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Erbin is required for myelination in regenerated axons after injury

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Abstract

Neuregulin 1 (NRG1) is an axon-derived factor that is critical for Schwann cell (SC) development and myelinogenesis in a manner dependent on transmembrane tyrosine kinases ErbB2 and ErbB3. Recent studies suggest that NRG1 signaling plays a role in remyelination of regenerated nerves after injury. In this study, we investigated the role of Erbin, a protein that interacts with ErbB2 in remyelination of injured nerves. We show that Erbin expression increased dramatically in injured nerves. Myelinated axons were fewer, and *g*-ratios of those that were myelinated were increased in *erbin*^{-/-} mice, which were impaired in functional recovery from nerve injury. These results indicate a necessary role of Erbin in remyelination of regenerating axons. Erbin ablation had little effect on numbers of BrdU-labeled and TUNEL-labeled SCs, suggesting mechanisms independent of altered proliferation or apoptosis. We demonstrated that Erbin mutant mice were impaired in raising or maintaining the levels of ErbB2 and in producing NRG1 in axons. Together, these observations demonstrate that Erbin is required for remyelination of regenerated axons after injury, probably by regulating ErbB2 and NRG1 levels, identifying a novel player in regulating remyelination.

Keywords

Erbin; Schwann cell; remyelination; Neuregulin; ErbB2

Introduction

SCs are critical for myelin sheath thickness and internodal distance, which are important determinants of nerve conduction velocity (Baumann and Pham-Dinh, 2001). They are also necessary for the recovery from nerve injury (Fu and Gordon, 1997; Suter and Scherer,

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2003; Chen et al., 2007; Nave et al., 2007; Fancy et al., 2011). After injury, axon segments distal to the injury site undergo Wallerian degeneration (Dubovy, 2011), a complex process characterized by axon disintegration, myelin breakdown, and debris phagocytosis by SCs and macrophages. Concomitantly, normally quiescent SCs dedifferentiate and proliferate to remyelinate axons sprouted from proximal segments. Unlike myelination that has been extensively studied, less is understood about regulatory mechanisms of remyelination.

NRG1 is a factor that regulates SC development and myelination of axons (Marchionni et al., 1993; Shah et al., 1994; Dong et al., 1995; Grinspan et al., 1996; Mahanthappa et al., 1996; Wolpowitz et al., 2000; Winseck et al., 2002; Michailov et al., 2004; Mei and Xiong, 2008). Of six types of NRG1 (Steinthorsdottir et al., 2004), type III appears to be the isoform implicated in axonal regulation of SC myelination (Michailov et al., 2004; Taveggia et al., 2005; Lemke, 2006; Nave and Salzer, 2006). The function of NRG1 is regulated by BACE1, an extracellular protease, whose mutation impairs myelination (Hu et al., 2006; Willem et al., 2006; Hu et al., 2008). NRG1 binds to ErbB3, which heterodimerizes with and thus activates ErbB2. NRG1 regulation of myelination requires ErbB2 and ErbB3 (Nave and Salzer, 2006; Mei and Xiong, 2008). However, NRG1 and ErbB2 seem to be dispensable for myelin maintenance (Atanasoski et al., 2006; Fricker et al., 2011).

Evidence suggests that NRG1 regulates remyelination after nerve injury. It promotes remyelination of regenerated axons and functional recovery after injury (Chen et al., 1998; Joung et al., 2010). It also increases the survival of terminal SCs after denervation (Trachtenberg and Thompson, 1996) and promotes their process extension (Hayworth et al., 2006). On the other hand, remyelination is delayed in peripheral nerves of BACE1 null mice (Hu et al., 2008). Recently, motoneuron-specific ablation of type III NRG1 impairs remyelination (Fricker et al., 2011), indicating a critical role of NRG1 in remyelination.

In the current study, we investigated how NRG1 signaling is regulated in remyelination of regenerated axons. We found that nerve injury increases the expression of Erbin, a binding protein that specifically interacts with the ErbB2 (Borg et al., 2000; Huang et al., 2001). Our earlier work demonstrated that Erbin is expressed in SCs and is critical for myelination and ensheathment of peripheral nerves during development (Tao et al., 2009). To study its role in remyelination, we compared myelination of regenerated axons after nerve injury between wild type and *erbin*^{-/-} mice, and found that Erbin is necessary for remyelination. We explored possible cellular and molecular mechanisms. Results suggest that Erbin is critical for remyelination by promoting SC differentiation and by maintaining ErbB2 levels. These observations identified a novel player in regulating remyelination.

Methods

Animals

erbin^{-/-} mice were described previously (Tao et al., 2009) and back crossed with C57BL/6 mice for multiple generations. Mice were housed in a room with a 12 hours light/dark cycle with access to food and water ad libitum. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Georgia Health Sciences University.

Antibodies and chemicals

Rabbit Erbin antibody was generated by GST-Erbin-PDZ as described previously (Huang et al., 2001) and purified by GST-fusion proteins immobilized on Affi-gel 10, following the manufacturer's instruction. Primary antibodies were: ErbB2 (1:500, sc-284, Santa Cruz Biotechnology, Santa Cruz, California), ErbB3 (1:500, sc-285, Santa Cruz Biotechnology, Santa Cruz, California), NRG1 (1:500, sc-348, Santa Cruz Biotechnology, Santa Cruz, California), Integrin β 4 (1:500, sc-9090, Santa Cruz Biotechnology, Santa Cruz, California),

MBP (1:250, M1891, Sigma Aldrich, St. Louis, MO), α -tubulin (1:1000, T5168, Sigma Aldrich, St. Louis, MO), β -actin (1:1000, A1978, Sigma Aldrich, St. Louis, MO), BrdU (1:1000, B2531, Sigma Aldrich, St. Louis, MO). Secondary antibodies for immunostaining were: Alexa Fluor 488 anti-rabbit (1:500, A11018, Invitrogen, Carlsbad, CA), Alexa Fluor 594 anti-mouse (1:500, A11015, Invitrogen, Carlsbad, CA).

Nerve crush injury and transplant

Animals (either sex, > 3 months old) were anesthetized with ketamine and xylazine (i.p., 100 mg/kg and 20 mg/kg, respectively) and right thighs were sterilized with 2% iodide and 70% alcohol. Right sciatic nerves at the mid-thigh were exposed. The tips of No. 5 Dumont microforceps (11252-00, F.S.T., Foster City, CA) which were pre-cooled with liquid nitrogen was used to clamp and crush the nerves three times (30 second each) as described previously (Hu et al., 2008). Skins were closed with steel clips (Leica 39465012, Buffalo Grove, IL). Animals were kept warm on thermostatic rubber pad (37°C) which was connected with Heat Therapy Pump (GAYMAR, Orchard Park, NY) during the whole process to increase the survival rate. In some experiments, segments of sciatic nerves (~10 mm) were isolated from wild type mice and transplanted into mutant mice or vice versa under Carl Zeiss surgical OPMI microscope. Nerve segments were sutured to recipient nerves in correct proximal–distal orientation with 10–0 nylon black (STREF: 03171 F.S.T., Foster City, CA).

Toe spreading analysis

Toe spreading analysis was performed as described previously (Wakatsuki et al., 2009). At designated time after nerve injury, the animals with palm and toes of the hindpaws painted with ink were allowed to walk on white paper along a 30×10 cm corridor. Each mouse was tested 3 times. The distances between the first and fifth toes were measured as toe spreading index.

Electron microscopy (EM)

Electron microscopic analysis of myelin was performed as described previously (Tao et al., 2009). Mice were anesthetized and cardiac perfused with 4% formaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (NaCac, pH 7.4). Sciatic nerves were removed and fixed overnight at 4 °C in the same perfusion fixative for 24 hours. They were washed by 0.1M NaCac and osmicated with 2% osmium tetroxide for 30–60 min at 4 °C, washed by 0.1 M NaCac for 30–60 min at 4 °C and by deionized H₂O for 30–60 min at 4 °C, and dehydrated in graded (30–70%) ethanol. Samples were stained with 2% uranyl acetate in 70% ethanol at 4 °C for 30 min followed by dehydration with 70–100% ethanol. Samples were incubated with propylene oxide for 10 min × 2 and embedded with resins. Ultrathin sections were photographed with Phillips 400 Transmission electron microscope. EM images were analyzed by Image J (NIH). To minimize the variation in different regions of nerves, cross sections of nerves were divided into 10 regions and two pictures were randomly taken from each region and analyzed by an individual blind to genotypes or treatments. *g*-ratios were calculated by the perimeters of axons (inner) divided by the perimeters of corresponding fibers (outer). Axonal diameters were normalized by perimeters through equation: diameter = perimeter/ π . This procedure allowed for inclusion of irregularly shaped axons and fibers and helped to eliminate biased measurement of diameters based on circularity.

Immunostaining, BrdU and TUNEL staining

Immunostaining was performed as previously described with modification (Tao et al., 2009). Sciatic nerves were embedded in OCT, frozen in liquid nitrogen, and cut into 20 μ m

sections that were mounted on SuperFrost plus slides. Sections and cultured cells on coverslips were fixed in 4% PB-buffered polyformaldehyde (PFA) and incubated at 4 °C for 30 min with 0.3% Triton-X-100 plus 5% goat serum in PBS (to permeabilize cells). They were incubated at 4 °C overnight with primary antibodies in PBS containing 5% goat serum. After washing 3 times with PBS, samples were incubated at room temperature for 1 hour with Alexa-488 goat anti-rabbit or Alexa-594 goat anti-mouse secondary antibody, and mounted with Vectashield mounting medium (Vector, Burlingame, CA). Images were taken by a Zeiss LSM510 confocal microscope and analyzed by Image J (NIH).

