Supporting Information

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SI Materials and Methods

Reagents. pFlag-ErbB2, pcDNA3-ErbB3, pEF6-Erbin, pRK5, pRK5-Erbin/PDZ (Erbin965), pU6-lacZshRNA and pU6–4049-shRNA are described in refs. 1 and 2. Antibodies (with catalog number) used are from Cell signaling Technology (pAkt, 9271; Akt, 9272; Erk, 9102), NeoMarkers (neurofilament, MS-359), Abcam (EBP50, ab3452; β-gal, ab616; Thy1.1, ab50200), BD Transduction Laboratories (δ-cateinin, C98320), Promega (pErk, Z803A), DakoCytomation (S-100, Z20311), Upstate (4G10, 16–105), and Santa Cruz Biotechnology (ErbB2, sc-284; ErbB3, sc-285; ErbB4, sc-283; P0, sc-18531; NRG1, sc-348; Integrinβ4, sc-9090). Fluorescence secondary antibodies were purchased from Molecular Probe, and HRP-conjugated ones were from Amersham Biosciences. Rabbit Erbin antibody has been described in ref. 3. NRG1 used is a recombinant polypeptide containing the entire *EGF* domain of the β-type NRG1 (rHRG β177–244) as a gift from Dr. Mark Sliwkowski (4). Cycloheximide was from Calbiochem; MG132 was from BostonBiochem. All other chemicals were from Sigma.

Generation of *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ Mice. To generate *erbin*$^{-/-}$ mice, exons 1 and 2 of *erbin* gene were replaced in *erbin*-targeting vector by a neomycin-resistant marker flanked with LoxP sites. Erbin null ES cells 129 Ola were used to generated *erbin*$^{-/-}$ mice. To generate *erbin*$^{\Delta C/\Delta C}$ mice, Bga258 ES cells that contain the *erbin* gene disrupted by gene trapping using the pGT2Lxf vector, were obtained from BayGenomics. ES cell clones were transferred into mouse blastocysts. Chimeras that were generated from ES cells were crossed to C57/Bl6 mice, and the resulting heterozygous animals were crossed to produce homozygous mice. Mouse genotyping were performed by PCR with genomic DNA extracted from mouse tail tips. Primers used for *erbin*$^{-/-}$ mice genotyping are: Primers N1 (forward): 5′-TTGTC AAGAC CGACC TGTCC GGTG; Primers N2 (reverse): 5′-ACGGG TAGCC AACGC TATGT CCTG; Primers E1 (Forward): 5′-CTAGT TCAAG GCCAG TCTGA; Primers E2 (reverse): 5′- CAGTT AGGGT TGCTG GATTA. Primers used for *erbin*$^{\Delta C/\Delta C}$ mice genotyping are: Primer 6: 5′-CACTC TGTAA TCAGT TCTTA GCAG; Primer 6′: 5′-GGTAA GACAG AAACT GGCAC CAG; and Primer 6‘‘: 5′-CACTC CAACC TCCGC AAACT C.

EM Studies. P30 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 perfusion fixative. Twenty-four hours later, samples were washed by 0.1 M NaCac and osmicated with 2% osmium tetroxide 30–60 min at 4 °C, washed by 0.1 M NaCac and by deionized H_2O 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 and embedded with embedding resins. Ultrathin sections were photographed with Phillips 400 Transmission electron microscope. EM images were analyzed by Image J (National Institutes of Health). To eliminate the bias on circularity, *g*-ratio of each axon was calculated by the perimeter of axons (inner) divided by the perimeter of corresponding fibers (outer). Axonal diameters were normalized by perimeters through equation: diameter = perimeter/π. This procedure allows for inclusion of irregularly-shaped axons and fibers and helps to eliminate biased measurement of diameters based on circularity. For quantitative analysis, cross sections of sciatic nerves were divided into 10 areas, and more than 2 images, randomly selected from each area, were examined.

Schwann Cell Culture. Primary Schwann cells were prepared by a modified protocol (5, 6). Briefly, rat sciatic nerves were digested in 3 mg/mL collagenase at 37 °C for 30 min, and incubated with 0.25% trypsin at 37 °C for 5 min. Digested nerves were passed through 18-G needles (15 times) and 20-G needles (7 times). Dissociated cells were isolated by centrifuging for 5 min at 150 g. Cell pellets were resuspended in DMEM/10%FBS and plated onto PLL-coated 100 mm dishes. AraC (5 μM) was added into media the next day for 72 h to suppress the growth of fibroblasts. Confluent cells were dissociated by trypsin digestion and subjected to immunopanning with Thy1.1 to eliminate fibroblasts. After replating, cells were cultured in media supplemented with 2 μM forskolin and 10 μg/mL insulin instead of NRG1, to avoid interfering ErbB signaling. More than 98% purity of SC cells, assayed by the SC specific marker S100, was obtained.

