A Dual Role of *erbB2* in Myelination and in Expansion of the Schwann Cell Precursor Pool

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Abstract. Neuregulin-1 provides an important axonally derived signal for the survival and growth of developing Schwann cells, which is transmitted by the ErbB2/ErbB3 receptor tyrosine kinases. Null mutations of the *neuregulin-1*, *erbB2*, or *erbB3* mouse genes cause severe deficits in early Schwann cell development. Here, we employ Cre-*loxP* technology to introduce *erbB2* mutations late in Schwann cell development, using a *Krox20-cre* allele. Cre-mediated *erbB2* ablation occurs perinatally in peripheral nerves, but already at E11 within spinal roots. The mutant mice exhibit a widespread peripheral neuropathy characterized by abnormally thin myelin sheaths, containing fewer myelin wraps. In addition, in spinal roots the Schwann cell precursor pool is not correctly established. Thus, the Neuregulin signaling system functions during multiple stages of Schwann cell development and is essential for correct myelination. The thickness of the myelin sheath is determined by the axon diameter, and we suggest that trophic signals provided by the nerve determine the number of times a Schwann cell wraps an axon.

Key words: cre-loxP • neuregulin • myelin • glia • neuropathy

Introduction

Neural crest cells constitute a migratory and pluripotent cell population that emerges from the dorsal neural tube. Upon reaching their target sites, neural crest cells differentiate into various cell types, including Schwann cells, which is a population of glial cells that ensheathes axons of sensory and motoneurons (Le Douarin, 1982). Schwann cell precursors migrate and proliferate along preexisting axon tracts during development, and progress through a series of defined stages that are characterized by the expression of specific genes encoding proteins such as P_0, S100, SCIP, or Krox20 (Mirsky and Jessen, 1996; Zorick and Lemke, 1996). Proliferation, survival, and differentiation of Schwann cell precursors depend critically on signals that are provided by the associated axons. During the first week of postnatal life, the majority of Schwann cells destined to myelinate can already be distinguished morphologically in mice. They establish a 1:1 relationship with the accompanying axon and cease to proliferate, but remain capable of entering the cell cycle, a property important in adult regeneration processes (Zorick and Lemke, 1996). The myelination program in Schwann cells is characterized by the expression of specific transcription factor genes like *SCIP* (also known as *Oct-6* or *Tst-1*) and *Krox20* (also known as *Egr-2*; Chavrier et al., 1988; He et al., 1989; Monuki et al., 1989; Wilkinson et al., 1989; Suzuki et al., 1990). Genetic analysis in mice shows that SCIP determines the correct onset of myelination, whereas Krox20 is required for proper ensheathment of the axon and for the expression of genes encoding myelin proteins (Topilko et al., 1994; Weinstein et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996; Zorick et al., 1999). Myelin proteins are major constituents of the myelin sheath, and the molecular composition of the sheath as well as its appropriate thickness are essential for the normal functioning of a nerve fiber. Mutations in humans and rodents can cause neuropathies accompanied by myelin defects, and are typically associated with a reduced thickness of myelin and a disturbed transmission of the action potential along the affected nerves (Nave, 1994; Martini and Schachner, 1997; Scherer, 1997; Suter, 1997).

Early Schwann cell precursors rely on axonal signals for maintenance, proliferation, and differentiation. One neuronal signal that controls survival and proliferation is provided by Neuregulin-1, also named GGF, for glial growth factor (Raff et al., 1978; Lemke and Brockes, 1984; Goodearl et al., 1993; Marchionni et al., 1993). Alternative names used for this factor are NDF (Neu differentiation factor), Heregulin, or ARIA (acetylcholine receptor inducing activity; for reviews see Alroy and Yarden, 1997;

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Burden and Yarden, 1997). Neuregulin-1 is produced by sensory and motoneurons as a transmembrane molecule inserted into axonal membranes (Ho et al., 1995; Bermingham-McDonogh et al., 1997; Yang et al., 1998). This axon-derived signal is recognized by Schwann cells via a receptor tyrosine kinase composed of a heterodimer of ErbB2 and ErbB3, which signals through tyrosine phosphorylation (Levi et al., 1995; Morrissey et al., 1995; Grinspan et al., 1996; Syroid et al., 1996; Vartanian et al., 1997). This concept is supported by genetic studies in the mouse. Mice with mutations in *neuregulin-1*, *erbB2*, or *erbB3* all show severe reductions in the numbers of early Schwann cell precursors; at later developmental stages, *erbB3* and *erbB2* mutants lack Schwann cells (Meyer and Birchmeier, 1995; Erickson et al., 1997; Meyer et al., 1997; Riethmacher et al., 1997; Britsch et al., 1998; Woldeyesus et al., 1999; Morris et al., 1999).

The time period during which Schwann cell precursors critically depend on Neuregulin-1 for proliferation and survival ends with the transition from an early precursor to a more mature, differentiating Schwann cell (Dong et al., 1995; Grinspan et al., 1996; Syroid et al., 1996; Murphy et al., 1996). After this transition, the differentiating Schwann cells generate survival factors in an autocrine loop, and become independent of Neuregulin-1, although they are still able to respond to the factor (Rosenbaum et al., 1997; Cheng et al., 1998; Meier et al., 1999; Syroid et al., 1999). Interestingly, *neuregulin*-1 continues to be expressed in sensory and motoneurons even in adulthood, and both differentiating and mature Schwann cells continue to express the Neuregulin receptor genes *erbB2* and *erbB3*, albeit *erbB2* is expressed at reduced levels (Chen et al., 1994; Corfas et al., 1995; Grinspan et al., 1996). We investigate here the functions of the Neuregulin signaling system in myelinating Schwann cells by the use of a Cre-recombinase–induced *erbB2* mutation. We observe severe defects in myelination, which results in the formation of abnormally thin myelin sheaths. This correlates with ataxia, tremor and wasting of the animals. Moreover, a postnatal loss of motor axons occurs. Thus, the Neuregulin signaling system not only regulates Schwann cell numbers, but is also necessary for formation of an adequate myelin sheath.

Materials and Methods

Generation of a Targeting Vector and erbB2^flox Strain of Mice

The isolation of genomic *erbB2* DNA derived from the 129 mouse strain has been described (Britsch et al., 1998). Oligonucleotides encoding the *loxP* sequence together with an additional EcoRV site were inserted 5′ of exon p. A neomycin cassette flanked by *loxP* sites was inserted 3′ of exon n (see Fig. 1 A). The *erbB2^flox^neo^flox^* targeting vector was electroporated into E14.1 embryonic stem (ES) cells; homologous recombination events were enriched by selection with G418, and identified by Southern blot hybridization using an external genomic probe located 5′ to exon r (data not shown). As described previously (Torres and Kühn, 1997), independent ES cell clones heterozygous for the *erbB2^flox^neo^flox^* allele (see Fig. 1 A) were electroporated with pICcre; colonies were screened by Southern blot hybridization using probe 1 (see Fig. 1 A). Two colonies that contained the *erbB2^flox^* allele derived from independent parental *erbB2^flox^neo^flox^* clones were used for a generation of mice that carry this allele as described (Riethmacher et al., 1997). Homozygous *erbB2^flox^* animals appeared normal and were fertile. To establish the *erbB2^Δ^* strain, *erbB2^flox^*