BrdU labeling was performed as previously described with modification (Xu et al., 2007). BrdU (B5002 Sigma-Aldrich, St. Louis, MO) was injected into mice 5 days after sciatic nerve crush (i.p., 100 mg/kg, three times with 6 hours interval). Two hours after the final injection, mice were cardiac perfused with 4% PFA and 4% sucrose in PB. Sciatic nerve were dissected out and subjected to cryostat section. Longitudinal or cross sections were permeabilized three times with 1% Triton-X100 for 5 min and incubated with 2N HCl for 2 hours at room temperature to expose BrdU-incorporated DNA. Samples were neutralized by Borate buffer (0.1 M, pH8.5) for 12 min and washed by PBS three times before immunostaining using anti-BrdU antibody (1:1000 in blocking buffer: PBS containing 5% goat serum).

A TUNEL apoptosis detection kit (T9162-80, US Biological, Swampscott, MA) was used for DNA fragmentation fluorescence staining according to the manufacturer's protocol with modification. Frozen tissue sections (15 days after crush) were fixed with 4% PFA overnight at 4°C. Samples were washed 10 min for three times with PBS and then permeabilized with 0.5% Triton-X100 in PBS overnight at 4°C. Samples were incubated with TUNEL reaction mixture (enzyme solution + labeling solution) for 60 min at 37°C and then washed with PBS, mounted by Vectorshield with DAPI and examined under a fluorescence microscope.

Western blotting

Western blotting was performed as described previously (Tao et al., 2009). Proteins were transferred from SDS-PAGE gel to nitrocellulose membrane, which was incubated in blocking buffer - PBS with 0.2% Tween-20 and 5% milk for 1 hour at room temperature and subsequently incubated with indicated antibodies in the blocking buffer overnight at 4°C, washed with PBS containing 0.3% Tween-20, and then incubated with HRP-conjugated secondary antibody for 1 hour at room temperature (Tao et al., 2009). Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce, 32106, Rockford, IL). Autoradiographic films were scanned with an Epson 1680 scanner, and the captured images were analyzed with Image J (NIH).

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from dissected sciatic nerves using Trizol (Invitrogen, Carlsbad, CA) and purified using RNeasy mini kit (Qiagen, Valencia, CA). RT-qPCR was performed as described previously (Liu et al., 2011). Equal amounts of total RNAs (1 µg) were reverse transcribed by random hexamer-primers using Maxima® Enzyme Mix (Fermentas, Glen Burnie, MD). Quantitative PCR reactions were run in Bio-rad MJ Research PTC-200 Peltier Thermal Cycler using SYBR Green/ROX (Fermentas, Glen Burnie, MD). For erbB2 transcripts, primers were 5'- CGCGG GTACC CAAGT GTGTA (forward) and 5'- CGTTG TCCAA AGGGT CTCG (reverse), which generated a product of 317 bp. For erbB3 transcripts, primers were 5'- CACAC CTGGT CATAG CGGTG AC (forward) and 5'- TGGGT CCAGA GGCTC GATGC TC (reverse), which generated a product of 152 bp. For erbB transcripts, primers were 5'- GCAAG GTTCC TCATG ACTG (forward) and 5'- CTCGA ATCTC TTGCT TTGCC (reverse), which generated a product of 540 bp. For

NRG1 type III transcripts, primers were 5'- GGACC CCTGA GGTGA GAACA (forward) and 5'- CAGTC GTGGA TGTCG ATGTG G (reverse), which generated a product of 102 bp.

Statistical analyses

Data were analyzed by paired or unpaired *t* test. Unless otherwise indicated, data were presented as mean \pm SEM. Statistical significance was considered when $p < 0.05$.

Results

Expression of Erbin is increased in SCs of injured sciatic nerves

To investigate if Erbin regulates remyelination, we determined expression of Erbin in injured sciatic nerves. Right sciatic nerves were injured by crushes with liquid-nitrogen-cooled microforceps (Hu et al., 2008), and distal segments of injured nerves were isolated for Western blot analysis. ErbB2 is known to be increased in injured nerves and are implicated in SC proliferation, differentiation and remyelination after injury (Carroll et al., 1997; Kwon et al., 1997; Atanasoski et al., 2006; Hu et al., 2008). Accordingly, as shown in Fig. 1, ErbB2 levels were increased in distal segments of injured sciatic nerves by 6.7 and 16.3 folds, respectively, at days 5 and 20 after injury. This increase was preceded by a brief reduction at days 1 and 3, probably due to nerve degeneration (Fig. 1A, B). Similar changes were observed for ErbB3 expression after nerve injury, except the increase was less than ErbB2. Interestingly, Erbin levels were also increased in distal segments of injured sciatic nerves (Fig. 1A, B). As control, Integrin β 4, a laminin receptor, was reduced in distal nerves after injury (Fig. 1A, B), confirming a previous report (Einheber et al., 1993), suggesting that the elevating effect on Erbin after nerve injury was specific. Together, these results indicate that Erbin expression was specifically increased in injured sciatic nerves.

To determine in which cells of sciatic nerves Erbin was increased, we stained longitudinal sections of injured nerves with purified anti-Erbin antibody that recognizes a single band on Western blot (data not shown). In proximal segments (20 days after injury), Erbin was present in longitudinal tracks with hallow space in between that is presumably occupied by axons (enlarged image, Fig. 1C), suggesting that Erbin was present in myelin, in agreement with previous report (Tao et al., 2009). Notice that SC nuclei in this segment were elongated in shape due to myelination. However, distal segments (20 days after injury) were filled with proliferating and differentiating SCs (as indicated by DAPI staining). Erbin immunoreactivity was increased (Fig. 1C), in agreement with results from Western blotting (Fig. 1A, B). Moreover, the immunoreactivity was in close proximity with cell nuclei (Fig. 1C), suggesting presence in regenerating SCs. The increase of Erbin in injured nerves started as early as day 3, two days ahead of those of ErbB2 and ErbB3 (Fig. 1A, B), suggesting a preceding role of Erbin in reparative process after nerve injury.

Erbin is required for remyelination after nerve injury

Nerve injury leads to Wallerian degeneration (Dubovy, 2011). To determine if Erbin mutation alters this process or regeneration thereafter, we divided nerve segments into different parts. As shown in Fig. 2A, proximal segments of nerves 2 mm from the injury site was designated as P2-3, whereas distal segments of nerves 2 mm and 4 mm from the injury site were designated as D2-3, and D4-5, respectively. Nerve segments were processed for electron microscopic (EM) analysis. As shown in Fig. 2B, both myelinated and unmyelinated axons of P2-3 segments in wild type and *erbin*^{-/-} mice were relatively intact 5 days after injury. Myelin thickness correlated with axonal sizes (Fig. 2C). The *g*-ratio of myelinated axons, obtained by dividing axon diameter by fiber (axon plus myelin) diameter, was higher in *erbin*^{-/-} P2-3 segments, compared to wild type controls. This result could

suggest that Erbin is involved in myelination development, in agreement with our earlier report (Tao et al., 2009). In contrast, the segments distal to the injury site underwent Wallerian degeneration. On day 5 after injury, in D2-3 segments, axonal degeneration and myelin breakdown or collapse was apparent, and the debris was phagocytosed by macrophages (Fig. 2B). Degeneration was less severe in D4-5 segments, which were farther away from the injury site: a few intact myelinated axons remained. The degeneration prevented accurate measurement of *g*-ratio of axons in distal segments on day 5 after injury. However, there was no difference in Wallerian degeneration between wild type and *erbin*^{-/-} mice, suggesting that Erbin ablation did not alter this process.

To explore the role of Erbin in nerve regeneration, we studied remyelination of injured sciatic nerves in *erbin*^{-/-} mice. After nerve injury, axons are regenerated from proximal ends and sprout into distal parts of injury site within 10–14 days (Fu and Gordon, 1997; Suter and Scherer, 2003; Chen et al., 2007; Nave et al., 2007; Fancy et al., 2011). As shown in Fig. 2B, on day 5, myelin sheathes collapsed and axons were degenerated in D2-3 segments of both control and Erbin mutant mice. However, on day 20, axons were detected in these segments in wild type mice, indicating that axons were regenerated (Fig. 3C). Moreover, these D2-3 axons were myelinated (Fig. 3C). However, fewer axons were detected and fewer axons were myelinated in distal segments in *erbin*^{-/-} mice, compared to wild type controls (Fig. 3G), indicating an impairment of axonal regeneration and remyelination when Erbin is ablated. This notion is supported by three additional pieces of evidence. First, many large D2-3 axons in *erbin*^{-/-} mice were not myelinated or were naked on day 20 after injury, in contrast to those in wild type controls (Fig. 3C). The number of un-myelinated axons with diameter of 1 μ m or larger was dramatically increased in *erbin*^{-/-} mice (Fig. 3H). Second, of those myelinated axons in *erbin*^{-/-} D2-3 segments, the *g*-ratio was significantly larger than that of wild type controls. Similar myelination defects were observed in D4-5 segments in *erbin*^{-/-} mice. Third, concomitantly with morphological changes were alterations of myelin basic protein (MBP), a major myelin component. On day 5 after injury, MBP levels were reduced in distal segments, compared to those in proximal segments of both wild type and *erbin*^{-/-} mice (Fig. 3J). This time course was consistent with axonal degeneration and myelin collapse in Wallerian degeneration (Carroll et al., 1997) (Fig. 2). On day 20, MBP levels were increased in wild type distal segments, compared to those on day 5 (lanes 7 and 3, respectively; Fig. 3I, J), in agreement with increased myelination on day 20. However, although MBP in *erbin*^{-/-} distal segments was also elevated (compared lanes 4 and 8), levels in distal segments were significantly lower in *erbin*^{-/-} mice, compared to wild type controls. These results suggest that Erbin ablation attenuates the ability of mutant mice to produce MBP. Together, these morphological and biochemical studies indicate that Erbin is essential for axonal remyelination.

