Erbin regulates NRG1 signaling and myelination

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Neuregulin 1 (NRG1) plays a critical role in myelination. However, little is known about regulatory mechanisms of NRG1 signaling. We show here that Erbin, a protein that contains leucine-rich repeats (LRR) and a PSD95-Dlg-Zol (PDZ) domain and that interacts specifically with ErbB2, is necessary for NRG1 signaling and myelination of peripheral nervous system (PNS). In Erbin null mice, myelinated axons were hypomyelinated with reduced expression of P0, a marker of mature myelinating Schwann cells (SCs), whereas unmyelinated axons were aberrantly ensheathed in Remak bundles, with increased numbers of axons in the bundles and in pockets. The morphological deficits were associated with decreased nerve conduction velocity and increased sensory threshold to mechanistic stimulation. These phenotypes were duplicated in *erbin*$^{\Delta C/\Delta C}$ mice, in which Erbin lost the PDZ domain to interact with ErbB2. Moreover, ErbB2 was reduced at protein levels in both Erbin mutant sciatic nerves, and ErbB2 became unstable and NRG1 signaling compromised when Erbin expression was suppressed. These observations indicate a critical role of Erbin in myelination and identify a regulatory mechanism of NRG1 signaling. Our results suggest that Erbin, via the PDZ domain, binds to and stabilizes ErbB2, which is necessary for NRG1 signaling that has been implicated in tumorigenesis, heart development, and neural function.

ErbB2 | PDZ | protein stability | Schwann cells | Akt

Myelin sheath wraps axons to ensure the velocity and timing of action potential propagation and insulates neuronal activity. Impaired myelin formation and maintenance have been implicated in various neurological and psychiatric disorders including schizophrenia, multiple sclerosis, and Charcot–Marie–Tooth neuropathy disease (1, 2). Myelination is a tightly controlled, complex process. In the peripheral nervous system (PNS), neuregulin 1 (NRG1) has emerged as a key axon-derived factor that regulates myelination. Disruption of NRG1 signaling by ablating either the *EGF* domain that is contained in all isoforms or type III isoform leads to an almost complete loss of SCs and of the sensory and motor neurons that they support (3, 4). Recent studies have suggested that the level of NRG1 in the axon is critical for myelination (5, 6).

NRG1 acts by stimulating a family of single transmembrane receptor tyrosine kinases called ErbB proteins (1). Although NRG1 was isolated as a "ligand" to activate ErbB2, it does not interact with the receptor (7). However, ErbB3 can bind NRG1 but its homodimers are catalytically inactive, indicating impaired kinase function (8). Therefore, ErbB2 and ErbB3, major ErbB proteins in SCs, need to form heterodimers with each other to be functional (9). This notion is supported by mouse genetic studies that mutation of NRG1, ErbB2, or ErbB3 genes causes severe deficits of peripheral neurons and SCs (3, 4, 10–13). Disruption of NRG1/ErbB signaling by a dominant negative approach led to deficits in myelinating and nonmyelinating SCs (14, 15). Intracellularly, NRG1 stimulates various cascades including the PI3K/Akt pathway that have been implicated in myelinaton (16). Although much is known about the role of NRG1 signaling in SC myelination, less is known about its regulatory mechanisms.

Erbin is an adapter protein of the LAP family that contains leucine rich repeats (LRR) and a PSD95-Dlg-Zol (PDZ) domain (17, 18). Via the PDZ domain, Erbin interacts specifically with ErbB2 (17, 18), and several other proteins including integrinβ4 (19) and δ-catenin (20), but not ErbB3 or ErbB4 (17, 18). The N-terminal LRR domain interacts with the Sur-8/Ras/Raf complex (21, 22) and Nod2, an intracellular sensor of specific bacterial cell wall components (23). The region between the LRR and PDZ interacts with EBP50 (24) and Smad3 (25). In vitro studies suggest that Erbin may have a broad range of functions by binding to various partners (26).

Here, we studied the role of Erbin in NRG1 signaling using peripheral myelination as a model. We examined axon myelination and ensheathment of sciatic nerves in Erbin null mutant mice and discovered deficits in both myelinated and unmyelinated axons, compromised nerve conduction and increased sensory threshold to mechanistic stimulation. Similar phenotypes were observed in *erbin*$^{\Delta C/\Delta C}$ mice that express an Erbin mutant without the PDZ domain. We have also investigated molecular mechanisms by a combination of in vivo and in vitro experiments. The results indicate that Erbin plays a pivotal role in NRG1 signaling and ErbB2 stability and identify a function of Erbin in peripheral myelination. In light of broad functions of NRG1, these results may have implications in tumorigenesis, heart development, and neural function.