homozygotes were crossed with *deleter* mice (Schwenk et al., 1995). Cre-mediated deletion of the floxed exons p-n removes 362 nucleotides of coding sequence and, thus, introduces a frameshift mutation. The predicted protein product encoded by the *erbB2^Δ^* allele contains the first 386 amino acids of ErbB2 followed by 51 amino acids from an altered reading frame. Mice heterozygous for the *erbB2^Δ^* allele were bred with *erbB2*+/− mice; all embryos with the genotype *erbB2^Δ^*/− identified at E11.5 were dead as judged by the absence of heartbeat and signs of resorption. Histological analysis of E10.5 *erbB2^Δ^*/− embryos showed the previously described phenotypes, i.e., lack of trabeculation and abnormal cranial sensory ganglia. All analyses of conditional *erbB2* animals were carried out on the mixed C57BL/6/129 background. As controls for *Krox20-cre*/+; *erbB2^flox^*/− animals, littermates with a genotype *Krox20-cre*/+; *erbB2^flox^*/+ were routinely used. Littermates with genotypes *Krox20*+/+; *erbB2^flox^*/−, or *Krox20*+/+; *erbB2^flox^*/+, and also *Krox20-cre*/+; *erbB2*+/− animals were analyzed by light microscopy and thin myelin was not apparent.

Determination of Recombination Specificity and Efficiency

Tissues from 6-wk-old mice double heterozygous for a reporter-*lacZ* allele and *Krox20-cre* were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)[1] as described (Akagi et al., 1997). Blue staining indicative of Cre-mediated recombination was observed in peripheral nerves, hair follicles, and cartilage in which *Krox20* expression has been described (Levi et al., 1996). Bundles of fibers from sciatic nerves were teased, wholemount stained with 4′,6-diamidino-2-phenylindole (DAPI), and cleared in 80% glycerol in PBS. Alternatively, the nerve sheaths were disrupted to enhance fixation and allow access of X-gal to Schwann cells. The nerves were osmicated and embedded in resin (see also below), and 1-μm sections were counterstained with neutral red and eosin. For immunohistochemical analysis, teased nerves from *Krox20-cre*/+; reporter-*lacZ* animals were fixed for 20 min at 4°C in 4% paraformaldehyde (PFA), blocked with 10% goat serum in PBS containing 0.5% Triton X-100, and incubated with monoclonal anti-myelin basic protein antibody (Boehringer Mannheim) and rabbit polyclonal anti–β-galactosidase antibody (ICN Cappel). FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (Dianova) were used as secondary antibodies and nuclei were counterstained with DAPI. Genomic DNA of tissues from adult *Krox20-cre*/+; *erbB2^flox^*/+ animals was used for Southern blot hybridization; spinal roots were pooled from several animals to allow detection of *erbB2* alleles.

Histology, Electron Microscopy, and Axon/Schwann Cell Counts

Nerves were isolated from animals perfused with 2.5% glutaraldehyde in phosphate buffer, postfixed, and contrasted with osmium tetroxide as described (Topilko et al., 1994). For light microscopy, nerves were embedded in Technovit 7100 resin (Kulzer); 1-μm sections were stained with toluidine blue. Preparation for electron microscopy was done as described (Topilko et al., 1994). Numbers of axons in the fifth lumbar segment (L5) ventral roots were determined from composite light micrographs. The axon numbers determined in individual mutant animals were 549, 679, 721, 739, 773, 778, 800, 855, 887, 1,014 and 1,096; in control animals, 956, 968, 974, 1,026, 1,092 and 1,103 axons were counted. Neuron numbers in the dorsal root ganglia were determined as described (Riethmacher et al., 1997). Numbers of Schwann cell nuclei associated with nerves were determined from 1-μm sections.

Determination of Apoptosis and Proliferation Rates in Nerves

Animals between postnatal day 3.5 and 15 (P3.5 and P15, respectively) were injected once intraperitoneally with bromodeoxyuridine (BrdU; 100 μg/g body weight) and killed 80 min after injection. To increase numbers of BrdU-positive cells in nerves from 5-wk-old and adult animals, multiple BrdU injections (1 each day, over 4 d) were used. Animals were perfused

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[1]*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; ES, embryonic stem; L4, L5, fourth and fifth lumbar segments, respectively; P3.5, P15, postnatal day 3.5 and 15, respectively; PFA, paraformaldehyde; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

with 4% PFA in PBS, nerves were dissected, postfixed for 2–4 h, and embedded in OCT compound (Sakura). Frozen sections (6 μm) were processed for TUNEL staining using the ApopTag kit (Oncor); BrdU-positive nuclei were detected using a monoclonal anti-BrdU antibody (Sigma Chemical Co.). Sections were counterstained with DAPI (Sigma Chemical Co.) to determine overall numbers of Schwann cell nuclei.

Protein Extraction and Western Blot Analysis

Sciatic nerves were dissected, flash-frozen in liquid nitrogen, and stored at –70°C. Total protein lysates of nerves were prepared by sonication in Laemmli sample buffer (Laemmli, 1970). Samples were separated in 12.5% acrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Western blotting was performed using rabbit polyclonal antibodies directed against Krox20, SCIP, or PMP22, or a mouse mAb directed against P_0; for detection, an ECL kit (Amersham) was used.

Preparation of Digital Images

Southern and Western blots, hybridized with ^{32}P-labeled probes, or incubated with ECL reagents, respectively, were exposed to Kodak Bio-Max film and scanned into Adobe Photoshop® 3.0. PhosphorImage scans of Southern blots were analyzed using the software package TINA 2.08e© 1993 Raytest Isotopenmeßgeräte GmbH. Adobe Photoshop® 3.0 was also used to prepare the optical and electron micrograph images.

Results

Conditional Mutagenesis of erbB2

We generated an *erbB2^{flox}* allele in which two *loxP* sites flank three exons encoding part of the extracellular domain of the receptor. We chose these exons since their deletion introduces a frameshift mutation, which results in the production of an mRNA that encodes a truncated receptor. A two-step procedure was employed to generate the floxed allele in ES cells (Fig. 1; see also Gu et al., 1993). The mutant ES cells were used to generate mice that carry the *erbB2^{flox}* allele. Mice homozygous for the *erbB2^{flox}* allele appeared normal and were fertile. The *erbB2^{flox}* allele was crossed into a *cre*-deleter strain (Schwenk et al., 1995). The resulting *erbB2$^{\Delta}$* allele (Fig. 1 A) was combined with the previously described genetical-null allele of *erbB2* (Britsch et al., 1998). *ErbB2−/erbB2$^{\Delta}$* mice die before E11.5 and are phenotypically indistinguishable from *erbB2−/−* mice, indicating that Cre-induced recombination of the *erbB2^{flox}* allele generates a nonfunctional *erbB2* gene.