We have also examined regenerated nerves on day 40. The *g*-ratios of both wild type and *erbin*^{-/-} regenerated axons on day 40 were reduced (Fig. 4A, B), compared to those on day 20 (Fig. 3C–F), suggesting recovery from nerve injury. However, myelin of regenerated axons in *erbin*^{-/-} mice remained thinner (with larger *g*-ratios) than wild type controls, suggesting again impaired remyelination. Quantitatively, compared to day 20, the number of myelinated axons was increased and that of unmyelinated axons were reduced in wild type mice (Fig. 4B). Myelinated axons were fewer and unmyelinated axons more in *erbin*^{-/-} mice than controls (Fig. 4B), supporting continuing impairment in remyelination on day 40. However, the numbers of large (> 1 μ m in diameters) unmyelinated axons in distal segments were similar between injured two genotypes, indicating delayed remyelination of large axons in *erbin*^{-/-} mice. These results corroborate that Erbin is necessary for remyelination of newly generated axons.

Notice that axons from proximal segments of injured nerves in both mice remained intact on day 20 and day 40 after injury (Fig. 3A, 4A), although axons with similar size in proximal nerves of *erbin*^{-/-} had thinner myelin and increased *g*-ratio (Figs. 3A, B and 4A, B), the same as previously reported during development (Tao et al., 2009). Intriguingly, the distribution curves of myelinated axon size in D4-5 segments shifted toward the right on both day 20 and day 40 (Fig. 4D), suggesting increased size of regenerating axons in *erbin*^{-/-} mice. We speculate that this may be due to loss of physical boundary by myelin or a compensatory mechanism.

Functional recovery after nerve injury is impaired in *erbin*^{-/-} mice

To determine whether impaired remyelination in *erbin*^{-/-} mice impedes functional recovery, we performed walking footprint analysis. As shown in Fig. 5A, on day 5 after injury, ink mark left by right heels (the side of sciatic nerve injury) was more intense, compared to normal left heels, indicating impaired gastrocnemius innervation (de Medinaceli et al., 1982; Brown et al., 1989; Haastert et al., 2006). This impairment was observed in both wild type and *erbin*^{-/-} mice. On day 20, the ink mark intensity of right heels became similar to that of left heels in wild type mice, indicating functional recovery of injured sciatic nerves. However, marks of right heels of *erbin*^{-/-} mice remained heavier than those of left heels on day 20 although they became similar by day 40. These results suggest a delay in functional recovery in *erbin*^{-/-} mice. On the other hand, upon contact with a surface, rodents spread the toes of hind feet, which is a reflex that requires sensory innervation (Contreras et al., 1995). Sciatic nerve injury attenuates the contraction of paw extensor and paw intrinsic muscles, and consequently, mice have difficulty to spread toes (Fig. 5B). The ability to spread the toes has been used to indicate the recovery of sensory function after sciatic nerve injury (de Medinaceli et al., 1982; Brown et al., 1989; Klapdor et al., 1997; Haastert et al., 2006). As shown in Fig. 5C, on day 5 after injury, the distance between the first and fifth toes was significantly smaller in the right paws, compared to left paw controls in both wild type and *erbin*^{-/-} mice, indicating impaired sensory function. Consistent with the heel preference, the toe distance of right hind paws recovered by day 20 after injury in wild type mice (Fig. 5C). In *erbin*^{-/-} mice, however, the distance remained abnormal even on day 40 after injury (Fig. 5C), suggesting that sensory function was not fully restored in *erbin*^{-/-} mice 40 days after injury. Together, these observations provide evidence that Erbin ablation inhibits functional recovery from nerve injury, corresponding to the morphological deficiency of remyelination in regenerating nerves of *erbin*^{-/-} mice.

Erbin is not required for proliferation and survival of regenerated SCs in injured nerves

To investigate mechanisms of Erbin in remyelination, we determined whether proliferation or survival of SCs was altered in injured nerves of *erbin*^{-/-} mice by BrdU labeling and TUNEL assays, respectively. Mice were injected BrdU on day 5 after nerve injury, and sciatic nerves were collected 24 hours later and stained with anti-BrdU antibody for BrdU incorporation. Few nuclei were labeled by BrdU in proximal segments of injured nerves or in uninjured sciatic nerves (Fig. 6A, C). No difference was observed between proximal segments from wild type and *erbin*^{-/-} mice. In contrast, BrdU incorporated-nuclei increased dramatically in distal segments of injured nerves of wild type mice (Fig. 6A, C), consistent with the concept that SCs undergo proliferation during Wallerian degeneration (Kwon et al., 1997; Adilakshmi et al., 2011). However, similar increase in BrdU-labeled nuclei was also observed in *erbin*^{-/-} mice, indicating that Erbin is not required for SC proliferation after nerve injury (Fig. 6A–C).

Next, we investigated whether ablation of Erbin affected SC survival in regenerating nerves. To this end, sections from proximal and distal sciatic nerves of injured and uninjured nerves were processed to fluorescence labeling of the terminal uridine nucleotides in apoptotic

nuclei. As shown in Fig. 6D–F, TUNEL-positive cells were in sections from distal segments of injured nerves in both control and *erbin*^{-/-} mice and there was no difference between control and *erbin*^{-/-} samples. These results suggest that Erbin is not required for SC survival in injured nerves. In agreement with the notion that SCs increase in number in injured nerves, the ratios of TUNEL-positive nuclei over total nuclei were reduced in distal segments, compared to uninjured or proximal segments (Fig. 6F). Together, these results demonstrate that Erbin is not required for SC proliferation or survival, suggesting that it may regulate the myelinating ability of regenerated SCs.

Requirement of Erbin for elevated ErbB2 expression in injured nerves

Erbin is implicated in ErbB2 stability in transfected cells and during myelination (Tao et al., 2009). We determined if nerve injury-induced ErbB2 expression requires Erbin. Wild type and *erbin*^{-/-} mice were subjected to nerve injury as described in Fig. 1. Proximal and distal segments of injured nerves were isolated for Western blot analysis. Compared to proximal segments in wild type mice, ErbB2 and ErbB3 levels in distal segments were significantly increased 5 days after injury (Fig. 7A, B). In *erbin*^{-/-} mice, however, the ErbB2 levels in distal segments reduced significantly to 19.2% and ErbB3 levels reduced to 40.0% of those in wild type (Fig. 7A, B), despite the similar proliferation and survival of SCs in distal segments (Fig. 6). These results indicated that the increase in ErbB2/3 in injured nerves requires Erbin. Notice that ErbB2 were reduced in *erbin*^{-/-} proximal segments (Fig. 7A, B), which did not exhibit consistent morphological changes after nerve injury (Fig. 2). This observation was in agreement with our previous study (Tao et al., 2009). Despite the reduction at absolute levels, ErbB2 levels were higher in distal segments of *erbin*^{-/-} injured nerves, compared to that in proximal segments, suggesting some mechanisms related to protein production may not be severely impaired by ablation of Erbin. We measured ErbB2 and ErbB3 mRNAs in injured nerves by quantitative RT-PCR. ErbB2 mRNA was increased in distal segments in both wild type and *erbin*^{-/-} mice in comparison to those in proximal segments, although the levels were significantly lower in *erbin*^{-/-} injured nerves (Fig. 7C), suggesting similar amplification of transcriptional machinery for ErbB2 production. Notably, the increase of ErbB2 at mRNA levels was only $70.1 \pm 7.82\%$, in contrast to $187 \pm 24.5\%$ at protein levels in distal segments in comparison to those in proximal segments of wild type nerves, implying a requirement for protein stabilizing mechanisms. Moreover, the reduction of ErbB2 or ErbB3 in injured nerves of *erbin*^{-/-} mice at mRNA levels was much less than that at protein levels. Together, these observations suggest that Erbin mutation prevents ErbB2 and ErbB3 increase in injured nerves, mainly at protein levels. This mechanism contributes to reduced NRG1/ErbB signaling in SCs which is necessary for remyelination.

Erbin regulates remyelination via influence on both axonal and SC factors

Myelination is determined by the interaction between axons and SCs (Jessen and Mirsky, 2005; Nave and Trapp, 2008; Salzer et al., 2008). Since both axonal (type III NRG1) and SC factors (ErbB2) were altered in injured nerves of *erbin*^{-/-} mice (see below), we have to determine which factor is the key for Erbin to control remyelination. To the end, we performed allograft transplantation – transplanting sciatic nerve segments of donor mice to replace segments in recipient mice. Nerve allografts not only provide a scaffold for host nerve regeneration, but also carry SCs of donor mice to host mice. Axon segments in allografts will be degenerated, whereas regenerating axons will grow through the allografts and interact with and eventually remyelinated by donor SCs. This method has been used to differentiate functions of axons and SCs of different genetic background or after different treatments in nerve regeneration (Aguayo et al., 1976; Haney et al., 1999). As shown in Fig. 8A and 8B, a sciatic nerve segment (~ 10 mm) from a wild type donor mouse was transplanted to a wild type recipient mouse (+/+ to +/+). Twenty days after transplantation, 1

mm segments from the middle of transplanted nerves were dissected out and processed to electron microscopy. As shown in Fig. 8, *C* and *D*, axons were evident in *+/+* allografts in *+/+* mice and were myelinated with a *g*-ratio of 0.7529 ± 0.00393 . Notice that these axons were newly regenerated because donor axons should have been degenerated within days after surgery.

Interestingly, when *erbin*^{-/-} allografts were transplanted to wild type mice (*-/-* to *+/+*), myelination of regenerated axons was impaired with *g*-ratios of 0.7664 ± 0.00493 (Fig. 8*D*) and myelinated axon number decreased (Fig. 8*E*). This result suggests that *erbin*^{-/-} SCs brought over by allografts were impaired in their ability to myelinate regenerated axons, in support of the hypothesis that Erbin in SCs is critical for remyelination. Unexpectedly, remyelination was also impaired in “*+/+* to *-/-*” allografts (Fig. 8*C-E*), suggesting a role of Erbin from cells of proximal segments in regulating axonal factors for remyelination. Total axon numbers reduced in allograft “*+/+* to *-/-*”, suggesting impaired axon regeneration when axotomized neurons were surrounded with Erbin-deficient supporting cells.