Nucleofection. Transfection was performed by nucleofection using Nucleofector II (Amaxa) per manufacture's instructions. In brief, 4–5 μg plasmids were nucleofected into 2–4 × 10^6 cells by using program Q-001 for HEK293, A-024 for COS7 and T-030 for rat SCs (7). Nucleofected SCs were plated on 6-well plates precoated with PLL and laminin. More than 90% transfection efficiency was routinely achieved in primary SCs. Cells were used for experiments 72 h after plating to allow knockdown of Erbin. Cells were starved in DMEM/1%FBS for 24 h and DMEM without serum for 4 h before NRG1 stimulation.

RT-PCR. Total RNA was extracted from pooled mouse sciatic nerves with TRIzol (Invitrogen) according to the manufacture's instruction. cDNA and PCR products were generated by SuperScript III One-Step RT-PCR System with Platinum TaqDNA Polymerase (Invitrogen). *erbin*$_{1\text{-}693}$*βgal* transcripts were detected using forward primer P20 on exon 20 (5′- GAAAA TGGCA GAGAT GCGAC CTCC) and reverse primer β-geo on pGT2Lxf trapping vector (5′- GACAG TATCG GCCTC AGGAA GATCG), which generated a product of 604 bp. For *erbB2* transcripts, primers were 5′- CGCGG GTACC CAAGT GTGTA (forward) and 5′- CGTTG TCCAA AGGGT CTCG (reverse), which generated a product of 326 bp.

Immunostaining and Immunoblotting. Tissue sections were fixed in 4% PBS-buffered polyformaldhyde solution, and permeabilized with 0.3% Triton-X 100 and 3% goat serum in PBS. Samples were incubated with primary antibodies diluted in PBS containing 3% goat serum at 4 °C overnight. After washing with PBS for 3 times, samples were incubated with Alexa Fluor 488 goat anti-Rabbit or Alexa Fluor 594 goat anti-mouse secondary antibody for 1 h at room temperature. Samples were mounted with Vectashield mounting medium (Vector) and images were taken by Zeiss LSM510 confocal microscope. For immunoblotting, cells were lyzed and tissues homogenized in modified RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM EDTA, 5 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitors). Lysates were resolved on SDS/PAGE and transferred to nitrocellulose membranes. The membranes were blocked in PBS with 0.1% Tween-20 and 5% milk and incubated with indicated antibodies overnight at 4 °C, and then with

HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce). Autoradiographic films were scanned with an Epson 1680 scanner, and the captured image was analyzed with Image Quant 5.2 (Molecular Dynamics).

Immunoprecipitation. SC lysates were cleaned by centrifugation at 16,000 × *g* for 15 min and were subjected to immunoprecipitation with polyclonal antibody against ErbB3 at 4 °C for overnight and protein-A agarose (Roche, Germany) at room temperature for 1 h. Bound proteins were resolved by SDS/PAGE and analyzed by immunoblotting with monoclonal antibody recognizing phosphotyrosine (4G10). Blots were reprobed with ErbB3 antibody to check immunoprecipitating efficiency.

Conduction Velocity and Von Frey Fiber/Sensory Test. Tail conduction velocity was recorded at stringently controlled tail surface temperatures (23–24 °C). The time between a stimulus and action potential includes nerve conduction and that of non-nerve tissues such as fat, muscle and skin tissues. To eliminate the variation by non-nerve tissues, tails were stimulated by 2 ring electrodes at different proximal locations. Conduction was recorded by an electrode placed at the distal region, with tail tips grounded by a clip electrode. Electrical pulses propagated on tail nerves were recorded by Nicolet VikingQuest. Conduction velocity was calculated by the program preset in VIASYS Healthcare NeuroCare. For sensory tests, hindpaws were stimulated by Von Frey Hair (Stoelting). The withdrawal of the paw or a brisk movement of mouse body was taken as positive response.

Endocytosis Assays. Surface proteins were biotinylated by 1.5 mg/mL sulfo-NHS-SS-biotin (Pierce) in DMEM at 4 °C for 60 min. After washing with cold HBSS (with Ca^{2+} and Mg^{2+}) 3 times, cells were incubated at 37 °C for indicated times in DMEM with or without NRG1 to allow endocytosis to occur. Remained surface biotin was cleaved by incubation (15 min each, twice) with the glutathione cleavage buffer (50 mM glutathione, 75 mM NaCl, 10 mM EDTA, 1% BSA, and 0.075 N NaOH). Cells were washed with cold HBSS and lyzed with modified RIPA buffer. Cell lysates were incubated with streptavidin beads (Pierce) on a rotating platform overnight at 4 °C. Precipitated biotinylated proteins were eluted by incubating the beads with 1X SDS-sample buffer and were analyzed by immunoblotting. All processes were performed with lysosome inhibitor leupeptin and proteasome inhibitor MG132.

Statistic Analysis. Data were analyzed using Excel software (Microsoft) and presented as mean ± SEM of 3 or more independent experiments. Student's *t* test was used for difference analysis.