The *Krox20* gene encodes a transcription factor expressed in promyelinating and myelinating Schwann cells and, thus, appears from ~E16 in peripheral nerves (Topilko et al., 1994). Furthermore, Schwann cell precursors that reside in the spinal roots express *Krox20* earlier, starting ~E11. To mutate *erbB2*, we used a mouse strain in which *cre* was introduced into the *Krox20* locus by homologous recombination. In this strain, *cre* expression faithfully reproduces the expression pattern of the endogenous *Krox20* gene; its detailed characterization is reported elsewhere (Voiculescu et al., 2000). The *Krox20-cre* allele was combined with a reporter allele, a *lacZ* gene which is expressed upon Cre-induced recombination (Akagi et al., 1997). In teased preparations of the sciatic and other nerves of such mice, we observed β-galactosidase staining in the majority of cells with elongated nuclei that were spaced at regular intervals along the nerve. In contrast, more closely and irregularly spaced cells were not stained

(Fig. 2, A and B). Sectioning of nerves, complemented by immunohistochemical analysis of teased fibers using antibodies directed against β-galactosidase and myelin basic protein confirmed the identity of the stained cells as myelinating Schwann cells (Fig. 2, C–E). Southern hybridization was used to quantify recombination of the *erbB2^{flox}* allele, and showed that recombination occurred in 40–50% of cells associated with sciatic nerves and spinal roots in animals with the genotype *Krox20-cre/+*; *erbB2^{flox}/+* (Fig. 2 F). It should be noted that these nerves contain significant numbers of nonmyelinating Schwann cells, connective tissue, blood cells, and vessels in which recombination does not occur. Recombination was also observed in the skin and ear: hair follicles express *Krox20*, accounting for the recombination in these tissues (Levi et al., 1996). No recombination was detected in the spinal cord.

ErbB2 Is Required for Correct Myelination of Peripheral Nerves

Conditional mutant animals with the genotype *Krox20-cre/+*; *erbB2^{flox}/−* were viable, but displayed various behavioral abnormalities within the first weeks of postnatal life. Alterations included kinked or serpentine tails, gait abnormalities, difficulties in hindlimb movement, and wasting associated with weight loss and premature death (5 out of a group of 37 mutants died within 6 mo). Such behavioral abnormalities were observed in all conditional mutants, but were variable in the time of onset and severity. Control animals, for instance animals with the genotype *Krox20-cre/+*; *erbB2+/−*, did not display these behavioral phenotypes.

Examination of the sciatic nerves from Cre-induced mutants showed no gross changes in morphology or histology at P3.5 (Fig. 3 A, compare to B), indicating that the Schwann cell precursor pool in this nerve is established. However, sciatic nerves of mutants at P15 were translucent and thin. Histologically, a strikingly reduced thickness of myelin sheaths was apparent (Fig. 3, C and D). Reduced thickness of the myelin sheaths persisted, and was also observed at 6 or 14 mo (Fig. 3, E–J, Fig. 4, arrowheads, and data not shown). Myelin of normal thickness (Fig. 3 E, arrow) was found only in $1.7 \pm 1.0\%$ of all myelinated axons in the sciatic nerve. Thus, the *Krox20-cre*–induced *erbB2* mutation reproducibly affected virtually all myelinating Schwann cells. Thin myelin was not observed in control mice with a genotype *Krox20-cre/+*; *erbB2+/−*, or *Krox20-cre/+*; *erbB2^{flox}/+*, or *Krox20+/+*; *erbB2^{flox}/−*, or *Krox20+/+*; *erbB2^{flox}/+*. Electron micrographs were used to quantify myelin thickness (Fig. 4). In 6-mo-old animals, the thickness of myelin was reduced two- to threefold. This is reflected in G ratios (ratios of axon diameters to fiber diameters) of 0.83 ± 0.04 in mutant, and 0.68 ± 0.04 in control sciatic nerves ($P < 10^{-6}$). Hypomyelination in sciatic, sural, saphenous nerves and nerves innervating lower leg muscles were observed in all mutant animals examined (28 animals). In the spinal roots, the phenotype was more dramatic (see below). The thin myelin was due to the presence of fewer wraps of myelin around the axon.

In addition, we observed occasionally large-caliber axons lacking myelin that were still surrounded by a Schwann cell basal lamina in the sciatic nerve (Fig. 4 C,
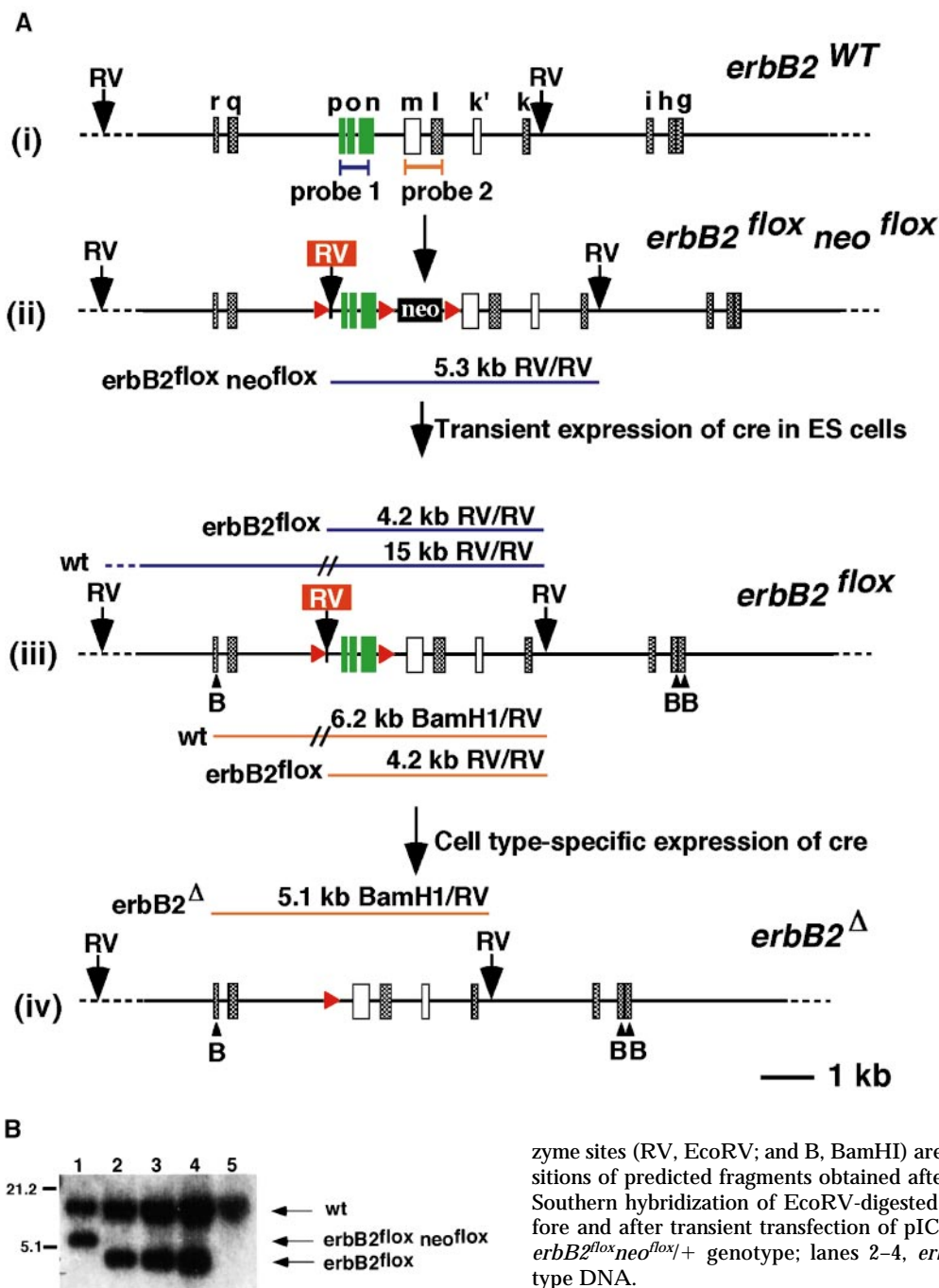
Figure 1. Strategy employed for the generation of the *erbB2^flox^* allele. (A) The structure of the wild-type erbB2 gene is shown at the top (i). The *erbB2^flox^neo^flox^* allele (ii) was generated by homologous recombination in ES cells. In this allele, three exons (green) are flanked by *loxP* sequences (red arrowheads). The 5′-most *loxP* sequence also contains, at its border, an EcoRV site shown in red. In addition, the neomycin resistance cassette (neo) is present that is also surrounded by *loxP* sequences. The *erbB2^flox^* allele (iii) was generated by transient expression of Cre in ES cells; in this allele, the neo-cassette has been removed, but two *loxP* sites that flank three exons of *erbB2* are retained. The *erbB2^Δ^* allele (iv) is obtained after Cre-induced recombination of *erbB2^flox^*. Exons are indicated by boxes and lettered (see Britsch et al., 1998); black hatched and green exons were sequenced directly, whereas the sequences of white exons were inferred from the cDNA. Probes 1 and 2 are indicated and were used for Southern hybridization in Fig. 1 B and Fig. 2 A, respectively. Restriction enzyme sites (RV, EcoRV; and B, BamHI) are indicated, as are the sizes and positions of predicted fragments obtained after digestion of genomic DNA. (B) Southern hybridization of EcoRV-digested genomic DNA from ES cells before and after transient transfection of pICcre. Lane 1, parental ES cell with *erbB2^flox^neo^flox^/+* genotype; lanes 2–4, *erbB2^flox^/+* cells; and lane 5, wild-type DNA.