Requirement of Erbin for elevated type III NRG1 expression in injured nerves

NRG1, in particular type III isoform, is a critical axonal factor for myelination during development and remyelination after injury (Michailov et al., 2004; Taveggia et al., 2005; Hu et al., 2006; Willem et al., 2006; Hu et al., 2008; Fricker et al., 2011). Two bands were detected in sciatic nerves using verified antibody to type III NRG1, representing the full length Pro-NRG1 (NRG-FL) and the C-terminal fragment (CTF), which was generated during NRG1 maturation (Hu et al., 2006; Willem et al., 2006; Hu et al., 2008; Tao et al., 2009) (Fig. 9*A, B*). In agreement, we also found increased NRG1 expression (both NRG1-FL and NRG-CTF) in injured nerves of wild type mice (Fig. 9*A, B*). The increase at the protein level was readily detectable on day 1, day 3 and day 20. The reduction of NRG1 in injured nerves on day 5 may be due to severe axon degeneration in response to nerve injury (Carroll et al., 1997; Wakatsuki et al., 2009). Intriguingly, the levels of NRG1 in distal segments of injured nerves were significantly lower than those in wild type controls (Fig. 9*C, D*). Notice that type III NRG1 levels were lower in *erbin*^{-/-} proximal segments than controls (Fig. 9*C, D*), implying a possible requirement of Erbin for neuronal NRG1 expression. These results suggest that Erbin mutation also prevents NRG1 increase in injured nerves, suggesting that NRG1/ErB signaling and/or Erbin activity in SCs may regulate NRG1 production in regenerating axons.

Discussion

Major findings of this paper include the following. First, Erbin expression increased dramatically in injured nerves. The increase was more in distal segments of the injury site and the increase was mainly in SCs (Fig. 1). Second, in *erbin*^{-/-} mice, myelinated axons were fewer, and *g*-ratios of those that were myelinated were increased (Figs. 3–4), indicating a necessary role of Erbin in remyelination of regenerating axons. In accordance with morphological myelin deficits, functional recovery from nerve injury was impaired in Erbin mutant mice (Fig. 5). Third, BrdU-labeled cells and TUNEL-labeled cells were not altered by Erbin ablation (Fig. 6), suggesting that impaired remyelination is not caused by reduced proliferation or increased apoptosis of SCs. Fourth, Erbin elevation was ahead of that of ErbB2/3 increase in injured nerves and Erbin mutation attenuates the elevation of ErbB2/3 and type III NRG1 in injured nerves, suggesting that the mutant mice are impaired in raising or maintaining NRG1/ErB signaling, which is critical for remyelination (Carroll et al., 1997; Hu et al., 2008; Fricker et al., 2011). Finally, Erbin mutation also prevents type III NRG1 increase in injured nerves, suggesting that NRG1/ErB signaling and/or Erbin activity in SCs may regulate NRG1 production in regenerating axons. Together, these

observations demonstrate that Erbin is required for remyelination of regenerated axons after injury.

Communication between SCs and axons controls myelin repair as well as development (Fu and Gordon, 1997; Jessen and Mirsky, 2005; Taveggia et al., 2010). NRG1 signaling appears to be critical for remyelination of injured nerves although it may be dispensable for myelin maintenance under physiological conditions (Atanasoski et al., 2006). First, expression of ErbB2 and ErbB3 in SCs and NRG1 (SC-derived type II and axon-derived type III) is increased in injured sciatic nerves (Carroll et al., 1997; Kwon et al., 1997; Atanasoski et al., 2006; Hu et al., 2008). Second, the increase is more in distal segments, where remyelination is active, than in proximal segments (Carroll et al., 1997; Hu et al., 2008). Third, NRG1-treated rodents showed earlier, and more robust remyelination of regenerated axons after injury and functional recovery (Chen et al., 1998; Joung et al., 2010). Moreover, addition of NRG1 could increase the survival of terminal Schwann cells at the neuromuscular junction (NMJ) after postnatal denervation (Trachtenberg and Thompson, 1996) and promote their process extension (Hayworth et al., 2006). Fourth, remyelination was delayed in peripheral nerves of BACE1 null mice, probably due to impairment in NRG1 cleavage and subsequent activation of PI3K/Akt which has been implicated in myelination (Ogata et al., 2004; Chen et al., 2006; Hu et al., 2008). Finally, neuron-specific ablation of type III NRG1 leads to hypomyelination or loss of myelin sheath in mutant mice (Fricker et al., 2011).

During development, NRG1 has been implicated in promoting SC proliferation (Dong et al., 1995). However, no change in BrdU-labeled SCs in *erbin*^{-/-} damaged nerves suggests that Erbin is not required for increased SC proliferation after injury. Impaired myelination did not appear to result from an increase in SC apoptosis because TUNEL-labeled SCs were not altered. This notion is in agreement with a report that NRG1/ErbB signaling was not required for SC proliferation or survival (Atanasoski et al., 2006). We propose that impairment in remyelination may be caused by compromised remyelinating ability of regenerated SCs. This notion is supported by the following additional evidence. First, Erbin is expressed mainly in SCs or myelin of the peripheral system under physiological conditions (Tao et al., 2009) as well as after nerve injury (Fig. 1C). Second, *erbin*^{-/-} SCs in “-/- to +/+” allografts are impaired to remyelinate regenerated axons in wild type recipient mice.

How does Erbin regulate remyelination? NRG1 signaling necessary for remyelination requires proper levels of ErbB2 for signaling. On the other hand, activation of ErbB kinases leads to endocytic removal of active ligand-receptor complexes from the cell surface, and subsequent sorting to proteasomes for degradation or endosomes for recycling (Waterman et al., 1998; Yang et al., 2005; Liu et al., 2007; Mei and Xiong, 2008). In the current study, we found that Erbin increases at protein levels ahead of ErbB2 (Fig. 1) and Erbin mutation prevents injury from elevating ErbB2 to similar levels as control wild type mice (Fig. 7). Moreover, in contrast to its effects on ErbB2 protein, Erbin mutation has less effect on mRNA levels of ErbB2, suggesting that a major mechanism of action is to increase the stability of ErbB2 proteins. In agreement, we showed previously that Erbin could prevent both constitutive and ligand-induced ErbB2 endocytosis (Tao et al., 2009). These observations suggest that Erbin regulates remyelination of regenerated axons mainly by stabilizing ErbB2 in SCs.

Intriguingly, remyelination was also impaired in “+/+ to -/-” allografts (Fig. 8C, D). This result suggests that axonal factors necessary for remyelination may be impaired in *erbin*^{-/-} mice. In response to nerve injury, axons increase the expression of various factors including cytokines and growth factors including type III NRG1 (Hammarberg et al., 1996; Kurek et

al., 1996; Hu et al., 2008) (Fig. 9). Axonal NRG1 promotes remyelination after nerve injury (Joung et al., 2010; Fricker et al., 2011). Interestingly, injury was unable to increase type III NRG1 to similar levels in *erbin*^{-/-} mice, compared to controls (Fig. 9), suggesting that Erbin is necessary for NRG1 production in axons after nerve injury. This result provides an additional mechanism for Erbin to regulate remyelination. Type III NRG1 mRNA was not detectable in proximal or distal segments of injured nerves (data not shown); therefore, increased axonal NRG1 in distal segments of wild type mice (Fig. 9) (Hu et al., 2008), where remyelination is active, is likely transported from soma of axotomized neurons.

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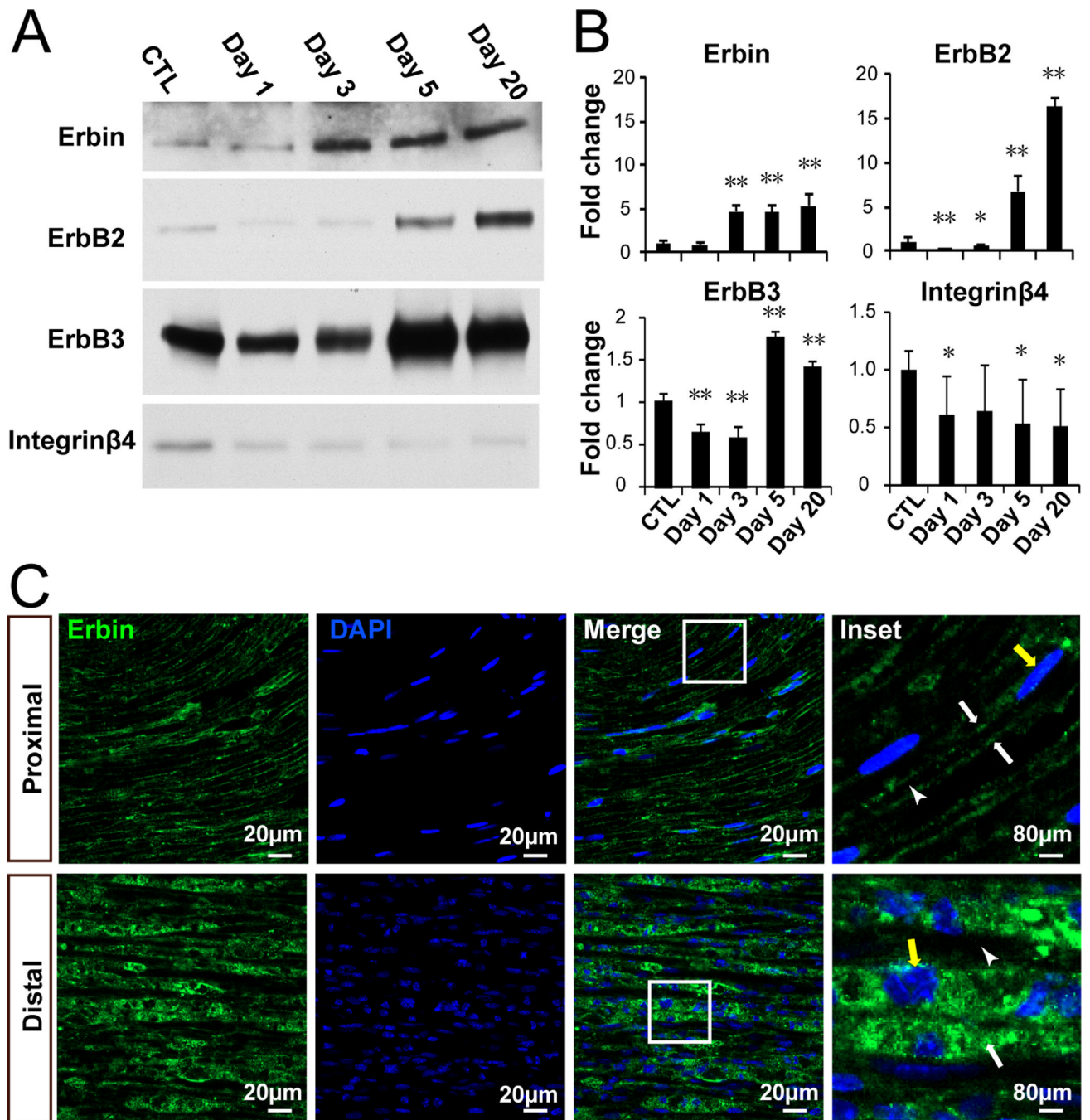