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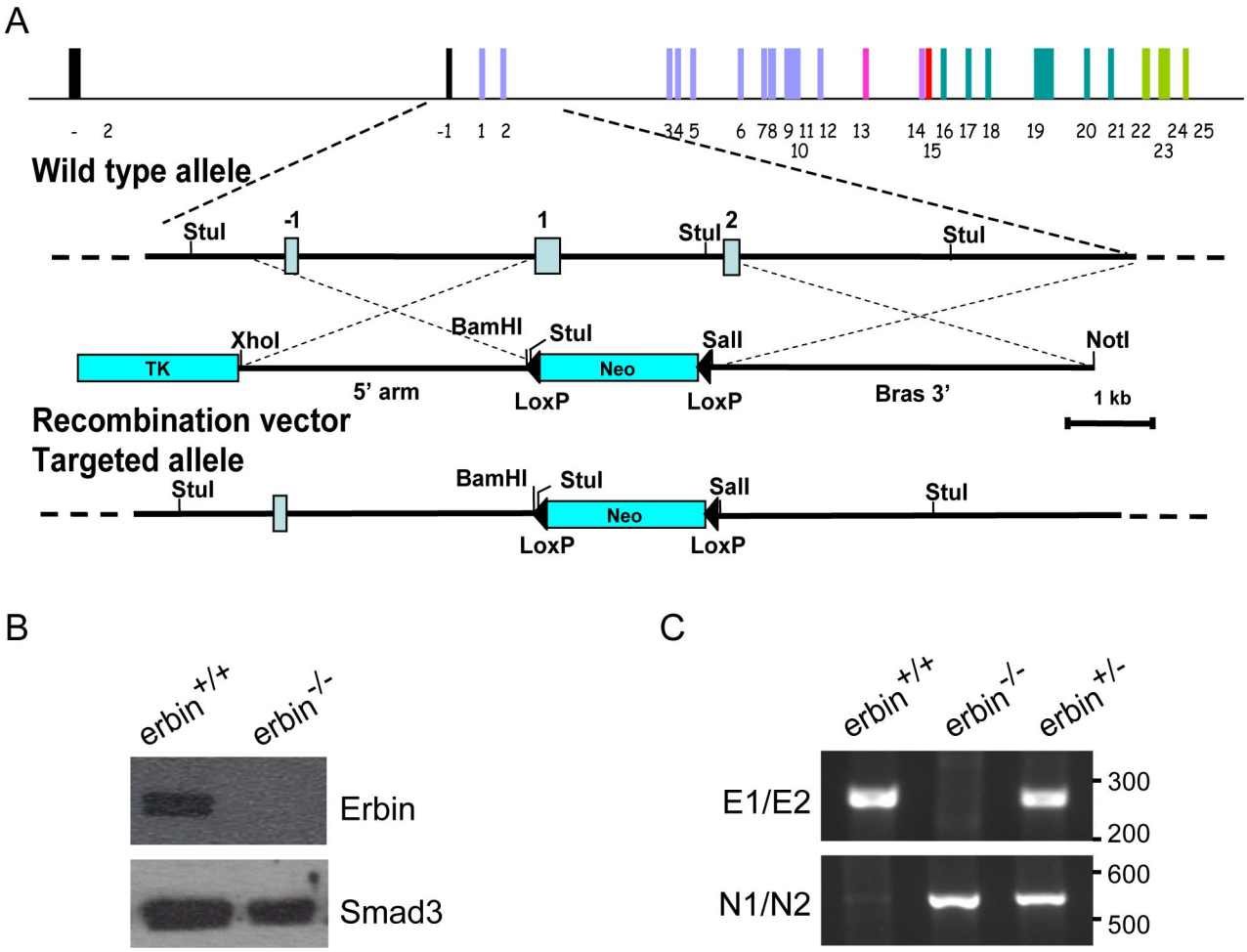

Fig. S1. Generation of Erbin null (*erbin*$^{-/-}$) mice. (*A*) Strategy to generate Erbin null allele. (*B*) Immunoblot analysis of embryonic fibroblasts derived from *erbin*$^{-/-}$ and control wild type (*erbin*$^{+/+}$) mice. Smad3 was probed to indicate equal loading of proteins. (*C*) Genotyping of *erbin*$^{+/+}$ and *erbin*$^{-/-}$ mice. The wild-type allele of *erbin* was amplified by primers E1 and E2, which generated 286 bp products; and the null-mutated allele was amplified by the primers N1 and N2 on *neo* gene, which yield 535 bp products.