arrow and inset). Such Schwann cells frequently ensheathed several axons (Fig. 3, G and I, arrows). These profiles were more frequent in nerves innervating muscle than in cutaneous sensory nerves. Extensive patches of amyelinated axons were observed in some Cre-induced mutants (Fig. 3 I). The histology of nonmyelinating bundles appeared normal (Fig. 4, asterisks).

In early postnatal stages or in the embryo, a more severe phenotype was observed in dorsal and ventral spinal roots. These nerves display a severe lack of Schwann cells in all animals with *Krox20-cre*–induced *erbB2* mutations at P3.5 and at P15 (10 examined; Table I, Fig. 5, and data not

shown). In accordance, myelin was absent in ventral roots, whereas thin myelin sheaths were surrounding a small proportion of axons in dorsal roots. This coincides with the early onset of *Krox20-cre* expression in these cells, revealing the early requirement for ErbB2 in establishment of the Schwann cell precursor pool. As animals reached adulthood, Schwann cell numbers increased, but the myelin sheaths formed were thin (Fig. 5). Localized patches of amyelinated axons persisted. Moreover, a single perineurium surrounds the entire spinal root in control animals, whereas perineurial sheaths compartmentalized the roots into six to seven branches in mutants (Fig. 5, arrowheads).
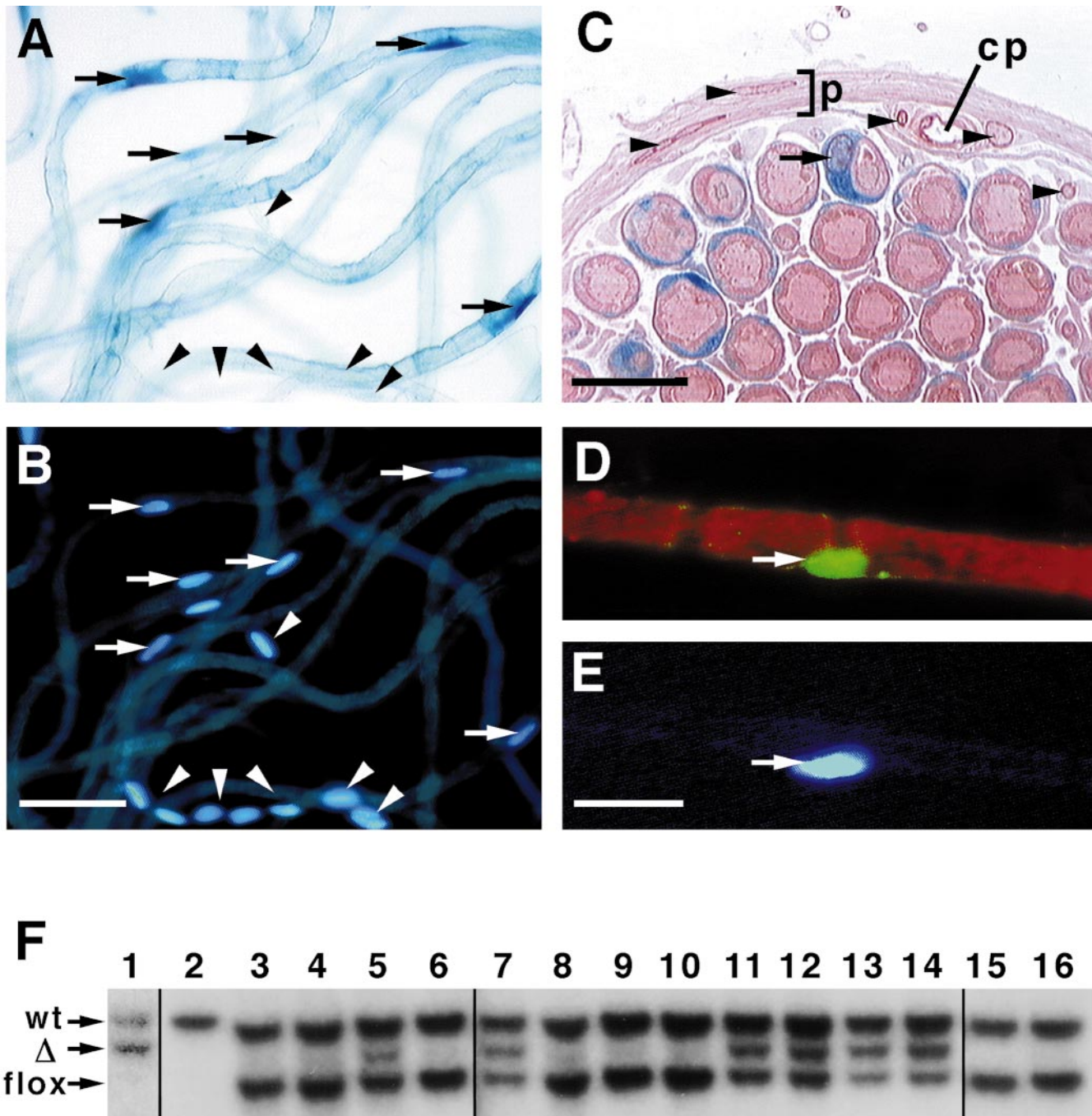
Figure 2. Tissue specificity of *Krox20-cre*–induced mutations. (A–E) Nerves from reporter *lacZ* mice (see Akagi et al., 1997) that also contain a *Krox20-cre* allele were stained for β-galactosidase or β-galactosidase activity. (A and B) Teased nerves stained with X-gal (A) and DAPI (B). (C) X-gal–stained nerves were also osmicated, sectioned, and counterstained with neutral red and eosin. The myelin and axon of an individual nerve fiber stained dark and light red, respectively. (D and E) Teased nerve fiber triple stained with antibodies against myelin basic protein (D, red), β-galactosidase (D, green), and DAPI (E, blue). Arrows point towards β-gal–positive Schwann cells; arrowheads point towards other cells and nuclei that do not stain; perineurium (p) and capillaries (cp). (F) Southern hybridization analysis of DNA digested with BamHI and EcoRV. Lane 1, *erbB2$^\Delta$/+*; lane 2, wild type; lanes 9 and 10, *erbB2^{flox}/+*; all other lanes contain DNA obtained from tissues of mice with a genotype *Krox20-cre/+*; *erbB2^{flox}/+*. Lane 3, liver; lane 4, muscle; lane 5, ear; lane 6, lung; lane 7, skin; lane 8, spleen; lanes 11 and 12, sciatic nerves; lanes 13 and 14 spinal roots; lane 15, spinal cord; and lane 16, ovaries. Densitometric analysis showed that recombination occurred in 40–50% of cells associated with peripheral nerves. Bars: (B) 50 μm; (C and E) 20 μm.