Figure 1. Increased expression of Erbin and ErbB receptors in injured nerves

A, Western blots showing increased expression of Erbin, ErbB2, and ErbB3 in distal segments of injured nerves. Distal segments at indicated days after injury were homogenized and subjected to Western blotting with indicated antibodies. **B**, Quantitative data of **A**. $n = 3$, $**p < 0.01$, $*p < 0.05$, compared to uninjured control (CTL). ErbB2 levels were increased in distal segments of injured sciatic nerves to 6.7 ± 1.67 and 16.3 ± 1.02 folds and ErbB3 to 1.8 ± 0.07 and 1.4 ± 0.08 folds, $n = 3$, $p < 0.01$, respectively, on days 5 and 20 after injury, while Erbin levels were increased to 4.7 ± 0.63 , 4.6 ± 0.86 , and 5.4 ± 1.30 folds for days 3, 5, and 20, $n = 3$, $p < 0.01$, respectively. **C**, Erbin expression was increased in regenerated

SCs. Longitudinal sections of injured distal segments on day 20 were co-stained with anti-Erbin antibody and DAPI. White arrows indicate Erbin staining; white arrowheads indicate hollow space between Erbin-stained myelin; yellow arrows indicate nuclei (elongated in proximal regions, but round in distal segments).

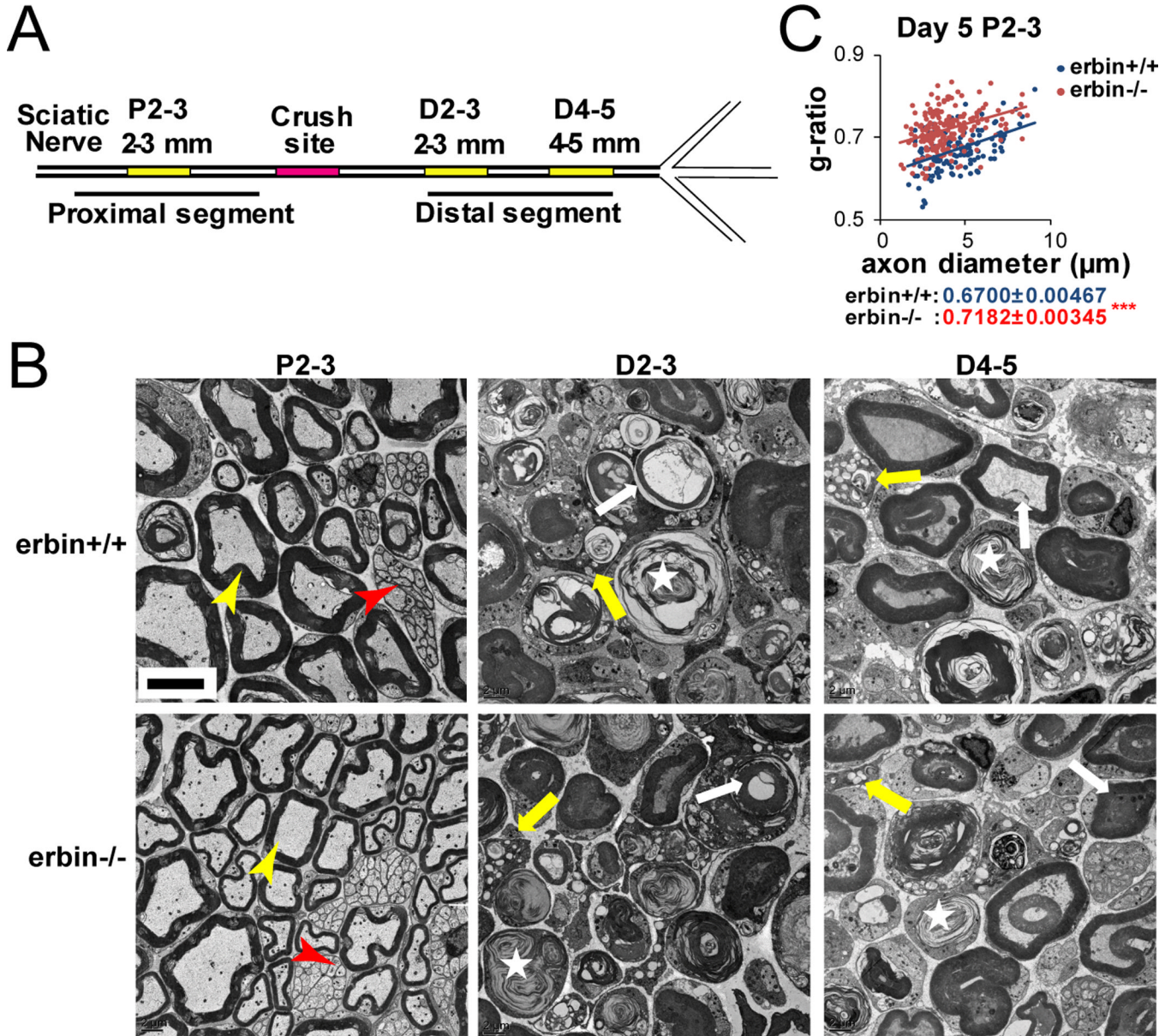


Figure 2. Wallerian degeneration of distal segments of injured nerves in both wild type and Erbin null mice

A, Schematic illustration of the injury site (red) and distal and proximal segments. Segments in yellow were analyzed by EM. P, proximal; D, distal. Numbers indicate distance from crush site in mm. **B**, Electron micrographs of P2-3 (left panels), D2-3 (middle panels), and D4-5 (right panels) of wild type (top panels) and *erbin*^{-/-} (bottom panels) mice on day 5 after injury. In P2-3: red arrowhead: un-myelinated axons; yellow arrowhead: myelinated axons. In D2-3 and D4-5: star: degenerated axons; white arrow: myelin debris; yellow arrow: macrophages with phagosomes. Scale bar, 5 μm . **C**, *g*-ratio of myelinated axons in P2-3 segments on day 5 after injury, plotted against axon diameter. Averaged *g*-ratio was presented as means \pm SEM. *** $p < 0.001$; $n = 20$ images per mouse, three mice per group.