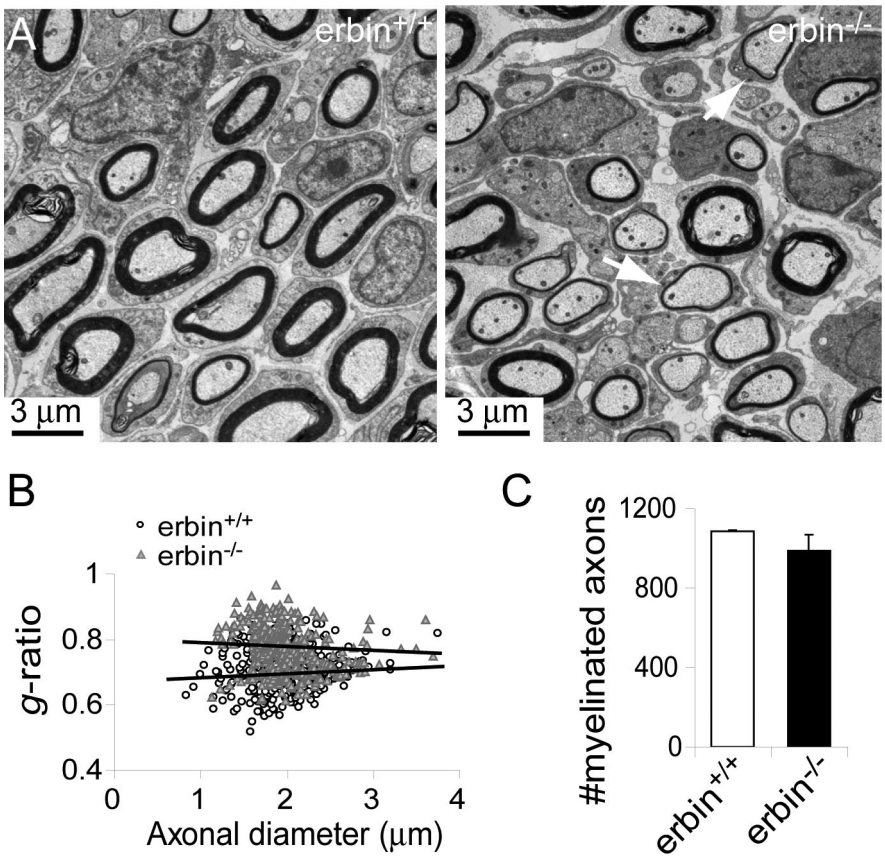

Fig. S2. Myelin deficits in *erbin*$^{-/-}$ sciatic nerves at P8. (*A*) Thinner myelin in *erbin*$^{-/-}$ sciatic nerves at P8. Shown are representative EM images of sciatic nerve cross sections from *erbin*$^{-/-}$ and their *erbin*$^{+/+}$ littermates at P8. White arrows indicate the large axons with extremely thin myelin. (*B*) Increased *g*-ratio of myelinated axons in *erbin*$^{-/-}$ sciatic nerves at P8. Shown are scattered plot of *g*-ratios corresponding to different axonal diameters. Averaged *g*-ratio changed from 0.712 ± 0.0139 ($n = 347$) in sciatic nerves of *erbin*$^{+/+}$ littermates to 0.766 ± 0.0136 ($n = 379$, $P < 0.001$) in those of *erbin*$^{-/-}$ mice at P8. (*C*) Similar number of myelinated axons in *erbin*$^{-/-}$ and *erbin*$^{+/+}$ littermate mice at P8. Cross sections of sciatic nerves were divided to 8 areas, and 2 EM pictures were randomly taken from each area. Myelinated axons were counted from all of the EM pictures for each mouse.