Results

To study the function of Erbin, we examined Erbin expression in various tissues. As shown in Fig. 1*A*, Erbin expression in sciatic nerves was higher than that in dorsal root ganglions (DRG); and in the brain, it was expressed at higher levels in the white matter than in the gray matter. These results suggest that Erbin is enriched in regions where myelinated axons are abundant. This notion was supported by immunohistochemical staining of transverse cross-sections of sciatic nerves. Erbin immunoreactivity was detected almost exclusively in myelin rings, not in axons, of sciatic nerves (Fig. 1*B*). To study the role of Erbin in myelination, we generated Erbin null mutant mice [*erbin*$^{-/-}$ (*SI Materials and Methods* and Fig. S1)] and examined electron micrographs of sciatic nerve cross sections of *erbin*$^{-/-}$ mice at 1 month old. Unless otherwise indicated, control mice were age-controlled wild-type littermates. Remarkably, myelin of axons was significantly thinner in *erbin*$^{-/-}$ mice in comparison with that of *erbin*$^{+/+}$ littermates (Fig. 1*C*), suggesting impaired myelination in Erbin mutant mice. To analyze the deficits quantitatively, we measured *g*-ratios, i.e., axon diameters/fiber diameters of myelinated axons. Averaged *g*-ratio in *erbin*$^{+/+}$ mice was 0.656 ± 0.0135 ($n = 278$), in agreement with the reports in refs. 5, 6, and 15. Significantly, it was increased to 0.740 ± 0.0153 in *erbin*$^{-/-}$ mice ($n = 206$, $P < 0.001$), indicating reduced myelin thickness in *erbin*$^{-/-}$ mice. The reduction in myelin thickness was observed in axons of different sizes ranging from 1 to 7 μm as revealed by the scatter plot of *g*-ratios of individual fibers versus of axon diameters (Fig. 1*D*). The ultrastructure and periodicity of

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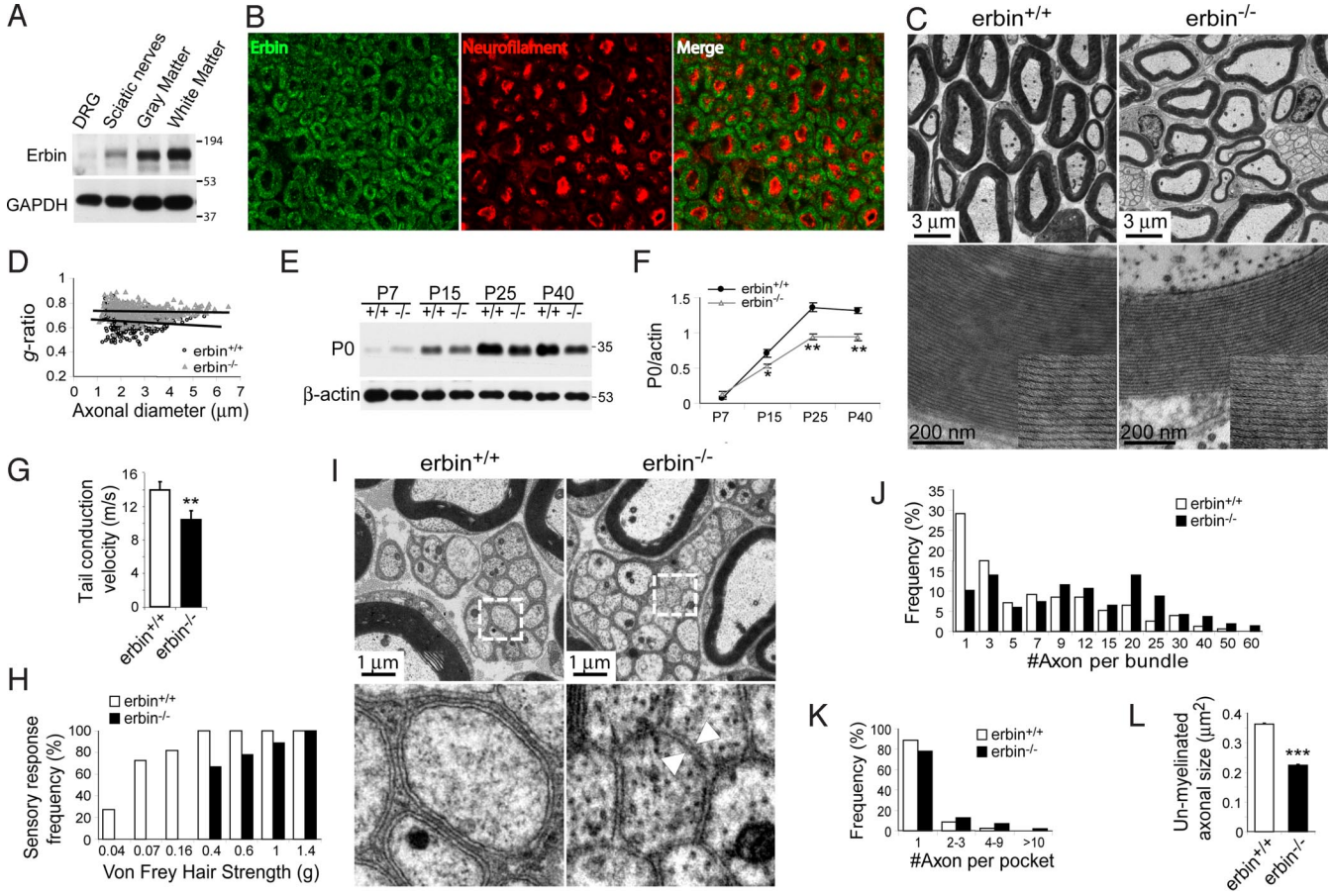