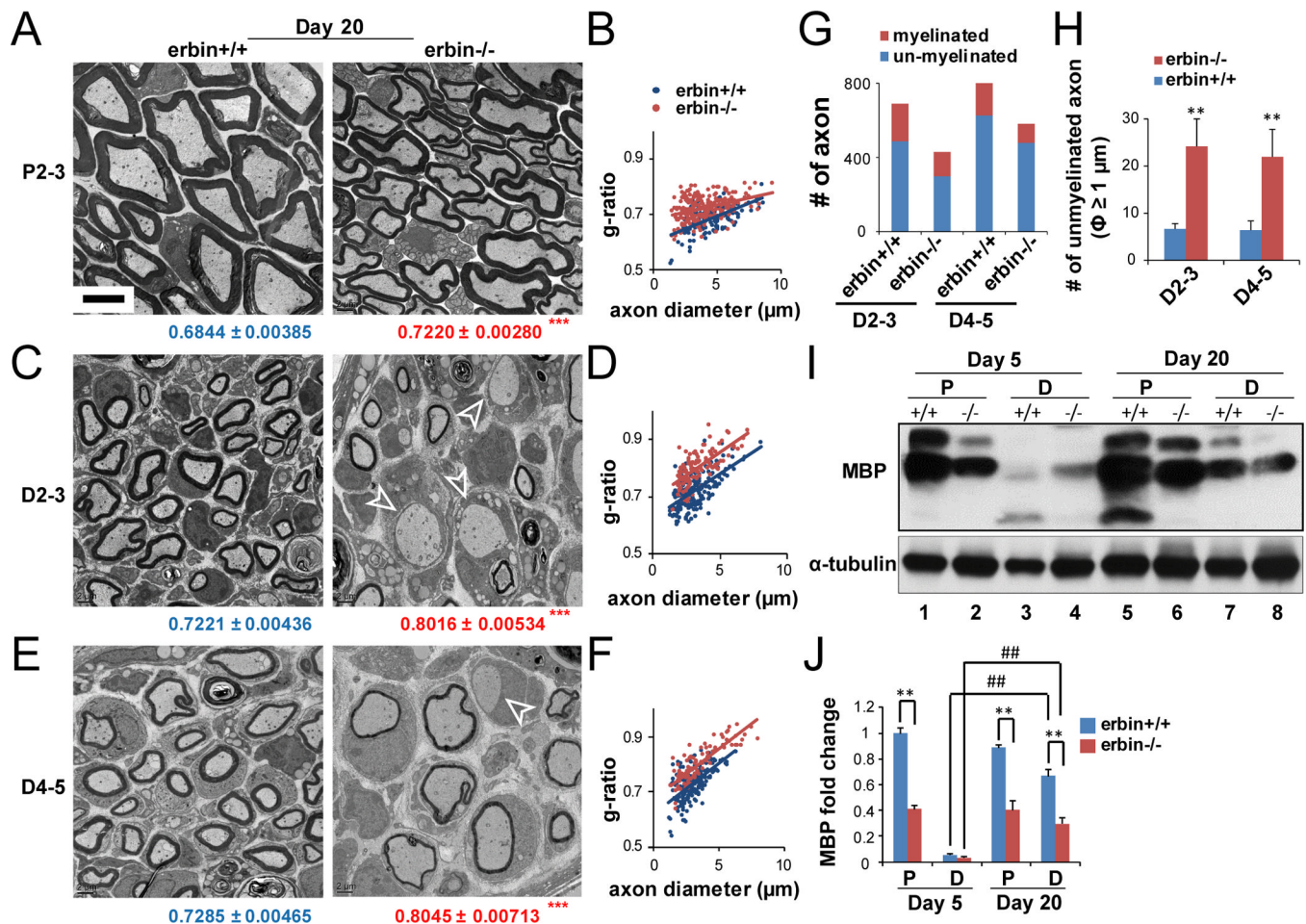


Figure 3. Deficient remyelination of regenerated axons in *erbin*^{-/-} mice on day 20 after nerve injury

Representative electron micrographs and *g*-ratios of respective segments of injured sciatic nerves of indicated genotypes on day 20 after injury. **A, B**, P2-3 segments. **C, D**, D2-3 segments. **E, F**, D4-5 segments. Empty arrowheads indicate naked axons with diameter equal to or bigger than 1 μm. Scale bar: 5 μm. Averaged *g*-ratio was presented as means ± SEM (n = 20 EM images of each mouse, three mice per genotype; *** *p* < 0.001). **G**, Fewer axons were myelinated in regenerating nerves of *erbin*^{-/-} mice. Scored were axons from over 20 EM images per mouse, 3 mice per genotype in an area of 0.01 mm². **H**, Increased number of unmyelinated axons with diameters equal to or larger than 1 μm in injured *erbin*^{-/-} sciatic nerves. Axons were scored as in **G**. ** *p* < 0.01. **I**, Reduced MBP levels in distal segments of injured sciatic nerves in *erbin*^{-/-} mice. Proximal and distal segments were isolated from injured sciatic nerves of indicated genotypes on days 5 and 20 after injury, and subjected to Western blotting with MBP antibody and α-tubulin (to indicate equal loading). **J**, Quantitative analyses of data in **I**. n = 3, ** *p* < 0.01, compared to wild type; ## *p* < 0.01, compared to data of distal segments on day 5.

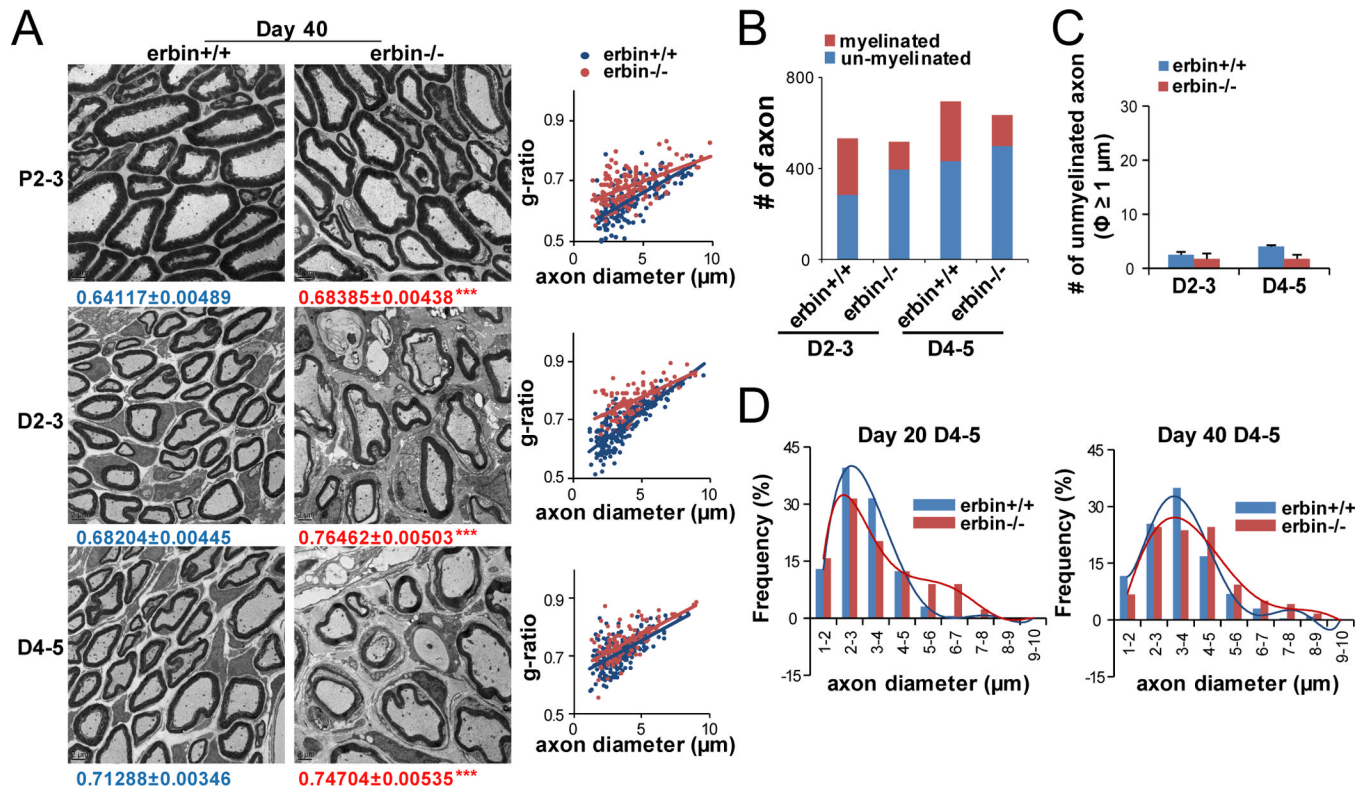


Figure 4. Deficient remyelination of regenerated axons in *erbin*^{-/-} mice on day 40 after nerve injury

A, Representative electron micrographs and g -ratios of respective segments of injured sciatic nerves of indicated genotypes on day 40 after injury. Averaged g -ratio was presented as means \pm SEM (n = 20 EM images per mouse, 3 mice per genotype; *** $p < 0.001$). **B**, Fewer axons were myelinated in regenerating nerves of *erbin*^{-/-} mice. Scored were axons from over 20 EM images per mouse, 3 mice per genotype in an area of 0.01 mm². **C**, No difference in un-myelinated axons with diameter of 1 μm or larger between wild type and *erbin*^{-/-} mice. Axons were scored as in B. **D**, Distribution curves of axon size of myelinated axons in D4-5 segments of wild type and *erbin*^{-/-} mice on day 20 and day 40 after injury.

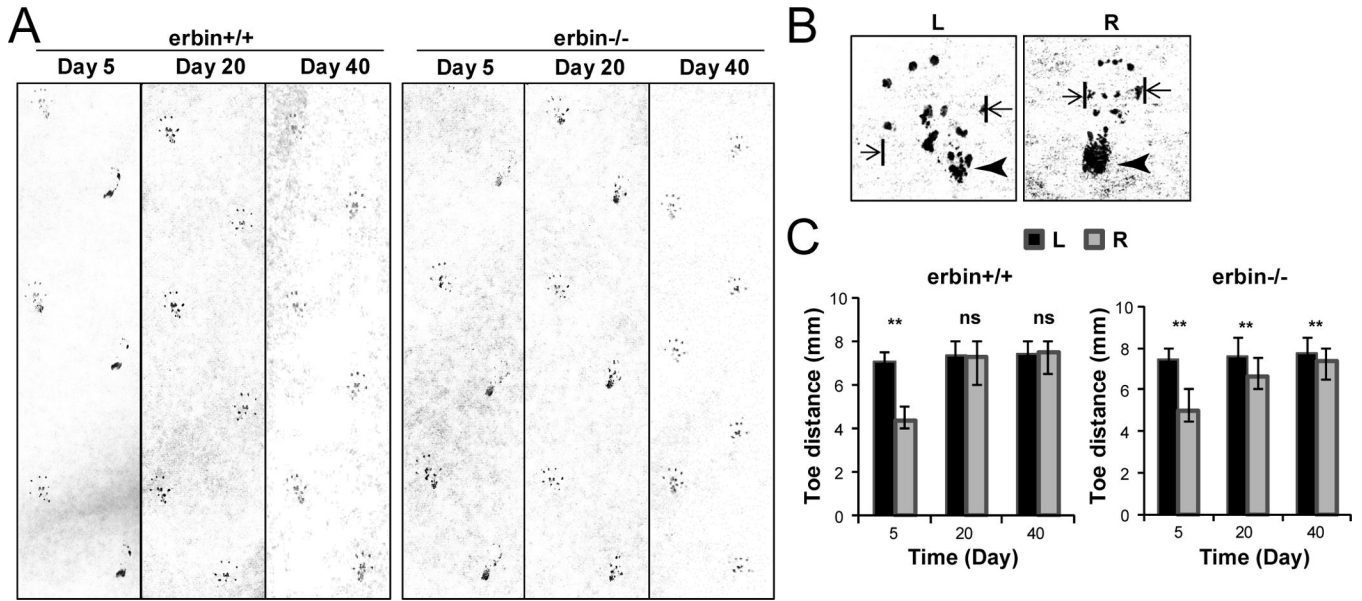


Figure 5. Delayed functional recovery of erbin^{-/-} mice after nerve injury

A, Gait patterns of wild type and erbin^{-/-} mice on indicated days after nerve injury. Paw prints were recorded by painting hind paws with black ink and having them walk on white paper along a 30 × 10 cm corridor (toward the top). Prints of the right feet (where sciatic nerves were injured) were on the right. **B**, Representative footprints of left (L) and right (R) hind paws of wild type mice, on day 5 after injury. Arrowhead indicates heel marks. Toe distance was measured between the first and fifth toes (indicated by arrows). **C**, Delayed recovery in toe distances in erbin^{-/-} mice. Toe distances were measured as indicated in panel B on different days after injury. n = 5, ***p* < 0.01, paired *t* test.