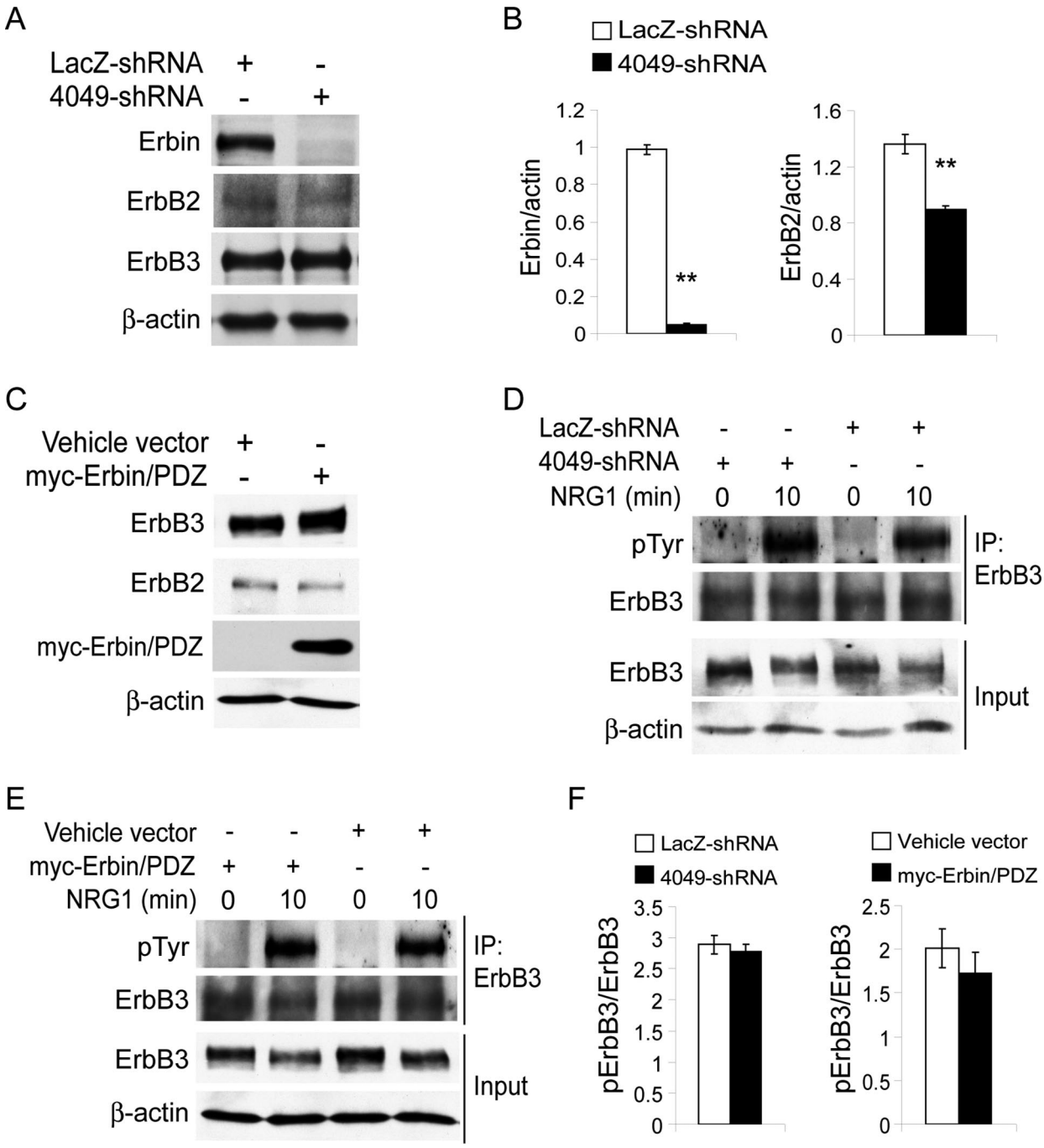

Fig. S3. ErbBs' levels and NRG1-induced ErbB activation in SCs transfected with shRNA or Erbin's PDZ domain. (*A*) Expression of Erbin, ErbB2 and ErbB3 in primary SCs transfected with lacZ-shRNA or 4049-shNRA. SCs were transfected by nucleofection with indicated constructs and analyzed for expression of indicated proteins and β-actin 72 h after transfection. Note that the experiment was done in the absence of NRG. (*B*) Quantitative analyses of data in *A*. $n = 3$; **, $P < 0.01$. Erbin expression was reduced by 91.2 $\pm$ 1.66% in 4049-transfected SCs in comparison with that in control lacZ-shRNA-transfected ones. (*C*) Expression of myc-Erbin/PDZ, ErbB2 and ErbB3 in primary SCs transfected with vehicle vector or myc-Erbin/PDZ. (*D* and *E*) NRG1-induced ErbB3 phosphorylation in transfected SCs. Lysates of control or NRG1 (10 min)-treated cells were subjected to immunoprecipitation and blotting with indicated antibodies. (*F*) Quantitative analyses of phosphorylated ErbB3 in *D* and *E*. $n = 3$.