Fig. 1. Hypomyelination and aberrant entheathment of axons in *erbin*$^{-/-}$ sciatic nerves. (*A*) Erbin expresses higher in myelin-enriched tissues. DRG, sciatic nerves, white matter (medulla and corpus callosum) and gray matter (top layers of cortex) were isolated from an adult mouse and homogenized. Homogenates were subjected to SDS/PAGE and probed for Erbin. GADPH was probed to indicate equal loading. (*B*) Localization of Erbin in myelin rings. Sciatic nerves were isolated from an adult mouse, cross-sectioned, and stained with polyclonal antibodies against Erbin and monoclonal antibodies against neurofilament. (*C*) Thinner myelin in *erbin*$^{-/-}$ sciatic nerves. EM images of sciatic nerve cross sections were shown at 2 different magnifications. (*Lower Insets*) Interperiod lines and ultrastructures of myelin sheath. (*D*) Increased *g*-ratio of myelinated axons in *erbin*$^{-/-}$ sciatic nerves. Three pairs of littermates, and ≈250 axons for each mouse, were analyzed as described in *SI Materials and Methods*. (*E*) Reduced expression of the P0 protein in *erbin*$^{-/-}$ sciatic nerves. Homogenates of sciatic nerves from *erbin*$^{-/-}$ or age-controlled littermates were subjected to SDS/PAGE and probed for the P0 protein, and β-actin to indicate equal loading. For all immunoblotting results, shown are representative blots from 3 or more independent experiments. (*F*) Quantitative analysis of data in *E*. $n = 3$, *, $P < 0.05$; **, $P < 0.01$. (*G*) Reduced nerve conduction velocity in *erbin*$^{-/-}$ mice. Nerve conduction was measured as described in *SI Materials and Methods*. $n = 4$, **, $P < 0.01$. (*H*) Increased threshold in sensory response. Von Frey Hair assays were performed as described in *SI Materials and Methods*. Shown are percentages of mice responding to Von Frey Hair stimulation at different strengths. $n = 8$ mice in each group. (*I*) Abnormal axonal segregation in Remak bundles in *erbin*$^{-/-}$ sciatic nerves. Shown are EM images of sciatic nerve cross sections. (*Lower*) Higher magnification of squared areas of *Upper*. Arrowheads indicate adjacent axons that were naked and without proper ensheathment. (*J*) Increased number of unmyelinated axons in Remak bundles in *erbin*$^{-/-}$ sciatic nerves. Remak bundles analyzed were 255 for *erbin*$^{+/+}$ and 216 for *erbin*$^{-/-}$. (*K*) Increased number of unmyelinated axons in SC pockets in *erbin*$^{-/-}$ sciatic nerves. Pockets analyzed were 1602 for *erbin*$^{+/+}$ and 1245 for *erbin*$^{-/-}$. (*L*) Reduced transverse surface areas of unmyelinated axons in *erbin*$^{-/-}$ mice. $n = 204$ for *erbin*$^{+/+}$, $n = 225$ for *erbin*$^{-/-}$. ***, $P < 0.001$. The age of mice was P30 days in *C*, *D*, and *I*-*L* and P60 in *G* and *H*.

compact myelin showed no apparent difference from that of control littermates (Fig. 1*C*, *Lower*), suggesting that impaired myelination in *erbin*$^{-/-}$ sciatic nerves results from the reduced number of myelin lamellae, but not radial sorting (the 1:1 association of SCs to axons), myelin initiation or compaction. In agreement, *erbin*$^{-/-}$ sciatic nerves at postnatal day 8 (P8) showed deficits in myelination and increased *g*-ratios (Fig. S2 *A* and *B*); however, many SCs associated with single axons, indicating proper radial sorting (Fig. S2*A*). Importantly, at this stage, the number of myelinated axons was similar between *erbin*$^{-/-}$ and control littermates (Fig. S2*C*), suggesting that myelin initiation might be normal in *erbin*$^{-/-}$ mutant mice.