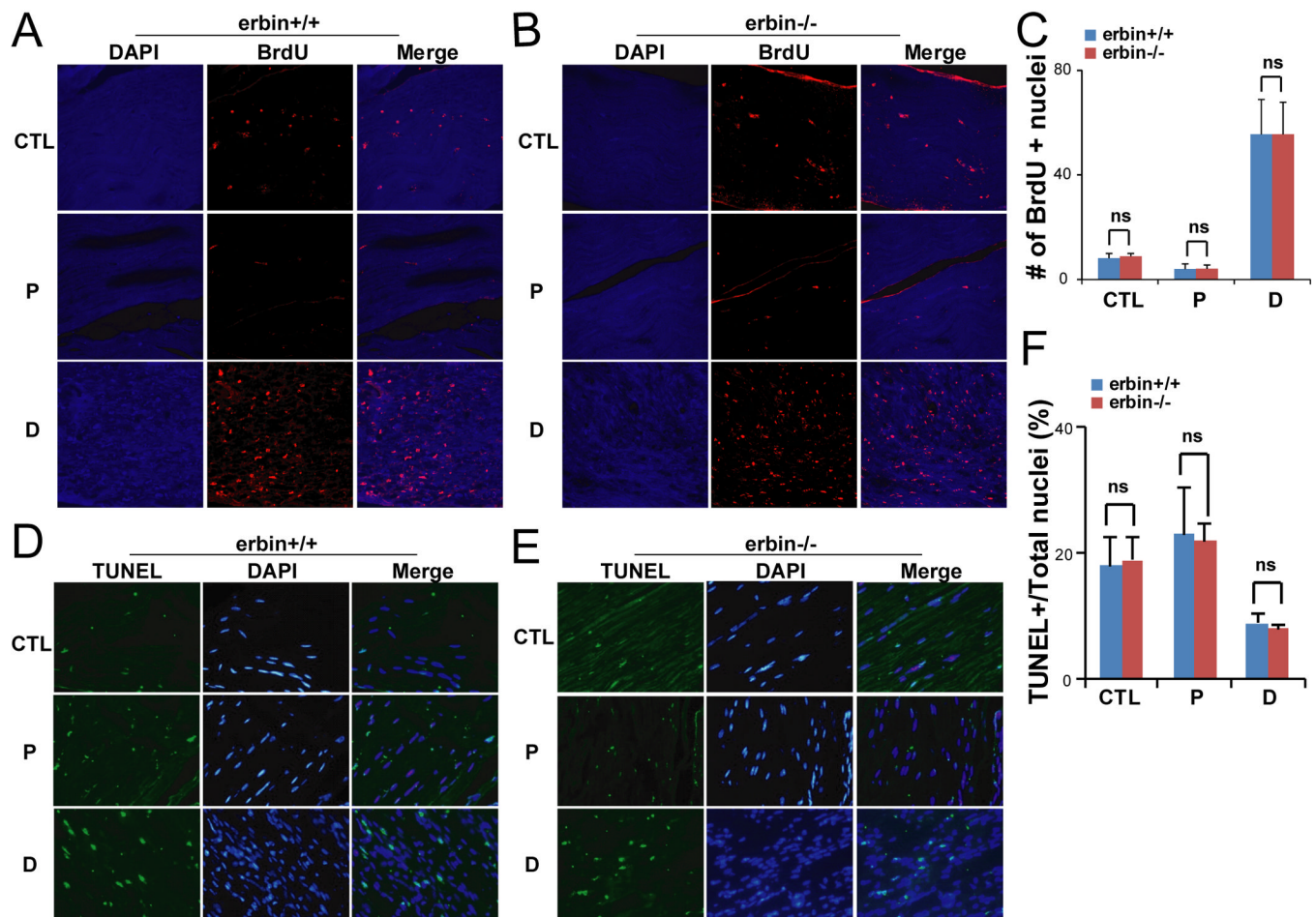


Figure 6. No effect by Erbin mutation on SC proliferation and survival in regenerating nerves after injury

A, B, Representative pictures of BrdU staining from intact nerves (CTL) or of different segments from injured nerves of wild type (A) or *erbin*^{-/-} mice (B). **C,** No difference in the number of BrdU labeled nuclei between wild type and *erbin*^{-/-}. Labeled nuclei in an area of 0.1 mm² were quantified. n = 3. **D, E,** Representative pictures of TUNEL staining from intact nerves (CTL) or of different segments from injured nerves of wild type (D) or *erbin*^{-/-} mice (E). **F,** No difference in the number of TUNEL-positive nuclei between wild type and *erbin*^{-/-}. TUNEL-positive nuclei were counted in an area of 0.1 mm² from different regions and normalized with total nuclear numbers. n = 3.

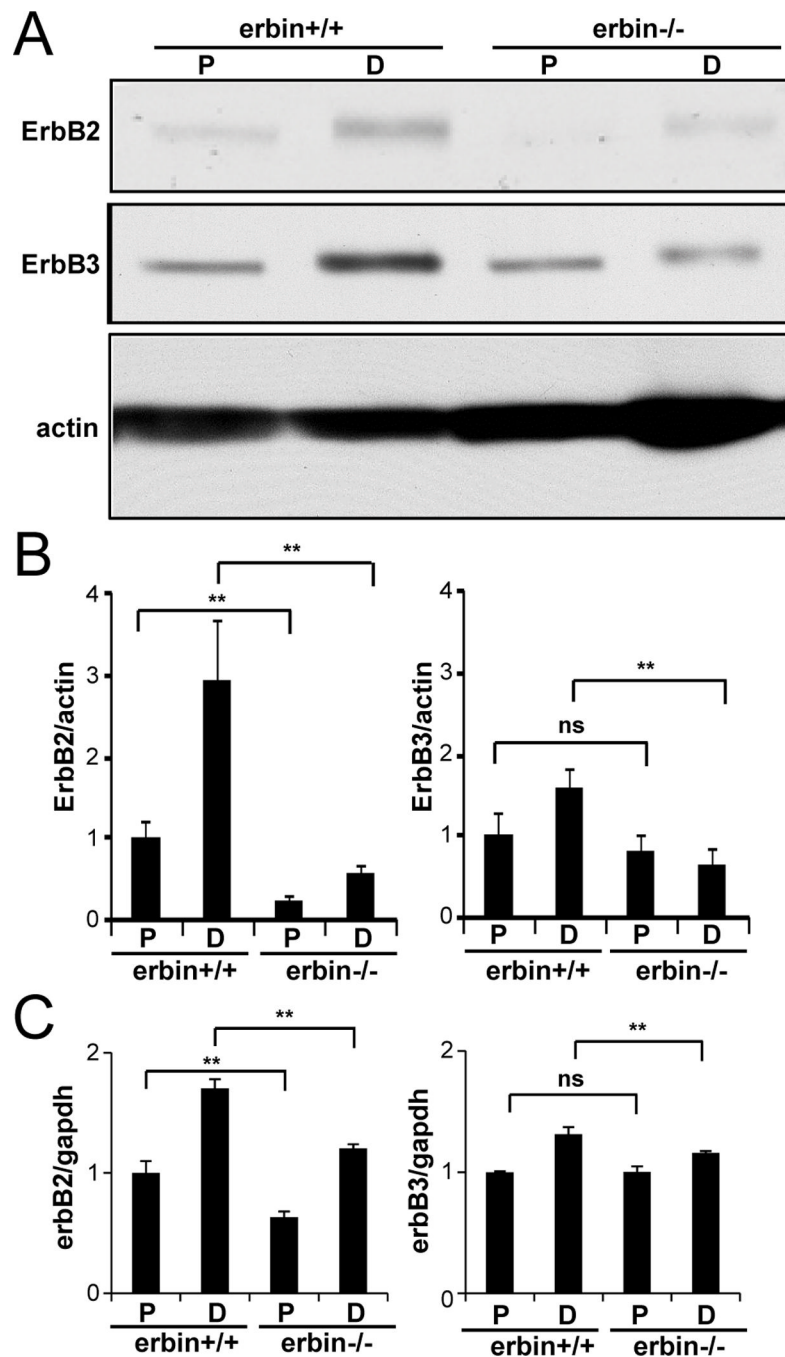


Figure 7. Injury-induced expression of ErbB2 was inhibited in erbin^{-/-} mice

A, Inability of injury to increase ErbB2 expression in erbin^{-/-} distal segments. 5 days after injury, proximal (P) and distal (D) segments were isolated and subjected to Western blot analysis for ErbB2 and ErbB3. **B**, Quantitative analysis of data in A. $n = 3$, $**p < 0.01$, compared to wild type controls. ErbB2 and ErbB3 levels were increased to 2.9 ± 0.25 and 1.6 ± 0.22 folds, respectively, above those in proximal segments in wild type mice ($n = 3$, $p < 0.01$). ErbB2 levels in erbin^{-/-} mice were 2.3 ± 0.46 folds ($n = 3$, $p < 0.05$) above those in proximal segments while ErbB3 had no increase. **C**, mRNA levels of erbB2 and erbB3 in

proximal and distal segments of injured sciatic nerves. mRNA was analyzed by quantitative RT-PCR, $n = 3$, $**p < 0.01$, compared to wild type controls.