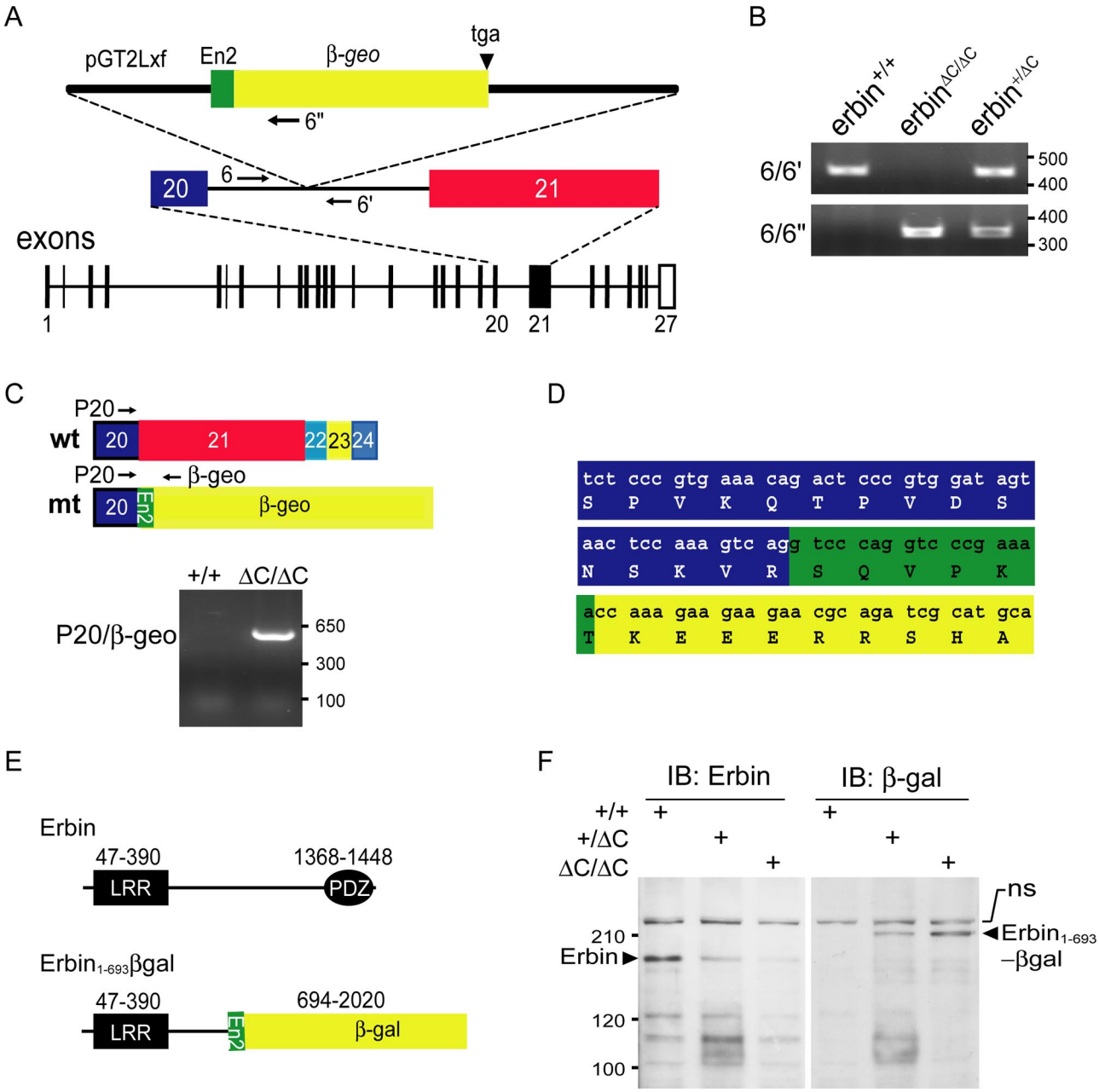

Fig. S4. Generation of *erbin*$^{\Delta C/\Delta C}$ mice. The β-gal gene (*β-geo*) was inserted downstream of exon 20 of the *erbin* gene, producing a chimerical mRNA of *erbin* and *β-geo* mRNAs. The mRNA encodes a fusion protein containing the N-terminal 693 aa residues and β-gal (thus named as Erbin$_{1-693}$βgal). (*A*) Schematic diagrams of the genetrap vector pGT2Lxf insertion between exons 20 and 21. pGT2Lxf contains En2, the splice acceptor/Engrailed-2 exon and *β-geo*. Arrows indicate the orientation of genotyping primers 6, 6′ and 6″. (*B*) Genotyping result. PCR products were generated by primers 6 and 6′ on intro 20 for wt, but not mutant (mt) allele; and by primers 6 and 6″ on *β-geo* for mt, but not wt, allele. (*C*) Analysis of mRNA sequences of wt and mt alleles. Total RNA was isolated from Erbin mutant mouse brain and resulting cDNA was used as template in PCR with primers P20 and β-geo. No PCR product was generated by this pair of primers for wt cDNA. (*D*) Sequences of the linking region between *erbin* and *β-geo*. The mutation generates a chimerical mRNA of exons 1–20 of *erbin* at 5′-end and *β-geo* at 3′-end. DNA sequencing revealed that the truncated Erbin (in blue) and β-gal (in yellow) are in the same reading frame, but the mutant fusion protein contains extra 6 aa residues (in green). (*E*) Domain structures of Erbin and Erbin$_{1-693}$βgal proteins. Mutant Erbin contains the N-terminal 693 aa residues, which are fused in frame with 6 amino acid residues encoded by En2 and β-gal and is thus named Erbin$_{1-693}$βgal. (*F*) Immunoblot analyses revealed gene-dosage-dependent expression of Erbin$_{1-693}$βgal at predicted 210 kDa. Brain homogenates were subjected to immunoblotting with antibody against the PDZ domain of Erbin or anti-βgal antibody. Erbin expression was reduced in *erbin*$^{+/\Delta C}$ (+/ΔC) and ablated in *erbin*$^{\Delta C/\Delta C}$ (ΔC/ΔC) samples whereas Erbin$_{1-693}$βgal was not detectable in *erbin*$^{+/+}$ (+/+) but in *erbin*$^{+/\Delta C}$ and *erbin*$^{\Delta C/\Delta C}$ samples. ns, non-specific band. In *A*, *C*, and *D*: blue indicates sequences of exon 20; green indicates sequences of En2; and yellow indicates sequences of β-gal.

