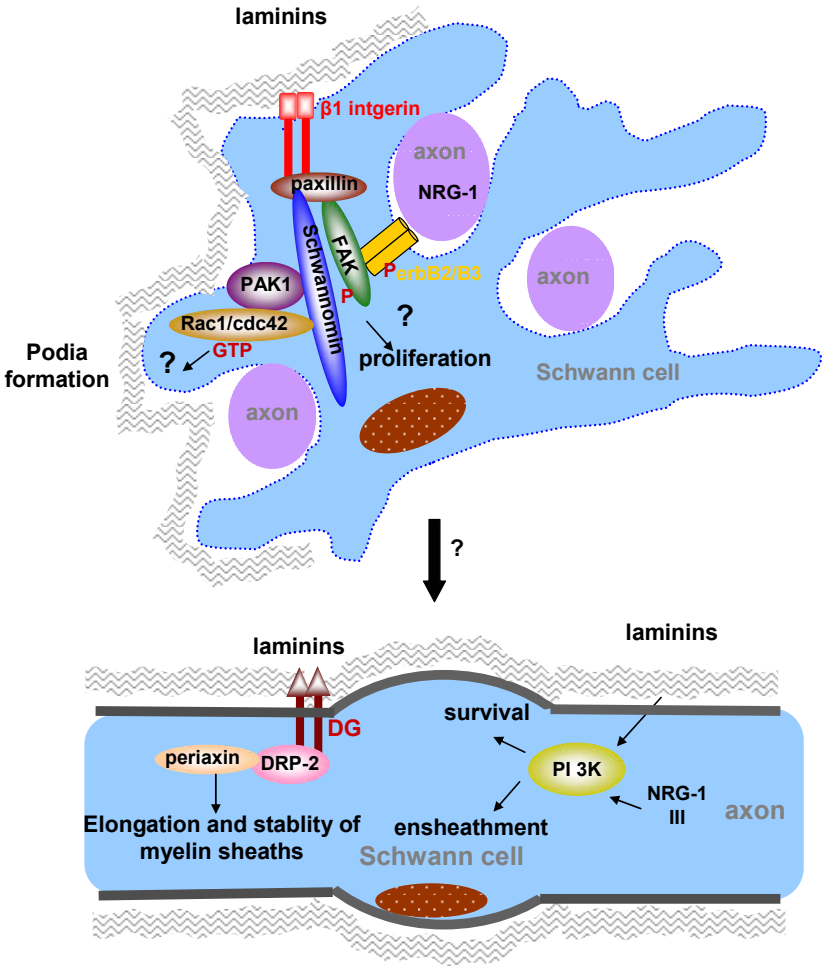


Figure 28. Proposed mechanism for the function of laminins in PNS development.

Laminin deposition enables Schwann cells to form processes that initiate axonal sorting and mediate axon-Schwann cell interaction. Axon-Schwann cell interactions allow Schwann cells to be exposed to axon-derived signals for proliferation and further differentiation. Schwann cell proliferation enables Schwann cells to establish a 1:1 relationship with an individual axon. Both the process formation and proliferation of Schwann cells could be regulated through the formation of a multiple molecular complex between β1 integrins and erbB receptors. β1 integrins expressed on Schwann cells interact with laminins to stabilize the cytoplasmic processes and facilitate the completion of the radial sorting of axons. After completion of axonal sorting and the initial stage of ensheathment, laminins may regulate Schwann cell elongation and stabilize myelin sheaths through the formation of a dystroglycan/Drp2/periaxin complex. Laminins in Schwann cell basal lamina also provide a long-term survival signal mediated by PI 3-kinase/Akt activities to maintain Schwann cell viability.