Biochemical Characteristics and Growth Properties of Schwann Cells in Conditional erbB2 Mutants

We determined the absolute numbers of Schwann cells in sciatic nerves and spinal roots. In the sciatic nerves of animals at all stages tested (P3.5, P15, and 6 mo), Schwann cell numbers were comparable in the control and Cre-induced mutant mice (Table I). In contrast, in dorsal and
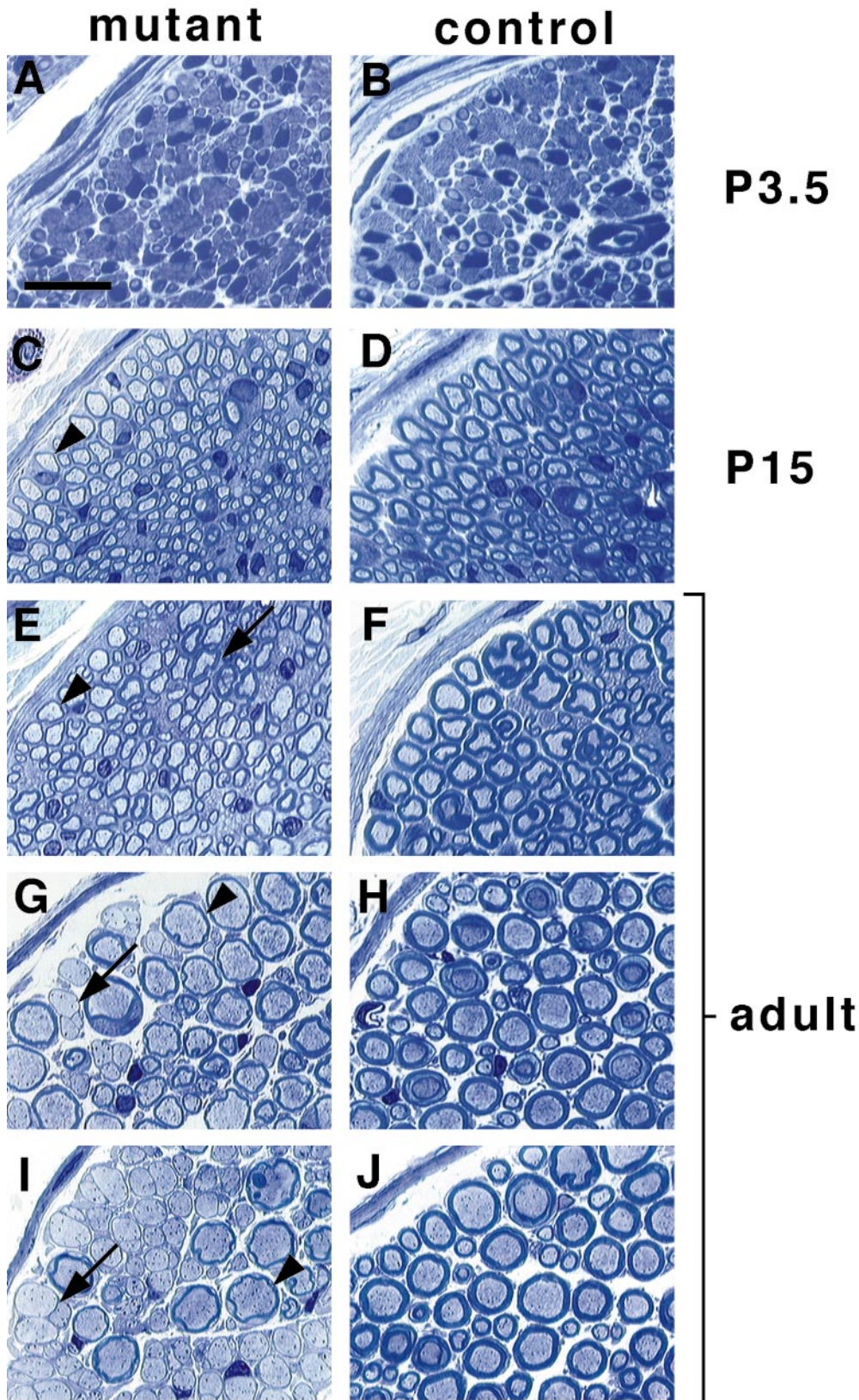
Figure 3. Peripheral nerve histology in mice with conditional *erbB2* mutation. Semithin sections of peripheral nerves from *Krox20-cre*/+; *erbB2flox*/− (A, C, E, G, and I) and control (B, D, F, H, and J) animals. The age of the animals is indicated. Shown are the tibial (A–D) and sural (E and F) branches of the sciatic nerve and nerves innervating lower leg muscles (G–J). Arrowheads (C, E, G, and I) point towards axons with abnormally thin myelin sheaths. The arrow in E points towards rare axons with myelin of normal thickness; arrows in G and I point towards large diameter axons that are not myelinated. Bar, 20 μm.

ventral roots, the numbers of cells were severely reduced. At P3.5, a 10-fold reduction was observed in the ventral, and a 5-fold reduction in the dorsal roots of the mutant mice. This reduction was still apparent at P15, and again more pronounced in ventral than in dorsal roots (Table I). At 6 mo, numbers were similar in the dorsal and ventral roots of control and mutant mice. Thus, compensatory mechanisms exist that allow repopulation of spinal roots