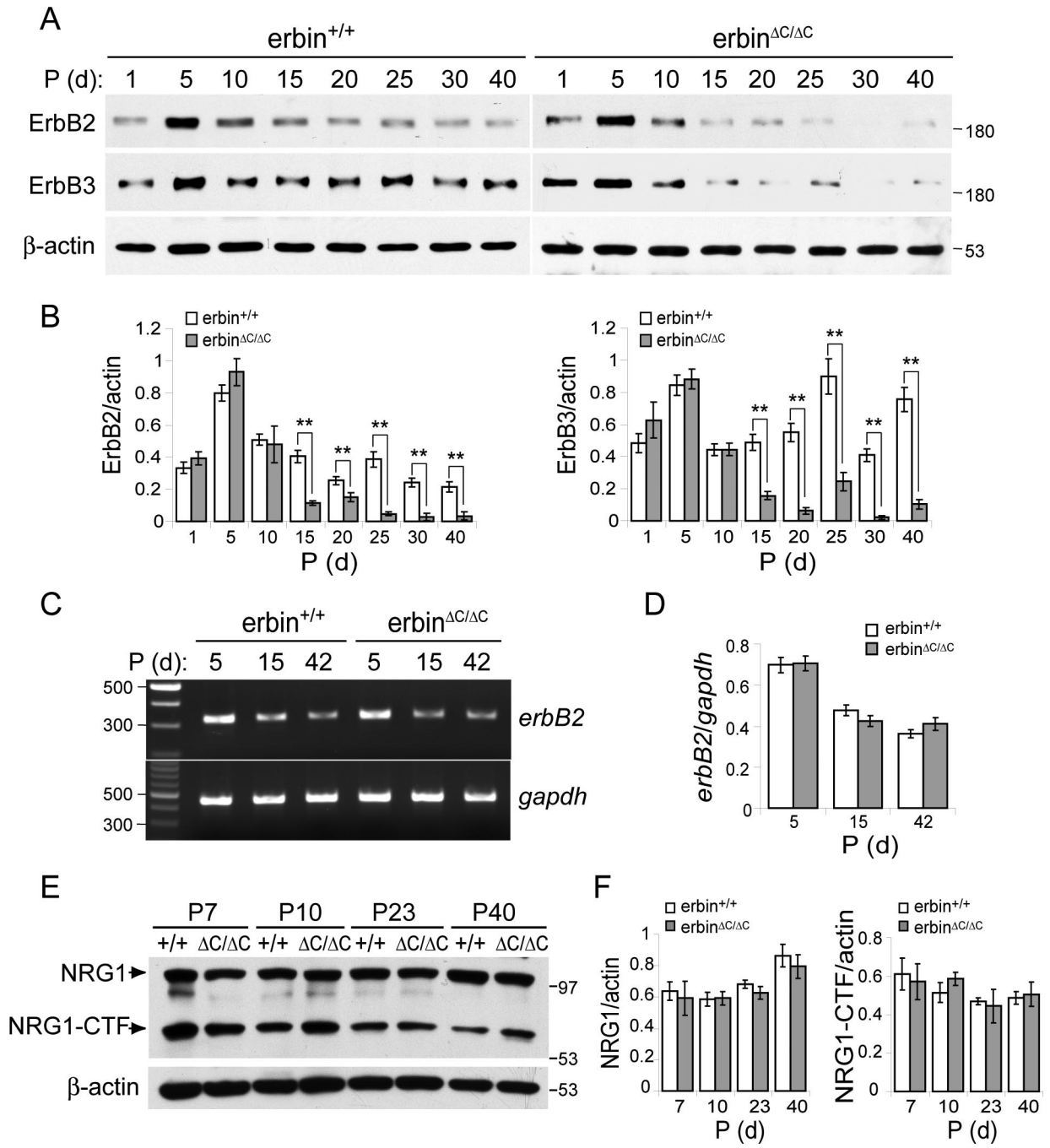

Fig. S5. Reduction of ErbB2 and ErbB3 in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. (*A*) Reduction of ErbB2 and ErbB3 in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Sciatic nerves at different ages were homogenized and analyzed for expression of indicated proteins by immunoblotting. (*B*) Quantitative analyses of data in *A*. $n = 3$, **, $P < 0.01$. (*C*) No difference of the *erbB2* mRNA level between *erbin*$^{\Delta C/\Delta C}$ and control sciatic nerves. Total RNA was purified and subjected to RT-PCR using specific primers of *erbB2* and *gapdh*. (*D*) Quantitative analyses of data in *C*. $n = 3$. (*E*) Similar levels of full length NRG1 and NRG1-CTF between *erbin*$^{\Delta C/\Delta C}$ and control sciatic nerves. (*F*) Quantitative analyses of data in *E*. $n = 3$.