Consistent with myelin deficits in morphologic studies, the expression of P0, a major structural protein for peripheral compact myelin (2), was significantly lower in *erbin*$^{-/-}$ sciatic nerves than that in wild-type littermates during development (Fig. 1 *E* and *F*). Thus, myelination is impaired when Erbin is mutated in mice. To investigate whether Erbin mutation alters nerve function, action potentials propagating along tail nerves were recorded under a condition that eliminated influence by nonnerve tissues and temperature variation. As shown in Fig. 1*G*, nerve conduction velocity was significantly reduced in *erbin*$^{-/-}$ mice, suggesting functional impairment. In addition, sensory threshold, smallest strength of Von Frey Hair to generate 80% hindpaw response, was increased in *erbin*$^{-/-}$ mice (Fig. 1*H*). Together, these results demonstrate that Erbin is essential for myelination and proper function of peripheral nerves.

In addition to large (≥1 μm), myelinated axons, there are small axons, such as C fibers that conduct nociceptive signal and are ensheathed by nonmyelinating SCs (6, 14). We investigated whether Erbin is necessary for ensheathment of unmyelinated axons. As shown in Fig. 1*I*, in wild-type mice, unmyelinated axons are clustered in Remak bundles that were sporadically located among myelinated fibers. Most bundles contained <20 axons; and 90% of axons were ensheathed into individual pockets (Fig. 1 *I* to *K*). In

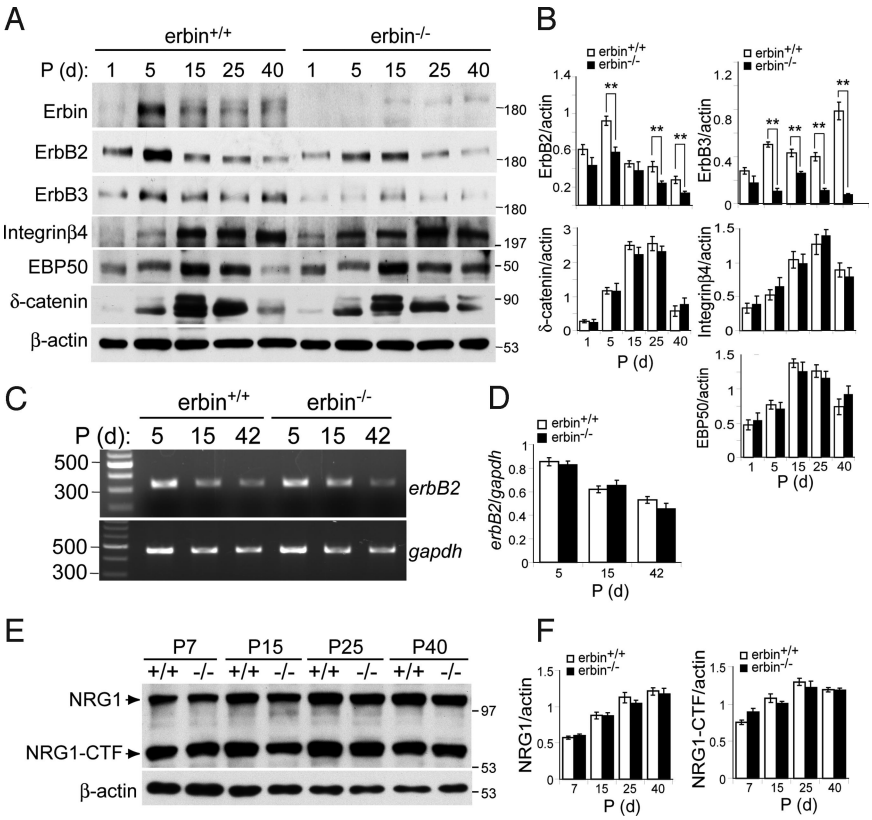

Fig. 2. Specific reduction of ErbB2 and ErbB3 in *erbin*$^{-/-}$ sciatic nerves. (*A*) Reduction of ErbB2 and ErbB3 in *erbin*$^{-/-}$ sciatic nerves. Sciatic nerves at different ages were homogenized and analyzed for expression of indicated proteins by immunoblotting. β-actin was probed to indicate equal loading. (*B*) Quantitative analyses of data in *A*. $n = 3$, **, $P < 0.01$. (*C*) No difference in the *erbB2* mRNA between *erbin*$^{-/-}$ and control littermate 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*$^{-/-}$ and control sciatic nerves. (*F*) Quantitative analyses of data in *E*. $n = 3$.