4.6 Differentiation of non-myelinating SCs in P_0/Cre:fLAMγ1 mice

Differentiation of non-myelinating SCs in mutant peripheral nerves was impaired (Fig. 26 and 27). There are several possibilities as to how the disruption of laminins may influence the differentiation of nonmyelinating SCs. First, the impaired radial sorting of axons impedes further differentiation of non-myelinating SCs. Non-myelinating SCs appear late in the PNS, at approximately P15-20 (Arroyo et al., 1998; Berti et al., 2006), and they can only ensheath a definite number of small caliber axons (5~30 axons) (Friede and Samorajski, 1968). Development of myelinating SCs precedes nonmyelinating SCs, and nonmyelinating SCs enwrap multiple small caliber axons only after the myelinating SCs reach a 1:1 ratio with individual large axons (Eccleston et al., 1987). The impaired radial sorting of axons may inhibit the "sorting out" of small caliber axons or the "presorting" of larger bundles to smaller bundles, and may therefore prevent the differentiation of nonmyelinating SCs. This possibility can be addressed by using an inducible P_0 promoter driving Cre expression (*P_0Cx-CreERT2*) (Leone et al., 2003) to specifically disrupt laminins in the PNS after radial sorting of axons is complete. Second, the failure of SCs to be exposed to axon-derived signals inhibits non-myelinating SC differentiation. The amount of NRG1 type III in axons determines the ensheathment fate of axons (Taveggia et al., 2005). Low levels of NRG1 type III are required for nonmyelinating SCs to ensheath several small axons, whereas high levels of NRG1 type III are required for myelinating SCs to myelinate large axons. Lack of provision of axon-derived NRG1 in laminin-deficient SCs (Fig. 14) may result in inappropriate differentiation

of both myelinating and nonmyelinating SCs (Figs. 8, 11, and 27). This issue can be addressed by over-expressing NRG1 in mutant peripheral nerves using a transgenic mouse line, *Tg(Thy1-Nrg1)* (Michailov et al., 2004). Third, laminins may mediate the clustering of L1 and N-CAM in the cell membrane during postnatal PNS development, which is an essential step for nonmyelinating SC differentiation. The homophilic and heterophilic interactions of L1 and N-CAM between unmyelinated axons and nonmyelinating SCs are important for non-myelinating SC differentiation (Haney et al., 1999; Martini and Schachner, 1986; Martini and Schachner, 1988). L1 interacts with integrins through the sixth Ig domain (L1-6D), and mice lacking the L1-6D lose L1-integrin interactions and L1-L1 homophilic adhesion (Itoh et al., 2005). Disruption of laminins in SCs may destabilize the homophilic and heterophilic interaction of L1 and N-CAM and sequester these adhesion molecules from the cell membrane (Fig. 27). To address this possibility, the integrity of the L1-integrin interaction can be assessed in SCs lacking laminins using co-immunoprecipitaion. The clustering of L1 and N-CAM in the SC membrane can be determined by comparing the fraction of membrane-bound L1/N-CAM with the intracellular fraction of L1/N-CAM using extracts from mutant nerves after *in vivo* biotinylation of L1/N-CAM (Roesli et al., 2006).

4.7 Laminins and their signaling components in human hereditary peripheral neuropathies

Mutations in laminin α2 cause Merosin Deficient Congenital Muscular Dystrophy in humans (Helbling-Leclerc et al., 1995) (CMD1A), which is the most common type of congenital muscular dystrophy. These mutations result in deficient or non-functional laminin 2. In CMD1A patients, both muscle and peripheral nerves are affected, and the phenotypes are a combination of nerve and muscular pathology. Muscle-specific expression of a human α2 laminin transgene in laminin α2-deficient (*dystrophic*) mice greatly improves muscle pathology. However, these animals still exhibit progressive hind limb paralysis, which may be due to the uncured peripheral nerve hypomyelination (Kuang et al., 1998). This suggests that deficiency of laminins contribute an important role to the pathogenesis of the PNS in this disease. In addition to CMD1A, mutations of laminin signaling components also contribute to the pathogenesis of other heritable peripheral neuropathies such as Charcot-Marie-Tooth 4F (mutations in *periaxin* gene) and neurofibromatosis (mutation in *NF2/schwannomin* gene) (Feltri and Wrabetz, 2005). Studies from mice and co-cultures lacking laminins in SCs provide extensive evidence that laminins play multiple essential roles during the various aspects of PNS development, including proliferation, survival, and differentiation of SCs. A better understanding of the mechanisms of how laminins affect PNS development and myelination could provide insight into these peripheral neuropathies and suggest new approaches to their therapies.

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