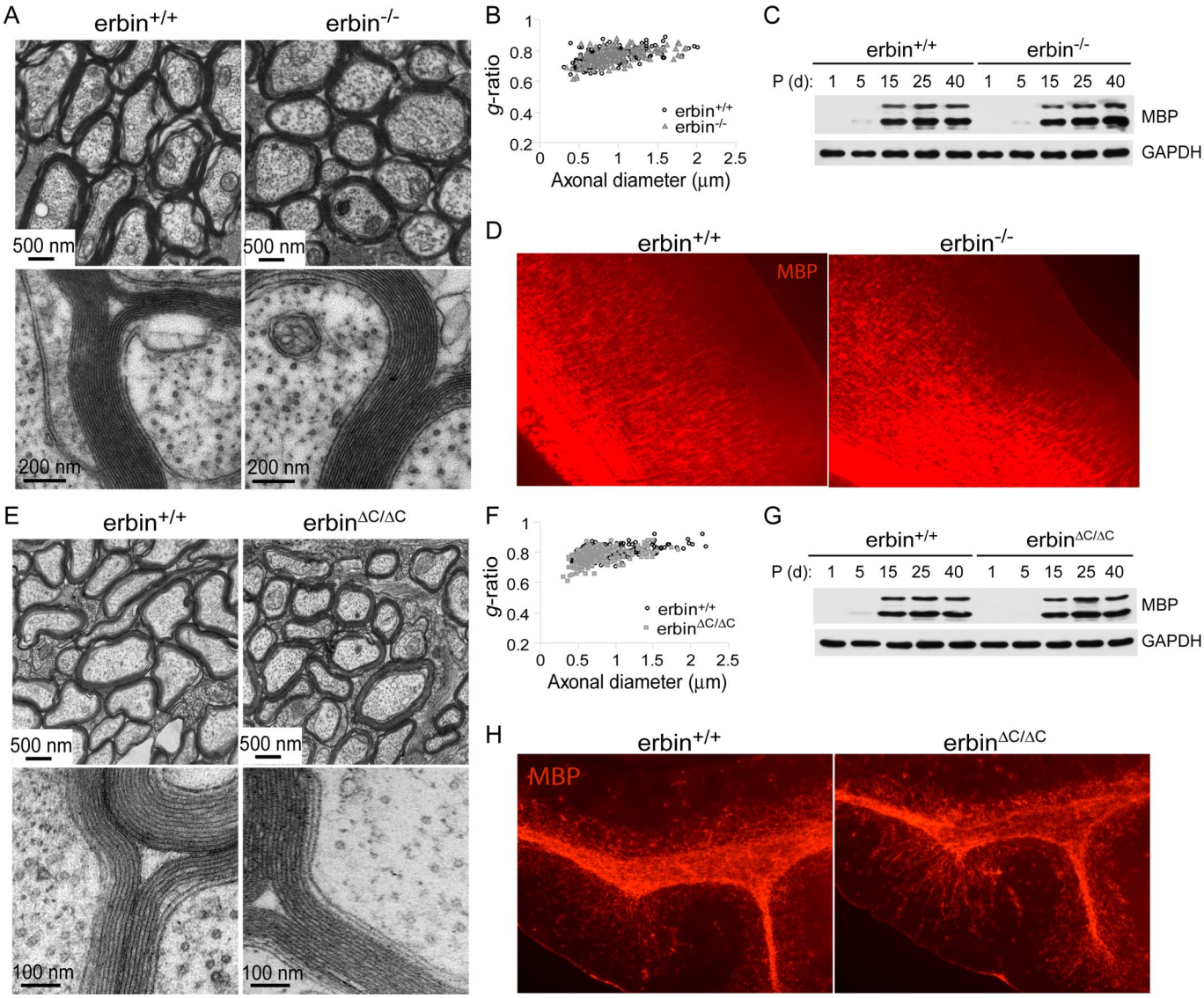

Fig. S6. CNS myelination were apparently normal in *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ mice. (*A*) Similar myelin in *erbin*$^{-/-}$ and control optic nerves. EM images of optic nerve cross-sections from P30 mice were shown at 2 different magnifications. (*Lower*) Images show similar interperiod lines and ultrastructures between mutant and control nerves. (*B*) Overlapped distribution of *g*-ratio in *erbin*$^{-/-}$ and control optic nerves. Three pairs of littermates, and ≈300 axons for each mouse, were analyzed as described in *SI Materials and Methods*. (*C*) Similar MBP levels in *erbin*$^{-/-}$ and control corpus callosum and medulla. Homogenates were subjected to SDS/PAGE and probed for MBP. Equal loading was indicated by GAPDH. (*D*) Similar MBP staining in corpus callosum of *erbin*$^{-/-}$ and control mice. (*E*) Similar myelin in *erbin*$^{\Delta C/\Delta C}$ and control optic nerves. EM images of optic nerve cross-sections from P30 mice were shown at 2 different magnifications. (*Lower*) Images show similar interperiod lines and ultrastructures between mutant and control nerves. (*F*) Overlapped distribution of *g*-ratio in *erbin*$^{\Delta C/\Delta C}$ and control optic nerves. Three pairs of littermates, and ≈300 axons for each mouse, were analyzed as described in *SI Materials and Methods*. (*G*) Similar MBP levels in *erbin*$^{\Delta C/\Delta C}$ and control corpus callosum and medulla. Homogenates were subjected to SDS/PAGE and probed for MBP. Equal loading was indicated by GAPDH. (*H*) Similar MBP staining in cerebellum of *erbin*$^{\Delta C/\Delta C}$ and control mice.