contrast, the number of unmyelinated axons was significantly increased in Remak bundles in *erbin*$^{-/-}$ sciatic nerves. Quantitative analyses indicate a right-ward shift of the distribution of the number of axons per bundle (Fig. 1*J*). Furthermore, the percentage of pockets containing more axons was significantly increased in *erbin*$^{-/-}$ sciatic nerves (Fig. 1*K*) where axons were compacted to each other and not completely segregated (Fig. 1*I Lower*, arrowheads). In addition, the averaged size of unmyelinated axons was smaller in sciatic nerves of *erbin*$^{-/-}$ mice (Fig. 1*L*), presumably because of lack of support from nonmyelinating SCs. These results indicate deficient ensheathment by nonmyelinating SCs in *erbin*$^{-/-}$ mice, suggesting a critical role of Erbin in this event.

To investigate mechanisms by which Erbin deficiency impairs myelination and ensheathment, we examined expression of Erbin-interacting proteins. Erbin, via the PDZ domain, interacts with integrinβ4, a receptor for laminins, which are the components of extracellular matrix (19), δ-catenin, a member of the p120 catenin family, which is critical for adherence junction formation (20), and ErbB2 (27), all of which are implicated in myelin formation or regeneration (10, 28, 29). It also interacts with EBP50, an adherence junction protein implicated in SC motility (24, 30). As shown in Fig. 2*A*, temporal expression of Erbin did not correlate with that of integrinβ4, δ-catenin, and EBP50 in developing wild-type sciatic nerves. Moreover, levels of the 3 proteins showed no difference between wild-type and *erbin*$^{-/-}$ sciatic nerves (Fig. 2 *A* and *B*). In contrast, ErbB2 expression pattern was similar to that of Erbin, both of which peaked at P5 and gradually reduced after that (Fig. 2*A*). Intriguingly, levels of ErbB2 were reduced in *erbin*$^{-/-}$ sciatic nerves (Fig. 2 *A* and *B*). Considering that ErbB2 is a key component of NRG1 receptor in SCs and NRG1 is important for myelination (1, 9), these results suggest that ErbB2 may be a target of Erbin deficiency. The reduction of ErbB2 did not appear to result from impaired transcription of the ErbB2 gene because ErbB2 mRNA levels were similar between wild-type and *erbin*$^{-/-}$ sciatic nerves (Fig. 2 *C* and *D*), suggesting that the reduction of ErbB2 proteins in *erbin*$^{-/-}$ sciatic nerves was due to a posttranscriptional mechanism. ErbB3, the other ErbB kinase in SCs that forms a heterodimer with ErbB2, was also reduced in *erbin*$^{-/-}$ sciatic nerves (Fig. 2 *A* and *B*). These results suggested that NRG1 signaling that is critical for SC development and myelination (5, 9) was compromised in the mutant mice. The idea was supported by observations that similar myelin deficits exhibit in type III NRG1 hypomorphic mice and mice that express a dominant negative (DN) ErbB4 mutant (5, 6, 14, 15). Note that Erbin deletion did not alter NRG1 expression (Fig. 2 *E* and *F*). Levels of full length NRG1 and its C-terminal fragment (NRG1-CTF) in *erbin*$^{-/-}$ sciatic nerves were similar to those in wild-type littermates. These results indicate that Erbin mutation specifically reduces NRG1 receptors in developing SCs.

To investigate mechanisms by which Erbin deficiency reduces ErbB2, Erbin levels were manipulated to examine if ErbB2 stability is altered. Cells were transfected with 4049-shRNA (21), short hairpin RNA that inhibits Erbin expression (Fig. 3*A* and *B*). Such transfected cells expressed less ErbB2 (Fig. 3 *A* and *B*), in agreement with in vivo studies. Remarkably, ErbB2 degraded faster in 4049-shRNA-expressing cells in comparison with cells transfected with control lacZ-shRNA (Fig. 3*C*). Quantitative analysis revealed that the half-life of ErbB2 in control cells was 7.99 ± 1.45 h ($n = 3$), which became significantly shorter (2.70 ± 0.374 h, $n = 3$, $P < 0.01$) in 4049-shRNA-transfected cells (Fig. 3*D*), indicating that ErbB2 was less stable in cells that express less Erbin. To test this hypothesis further, we transfected cells with Erbin and found that ErbB2 became more stable in cells that overexpressed Erbin (Fig. 3 *C* and *D*). Its levels barely changed within 4 h of experiments. The positive correlation between Erbin levels and ErbB2 stability suggest a necessary role of Erbin in maintaining ErbB2 stability.