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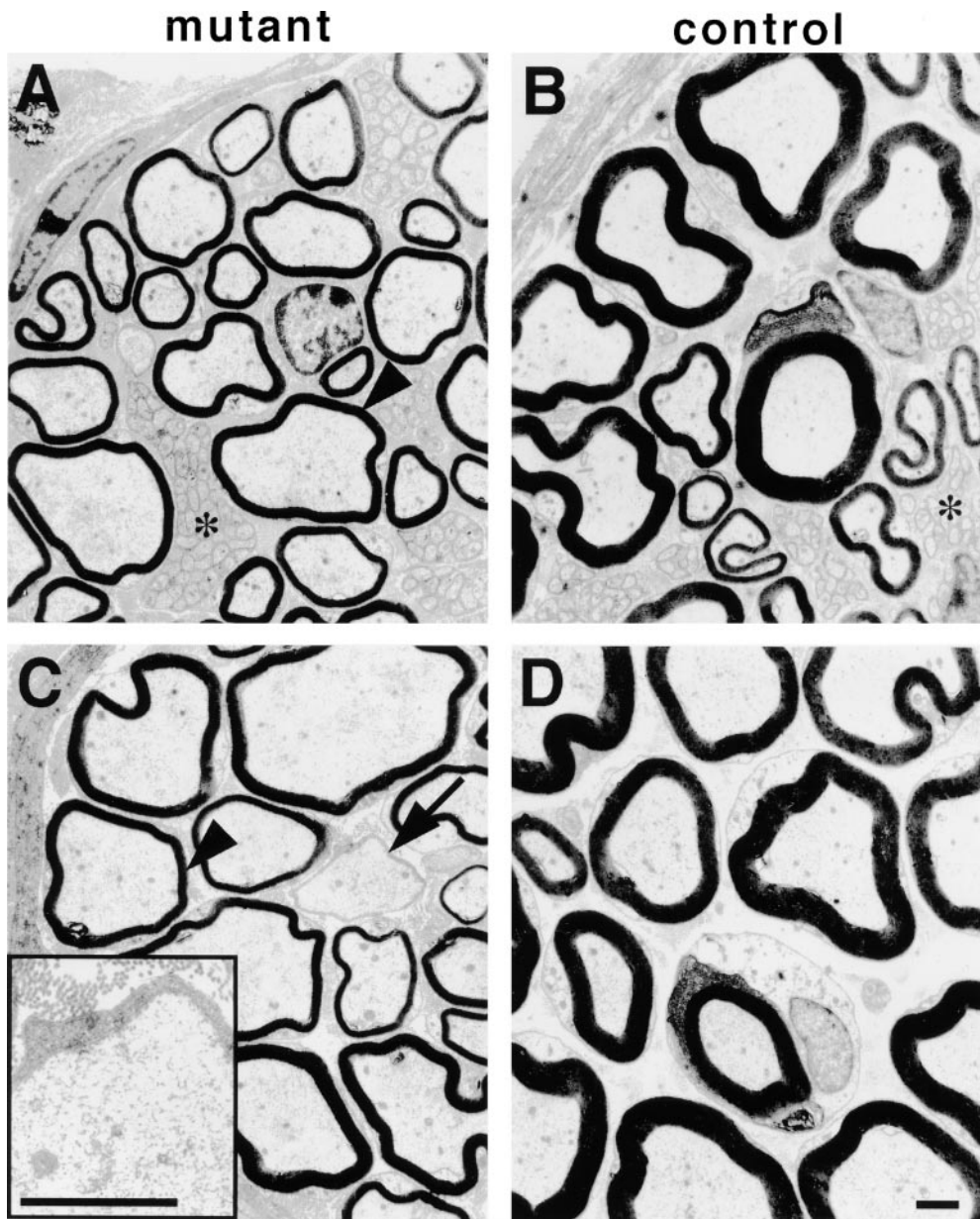
mutant

control

Figure 4. Electron microscopy of sciatic nerve branches from conditional *erbB2* mutants. Electron microscopic analysis of peripheral nerves from *Krox20-cre*/+; *erbB2*^{flox}/− (A and C) and control (B and D) animals at 6 mo of age. Shown are sural nerves (A and B) and nerves innervating lower leg muscles (C and D). Arrowheads (A and C) point towards abnormally thin myelin sheaths in nerves from mutant mice; the arrow in C indicates a large diameter axon surrounded by a basal lamina, which is also shown enlarged in the inset. Asterisks denote bundles of non-myelinated small diameter axons. Bars, 1 μm.

with Schwann cells; this repopulation occurs earlier in dorsal than in ventral roots.

Schwann cell proliferation and apoptosis determine the absolute numbers of Schwann cells, and were analyzed using BrdU incorporation and TUNEL labeling, respectively. Compared with the controls, the numbers of BrdU-positive cells were increased in the sciatic nerves of mutants at P3.5 and 5 wk (Fig. 6 A). The numbers of apoptotic nuclei were marginally increased at P3.5 (Fig. 6 B). In adults (age 6 mo), proliferation as well as apoptosis frequencies were below detection limits. Increased cell proliferation was also observed in ventral roots of 5-wk-old animals.

To assess the effect of the conditional *erbB2* mutation induced by *Krox20-cre* on Krox20 and SCIP protein levels, sciatic nerve extracts were analyzed by Western blotting (Fig. 6 C). The level of Krox20 protein was similar in the nerves of control and *Krox20-cre*–induced *erbB2* mutants.

The level of SCIP protein was slightly increased in mutant nerves at P15, but levels attained baseline in control and mutant nerves at later stages. Levels of myelin protein were reduced in conditional mutants, in accordance with the histology.

Table I. Effect of Krox20-cre–induced Mutation of erbB2 on Schwann Cell Number

	Sciatic		Ventral root		Dorsal root	
	Mutant	Control	Mutant	Control	Mutant	Control
P3.5	564 ± 31	588 ± 27	5 ± 2	51 ± 3	6 ± 2	33 ± 4
P15	395 ± 12	409 ± 21	18 ± 3	74 ± 4	96 ± 10	179 ± 5
P 6 mo	209 ± 33	204 ± 20	40 ± 13	41 ± 7	101 ± 9	112 ± 8

Schwann cell nuclei were counted from semi-thin sections of nerves at the indicated ages; values are displayed as numbers of nuclei per section. The number of nuclei within spinal roots at P3.5 was determined from sections of lumbar blocks (L4-L5 level); at later stages, L5 roots were examined. Data are shown as mean ± SD.

mutant   control

A   B   P15
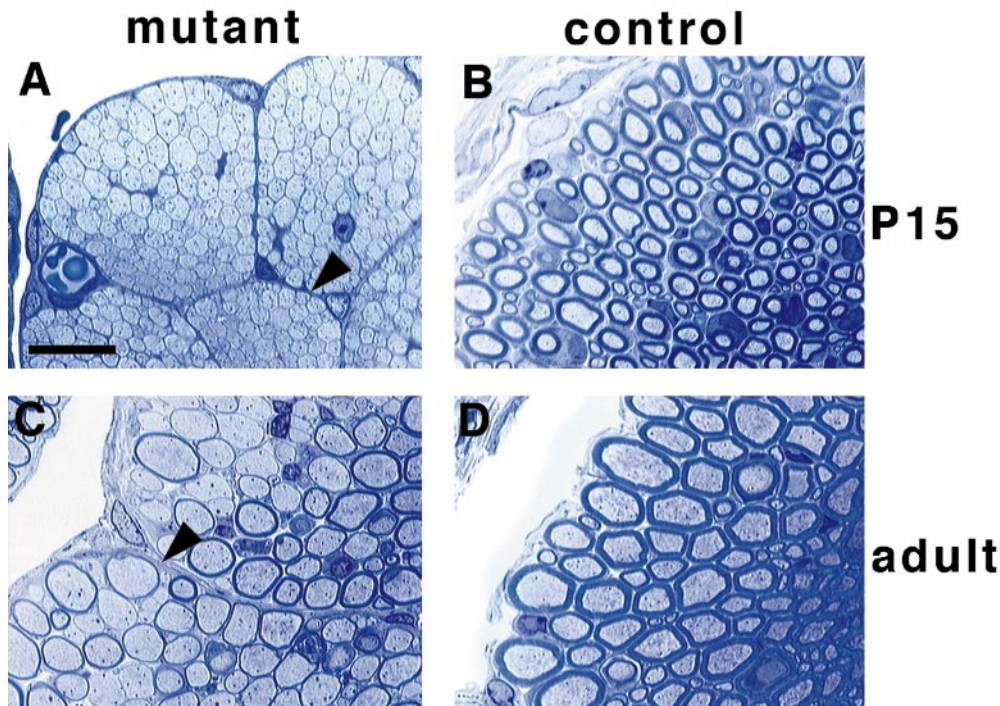
C   D   adult

Figure 5. Histology of spinal nerve roots from conditional *erbB2* mutants. Semi-thin sections of ventral roots from *Krox20-cre*/+; *erbB2*^{flox}/− (A and C) and control (B and D) animals at P15 (A and B) and adult stages (C and D). Arrowheads (A and C) point towards the abnormal perineuria observed in mutant animals. Note that in mutant animals, the extreme lack of Schwann cells observed at P15 is compensated at later stages, although myelin sheaths remain abnormally thin. Bar, 20 μm.