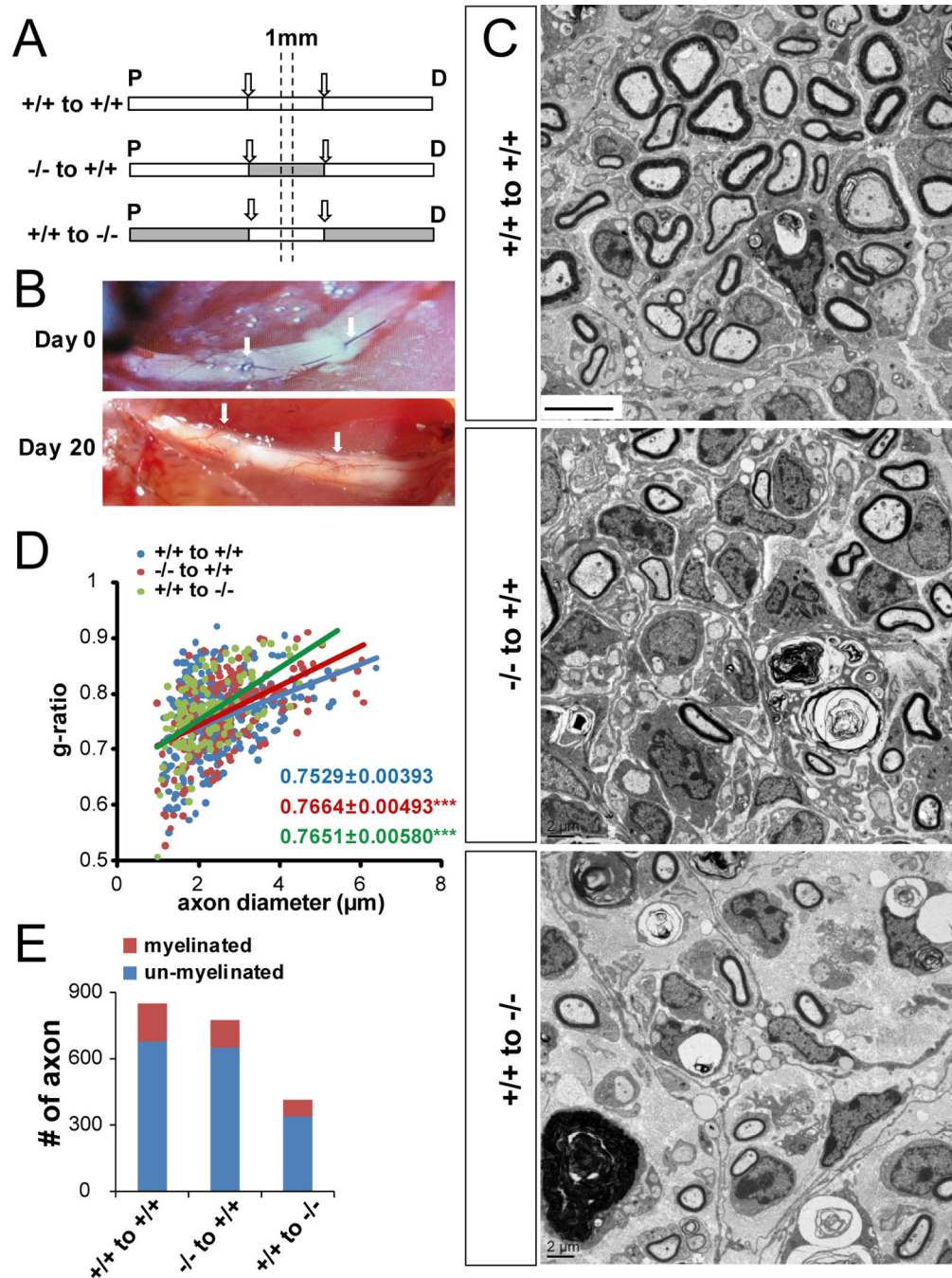


Figure 8. Impaired remyelination in allografts of different genotypes

A, Schematic illustration of allograft transplant experiments between wild type and *erbin*^{-/-} sciatic nerves. Arrows mark the allograft segment. Dashed lines mark the central region in allografts for EM examination. **B**, Sciatic nerves on day 0 and day 20 after transplant surgery. Arrows indicate allografts. **C**, Representative EM images from indicated allograft/transplant groups. Scale bar, 5 μm . **D**, Impaired myelination of *-/-* to *+/+* and *+/+* to *-/-* allograft axons. *g*-ratios of myelinated axons in allografts were plotted against axonal diameters. $n = 20$ images per mouse, 3 mice per group; $***p < 0.001$, compared to *+/+* to *+/+*. **E**, Reduced myelinated axons in *-/-* to *+/+* and *+/+* to *-/-* allograft axons. Scored

were axons from over 20 EM images per mouse, 3 mice per genotype in an area of 0.01 mm².

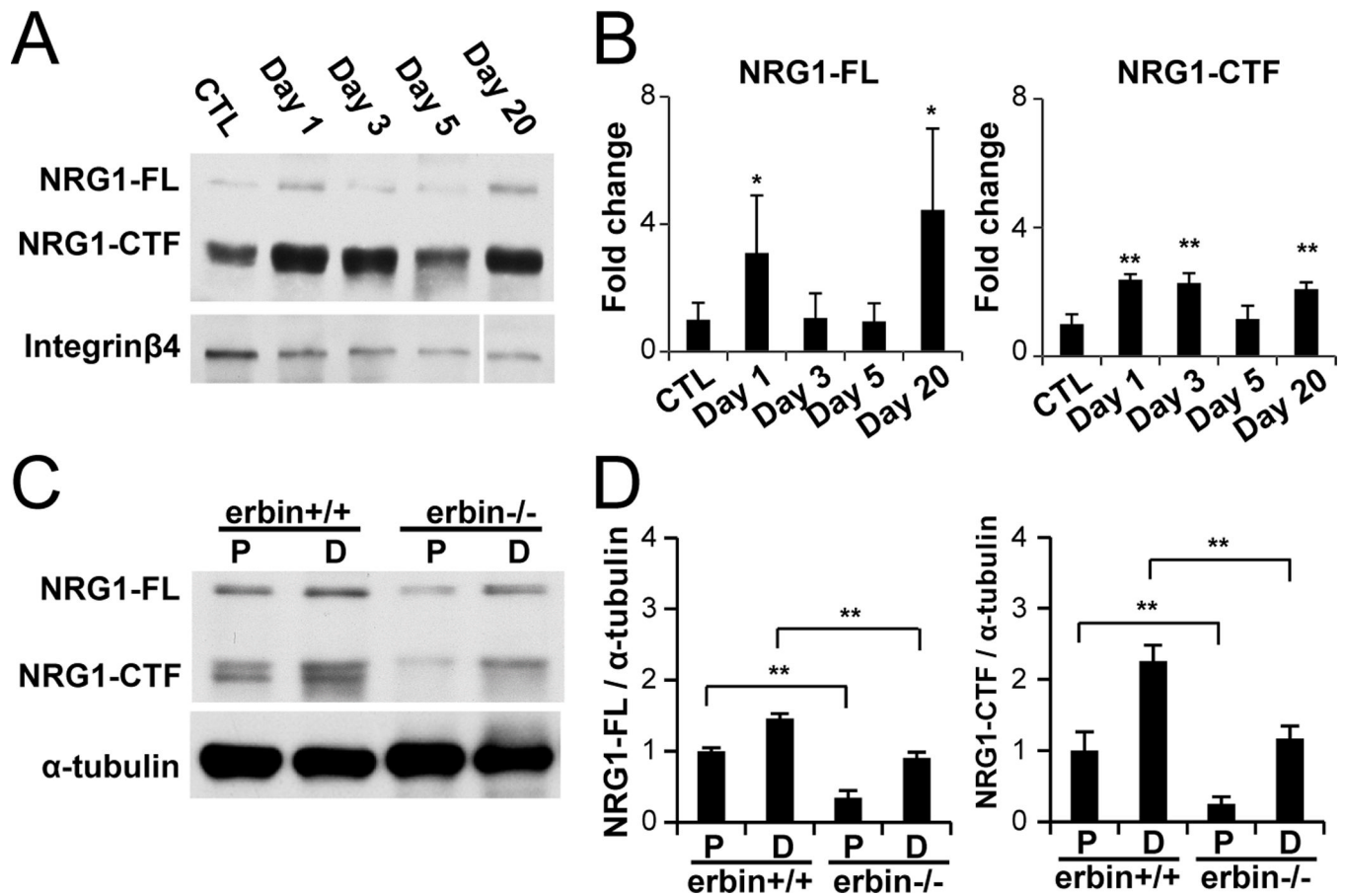


Figure 9. Requirement of Erbin for elevated type III NRG1 expression in injured nerves
A, Western blotting results showing increased type III NRG1 expression in wild type mice at indicated times after injury. **B**, Quantitative analysis of data in **A**. $n = 3$, $*p < 0.05$; $**p < 0.01$, compared to uninjured controls. **C**, Reduced type III NRG1 levels in injured distal segments in $erbin^{-/-}$ mice. Proximal (P) and distal (D) segments were isolated 5 days after injury and subjected to Western blot analysis. **D**, Quantitative analyses of data in **C**. $n = 3$, $**p < 0.01$, compared to wild type controls.