Degradation of transmembrane proteins is initiated by internalization (27). To explore mechanisms by which Erbin regulates ErbB2 stability, we examined if ErbB2 internalization changes with Erbin levels. In the absence of NRG1, internalized ErbB2 was

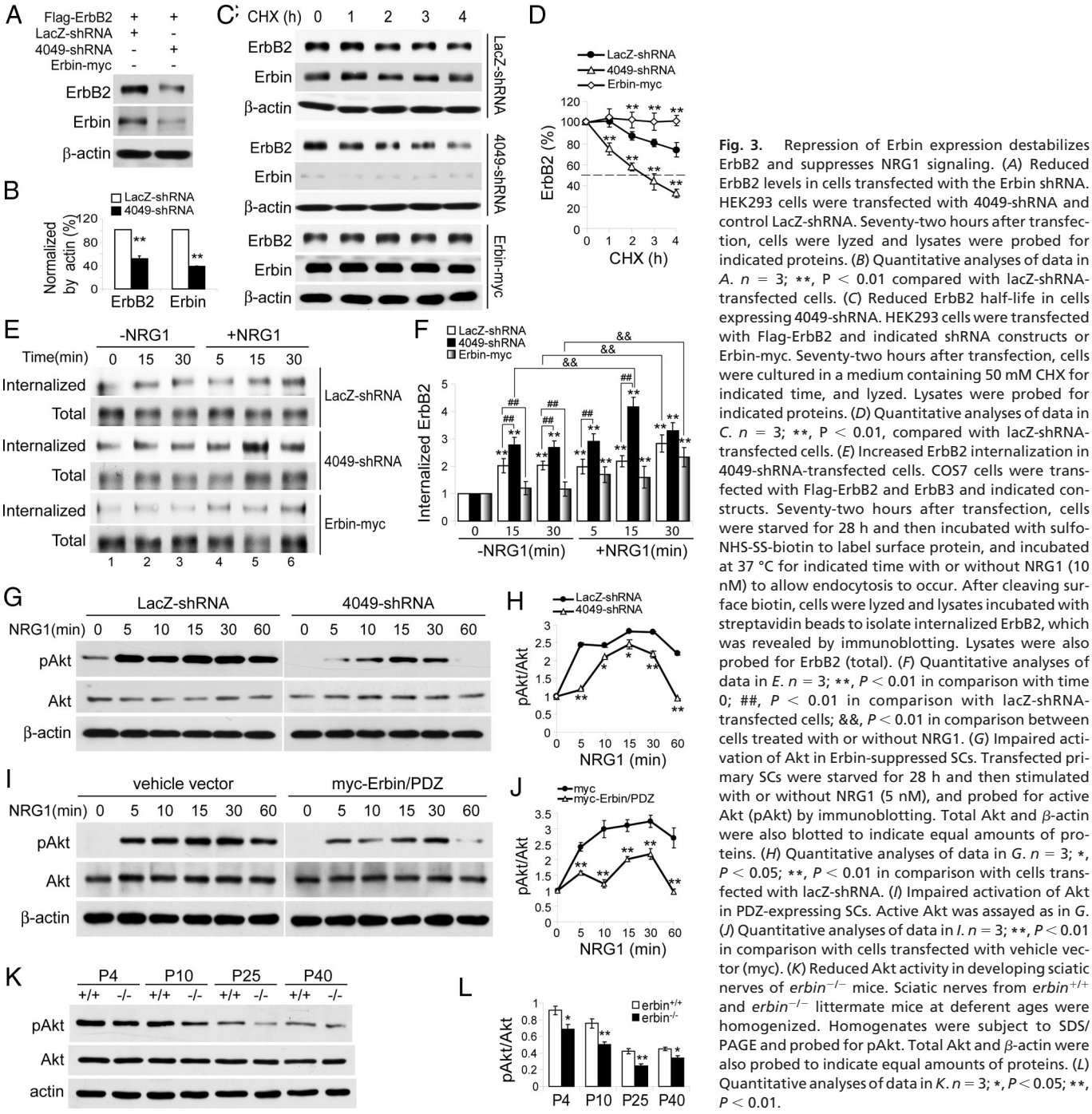

Fig. 3. Repression of Erbin expression destabilizes ErbB2 and suppresses NRG1 signaling. (*A*) Reduced ErbB2 levels in cells transfected with the Erbin shRNA. HEK293 cells were transfected with 4049-shRNA and control LacZ-shRNA. Seventy-two hours after transfection, cells were lyzed and lysates were probed for indicated proteins. (*B*) Quantitative analyses of data in *A*. $n = 3$; **, $P < 0.01$ compared with lacZ-shRNA-transfected cells. (*C*) Reduced ErbB2 half-life in cells expressing 4049-shRNA. HEK293 cells were transfected with Flag-ErbB2 and indicated shRNA constructs or Erbin-myc. Seventy-two hours after transfection, cells were cultured in a medium containing 50 mM CHX for indicated time, and lyzed. Lysates were probed for indicated proteins. (*D*) Quantitative analyses of data in *C*. $n = 3$; **, $P < 0.01$, compared with lacZ-shRNA-transfected cells. (*E*) Increased ErbB2 internalization in 4049-shRNA-transfected cells. COS7 cells were transfected with Flag-ErbB2 and ErbB3 and indicated constructs. Seventy-two hours after transfection, cells were starved for 28 h and then incubated with sulfo-NHS-SS-biotin to label surface protein, and incubated at 37 °C for indicated time with or without NRG1 (10 nM) to allow endocytosis to occur. After cleaving surface biotin, cells were lyzed and lysates incubated with streptavidin beads to isolate internalized ErbB2, which was revealed by immunoblotting. Lysates were also probed for ErbB2 (total). (*F*) Quantitative analyses of data in *E*. $n = 3$; **, $P < 0.01$ in comparison with time 0; ##, $P < 0.01$ in comparison with lacZ-shRNA-transfected cells; &&, $P < 0.01$ in comparison between cells treated with or without NRG1. (*G*) Impaired activation of Akt in