Neuropathology in Krox20-cre–induced erbB2 Mutants

To assess whether neuron numbers were altered in *Krox20-cre*–induced *erbB2* mutant mice, we counted axons in the L5 ventral root, which contains mainly motor axons. The numbers of such axons were frequently reduced in mutant animals, amounting to 20% loss on average. From 11 mutant animals, and 6 control animals examined, the number of axons in the mutant was 808 ± 152, compared with $1,020 \pm 65$ in control nerves ($P < 0.002$). The numbers of neurons in L5 dorsal root ganglia were similar in the control and mutant animals (not shown).

Discussion

The role of Neuregulin-1 as a growth and survival factor during Schwann cell development is well established (Zorick and Lemke, 1996; Jessen and Mirsky, 1999). Neuregulin-1 provides an axonally derived signal important in establishing the Schwann cell precursor pool, which is received in precursor cells by the Neuregulin-1 receptors ErbB2 and ErbB3 (for review see Adlkofer and Lai, 2000). We show here that in addition to its function in the establishment of the precursor pool, the Neuregulin signaling system also plays a role in myelination: abnormally thin myelin sheaths are formed in mice with conditional mutations of *erbB2* in myelinating Schwann cells. To introduce the conditional *erbB2* mutation, we used a *Krox20-cre* allele, but it should be noted that thin myelin is also observed when the mutation is introduced by a *P$_0$-cre* transgene (Garratt, A.N., unpublished observations). The conditional *erbB2* mutations also result in an altered behavior of the affected animals, which display movement abnormalities and loss of motoneurons.

The Role of ErbB2 in Myelination

Mutations that cause an abnormal myelin thickness and neuropathy have been extensively characterized (for reviews see Nave, 1994; Martini and Schachner, 1997; Scherer, 1997; Suter, 1997; Warner et al., 1999). Peripheral hypo- or hypermyelination are observed in patients with Charcot Marie Tooth disease, and can be caused by mutations in genes encoding the myelin proteins PMP22 and P$_0$, the gap junction protein connexin-32 or the transcription factor Krox20. Even incorrect *PMP22* dosage can cause changes in myelination and neuropathy. Mice with targeted or spontaneous mutations in these genes display similar phenotypes. Thus, the thickness of the myelin sheath is strictly controlled, and molecular mechanisms that cause pathological changes are manifold. We show here that hypomyelination and peripheral neuropathy can arise by a novel molecular mechanism, the ablation of the ErbB2 receptor tyrosine kinase. Mutations in signaling molecules have not been observed in patients with hereditary neuropathy. However, afflicted individuals frequently do not carry mutations in known disease causing genes.

ErbB receptor tyrosine kinases modulate the activity of various signaling pathways and are known to affect expression and activity of transcription factors (for reviews see Dougall et al., 1994; Schweitzer and Shilo, 1997; Alroy and Yarden, 1997, Fromm and Burden, 1998; Moghal and Sternberg, 1999). We tested whether the conditional *erbB2* mutation affects the levels of transcription factors known to regulate myelination. Schwann cells arrest wrapping of axons in the absence of Krox20 (Topilko et al., 1994; Zorick et al., 1999). In vitro, Neuregulin-1 does not affect *Krox20* expression directly, but influences the transition from a precursor to a committed Schwann cell that expresses *Krox20* (Murphy et al., 1996). In accordance,
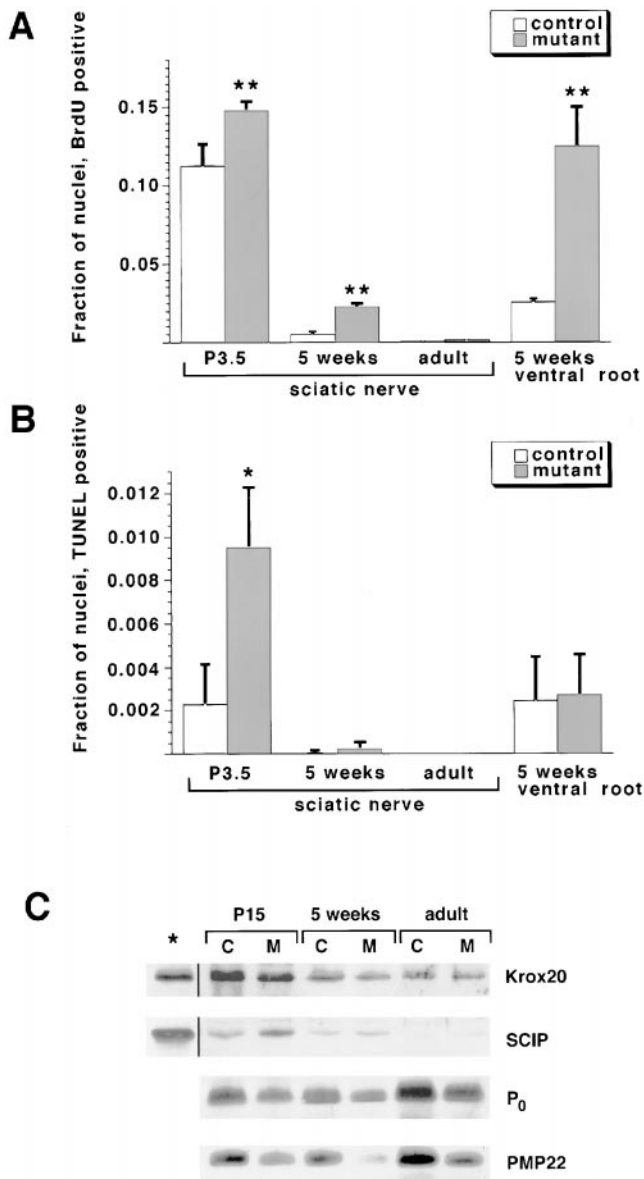
Figure 6. Growth and biochemical properties of Schwann cells in conditional *erbB2* mutants. Fractions of nuclei, which were positive for BrdU (A) or TUNEL (B) in sciatic nerves or in the ventral roots, were determined from control or *Krox20-cre*/+; *erbB2^{flox}*/− mice at the indicated ages. Data are shown as mean ± SD; *$P < 0.17$; **$P < 0.01$. (C) Western analysis of extracts from sciatic nerves using antibodies directed against Krox20, SCIP, P_0, and PMP22. Ages of control (C) and *Krox20-cre*–induced mutant (M) animals are indicated. Asterisk denotes lanes loaded with extracts from COS cells transfected with Krox20 or SCIP expression plasmids.

levels of Krox20 protein are not altered in sciatic nerves of mice with *Krox20-cre*–induced loss of *erbB2*. It should be noted that the conditional *erbB2* ablation occurs after the transition from a precursor to a committed Schwann cell. In *SCIP* mutant mice, myelination is delayed. *SCIP* is expressed in precursors and in Schwann cells during the myelination process, but is downregulated when myelination is completed (Monuki et al., 1989; Jaegle et al., 1996; Zorick et al., 1999). Downregulation of SCIP is delayed in

the sciatic nerves of conditional *erbB2* mutant mice. This correlates with the prolonged growth phase of Schwann cells, and might not reflect a direct effect of ErbB2 on *SCIP* expression.