Erbin-suppressed SCs. Transfected primary SCs were starved for 28 h and then stimulated with or without NRG1 (5 nM), and probed for active Akt (pAkt) by immunoblotting. Total Akt and β-actin were also blotted to indicate equal amounts of proteins. (*H*) Quantitative analyses of data in *G*. $n = 3$; *, $P < 0.05$; **, $P < 0.01$ in comparison with cells transfected with lacZ-shRNA. (*I*) Impaired activation of Akt in PDZ-expressing SCs. Active Akt was assayed as in *G*. (*J*) Quantitative analyses of data in *I*. $n = 3$; **, $P < 0.01$ in comparison with cells transfected with vehicle vector (myc). (*K*) Reduced Akt activity in developing sciatic nerves of *erbin*$^{-/-}$ mice. Sciatic nerves from *erbin*$^{+/+}$ and *erbin*$^{-/-}$ littermate mice at deferent ages were homogenized. Homogenates were subject to SDS/PAGE and probed for pAkt. Total Akt and β-actin were also probed to indicate equal amounts of proteins. (*L*) Quantitative analyses of data in *K*. $n = 3$; *, $P < 0.05$; **, $P < 0.01$.

higher in 4049-shRNA-transfected cells in comparison with that in lacZ-shRNA-transfected cells (Fig. 3 *E* and *F*). In contrast, less ErbB2 was internalized in cells overexpressing Erbin (Fig. 3 *E* and *F*). These results suggest Erbin could regulate constitutive ErbB2 internalization. To investigate whether Erbin also regulates NRG1-induced ErbB2 endocytosis, cells were stimulated by NRG1 for indicated time before biotin cleavage. In agreement with previous reports (31), NRG1 stimulated ErbB2 internalization (Fig. 3 *E* and *F*). Intriguingly, NRG1-induced ErbB2 internalization was accelerated and enhanced in 4049-shRNA-transfected cells in comparison with that in control cells (Fig. 3 *E* and *F*). The amounts of NRG1-induced endocytosed ErbB2 in Erbin-overexpressing cells appeared to be less than those in control cells although no statistical significance was observed in quantitative analysis. These results suggest that the level of Erbin regulates both constitutive and NRG1-stimulated ErbB2 internalization.