The number of myelin wraps a Schwann cell forms is determined by the diameter of the accompanying axon. Thus, axons not only provide signals that control growth or differentiation, but also instructive signals that determine the size of the membrane surface of Schwann cells and, thus, the number of myelin wraps. In general, the number of myelin wraps might be controlled by trophic signals provided by the axon. Neuregulin provides such a signal, as wrap numbers are reduced in conditional erbB2 mutants. Large diameter axons, because of the enlarged surface area, can present greater amounts of trophic factors and might, therefore, instruct the Schwann cell to form more wraps. Other trophic signals, in addition to the one provided by the Neuregulin signaling system, might be operative, and the sum of these signals would determine myelin thickness and the number of wraps. It is interesting to note that the *Drosophila* gene *DPTEN* that encodes a protein and PIP3 lipid phosphatase controls both cell and body size (Goberdhan et al., 1999).

Neuregulin Signaling System in Myelinating Schwann Cells

The balance of cell death and growth in peripheral nerves during the early postnatal phase reflects an adjustment of the numbers of Schwann cell precursors (Grinspan et al., 1996; Syroid et al., 1996; Zorick et al., 1999). The correct 1:1 relationship between axons and myelinating Schwann cells is thus attained, supernumerary cells are removed and areas deficient in Schwann cells are replenished. In early postnatal nerves, Schwann cell survival is regulated in part by access to axonally derived Neuregulin-1, and axotomy-induced death of pre- and perisynaptic Schwann cells can be rescued by exogenous Neuregulin-1 (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). In our conditional mutants, the importance of ErbB2/Neuregulin-1 signaling in Schwann cell survival and growth is particularly apparent in spinal roots. There, *Krox20* is expressed earlier (~E11), and the *Krox20-cre* driven mutations are induced earlier than in other peripheral nerves (Topilko et al., 1994; Voiculescu et al., 2000). In the spinal roots, Schwann cells are almost completely absent late in development and into the perinatal period. However, this deficiency is compensated at later stages.

The apoptotic response of Schwann cells to denervation is pronounced in early postnatal life but declines with increasing age (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). In culture, Schwann cells from the postnatal nerve survive without Neuregulin-1, but secrete survival-promoting growth factors like PDGF, neurotrophin-3, and insulin-like growth factor in an autocrine manner (Porter et al., 1987; Meier et al., 1999; Syroid et al., 1999). Autocrine expression of particular isoforms of *neuregulin*-1 also has been reported (Rosenbaum et al., 1997; Carroll et al., 1997). In adult mice with *Krox20-cre*–induced *erbB2* mutations, mature myelinating Schwann cells survive, and we do not detect apoptotic nuclei in the nerves. Thus, neither paracrine nor autocrine

activation of ErbB2 is essential for survival of mature myelinated Schwann cells.

Regeneration of Schwann Cells

The ability of Schwann cells to regenerate is enormous. In experimentally induced allergic neuritis, myelinating Schwann cells are destroyed, but can be effectively regenerated (Saida et al., 1980). This regenerative capacity is particularly evident in mice that express diphtheria toxin A-chain in myelinating Schwann cells. In such animals, myelinating Schwann cells die and are continuously regenerated through proliferation of the nonmyelinating compartment (Messing et al., 1992). In conditional *erbB2* mutant mice, we can still detect single Schwann cells that wrap several large-caliber axons in the adult. Such lesions are not repaired.

We observe a pronounced reduction of Schwann cell numbers in spinal roots of mice with *Krox20-cre*-induced *erbB2* mutations. This severe lack of Schwann cells is compensated during postnatal life, although local patches devoid of Schwann cells remain unrepaired. Interestingly, *Krox20* is not expressed in satellite cells of dorsal root ganglia (Murphy et al., 1996); nevertheless, proliferation is increased in dorsal root ganglia of conditional *erbB2* mutants (Garratt, A.N., unpublished observations). Thus, satellite cells might replenish Schwann cells in the mutants, particularly in the projections of dorsal root ganglia neurons (dorsal roots) where regeneration is faster than in ventral roots.

Neuropathology in Conditional erbB2 Mutant Mice

The conditional mutation of *erbB2* in myelinating Schwann cells results in a moderate loss of motoneurons. Since *erbB2* is not mutated in cells of the spinal cord, this is caused by indirect mechanisms. Axonal retractions were previously noted in mice with defective myelination (Giese et al., 1992; Adlkofer et al., 1995; Griffiths et al., 1998; Frei et al., 1999). In mice with *Krox20-cre*–induced mutations, motoneurons are lost, but sensory neuron numbers are not altered. We observe thin myelin in all those animals and in all nerves. A lack of myelin surrounding large-caliber axons is, however, mainly found in nerves innervating muscle, and is variable in extent in different animals, as is the extent of motoneuron loss. Thus, not thin myelin, but a local lack of myelin in parts of the nerve might cause this damage.

Multiple Functions of Neuregulin in the Schwann Cell Lineage

Neuregulin-1 and its receptors serve several functions in the Schwann cell lineage. The first stage in which this signaling system is required occurs early during development of the lineage. In *neuregulin-1*, *erbB2*, or *erbB3* mutant mice, the population of early Schwann cell precursors is already diminished as they start to populate the spinal nerves (Meyer and Birchmeier, 1995; Erickson et al., 1997; Riethmacher et al., 1997; Britsch et al., 1998; Morris et al., 1999; Woldeyesus et al., 1999). Defective migration of the precursors along the axon might be responsible. It is interesting to note that neural crest cells that form sympathetic

ganglia require *neuregulin*-1, *erbB2*, and *erbB3* for migration (Britsch et al., 1998). However, increased apoptosis and/or a lack of proliferation contribute to the severe Schwann cell phenotype apparent at late stages in null mutants, since precursors that form do not expand. The severe reduction of Schwann cells in spinal roots of conditional *erbB2* mutants appears to be caused by cell death, since *Krox20* expression and, thus, the *Krox20-cre*–induced mutation occurs after precursors reach the roots.

Importantly, we demonstrate here that *erbB2* is also required for correct myelination in Schwann cells. The thin myelin observed in conditional *erbB2* mutant mice is stable, and not repaired. Similarly, local deficits in Schwann cell numbers in peripheral motor nerves are not repaired. Thus, this and other studies provide evidence for a role of *erbB2* during repair of dysmyelinated lesions and regeneration of damaged Schwann cells (Carroll et al., 1997; Kwon et al., 1997; Li et al., 1997; Chen et al., 1998). Therefore, Neuregulin/ErbB signaling functions during the entire life span of a myelinating Schwann cell.

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