NRG1 activates various intracellular pathways including PI3K/Akt, MAPK, and JNK (1). Among them, the PI3K/Akt pathway appears to be a major effecter of NRG1 to regulate myelination (16). Having demonstrated that Erbin regulates ErbB2 stability and internalization, we next tested if Erbin deficiency alters intracellular signaling by NRG1. Primary SCs were transfected with control shRNA or 4049-shRNA, which suppressed Erbin expression in SCs (Fig. S3 *A* and *B*). Akt activation was assayed by specific antibody against active Akt. Expression of any of the constructs had no effect on Akt levels (Fig. 3 *G* and *H*). In cells transfected with control

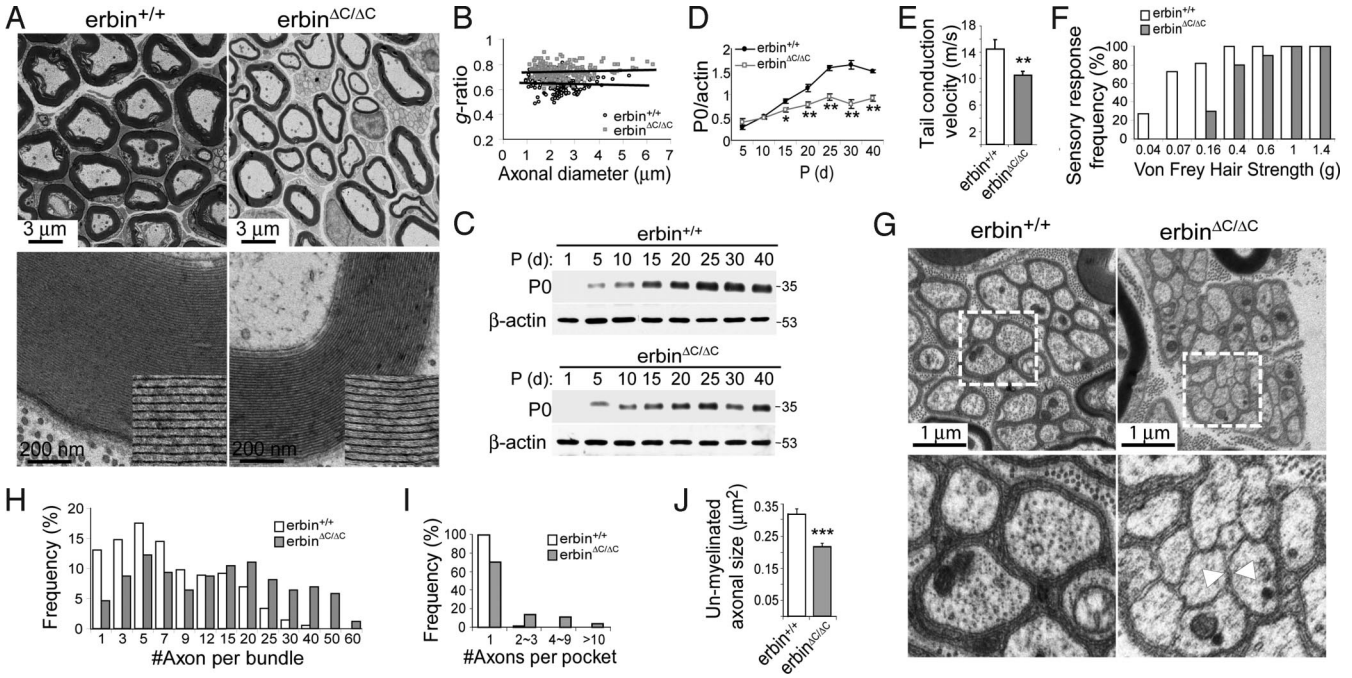

Fig. 4. Similar deficits in myelination and entheathment of axons in *erbin*$^{\Delta C/\Delta C}$ mice. (*A*) Thinner myelin in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. EM images of sciatic nerve cross-sections were shown at 2 different magnifications. (*Lower Insets*) Interperiod lines and ultrastructures of myelin sheath. (*B*) Increased *g*-ratio of myelinated axons in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Two pairs of littermates, and ≈250 axons for each mouse, were analyzed as described in *SI Materials and Methods*. (*C*) Reduced expression of the P0 protein in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Homogenates of sciatic nerves from *erbin*$^{\Delta C/\Delta C}$ or control littermates were subjected to SDS/PAGE and probed for the P0 protein, and β-actin was used to be loading control. (*D*) Quantitative analysis of data in *C*. $n = 3$, *, $P < 0.05$, **, $P < 0.01$. (*E*) Reduced nerve conduction velocity in *erbin*$^{\Delta C/\Delta C}$ mice. Nerve conduction was measured as described in *SI Materials and Methods*. $n = 4$, **, $P < 0.01$. (*F*) Increased threshold in sensory response. Von Frey Hair assays were performed as described in *SI Materials and Methods*. Shown are percentages of mice responding to Von Frey Hair stimulation at different strengths. $n = 8$ mice in each group. (*G*) Abnormal axonal segregation in Remak bundles in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Shown are EM images of sciatic nerve cross sections. (*Lower*) Higher magnification of squared areas of *Upper*. Arrowheads indicate adjacent axons that were naked and without proper ensheathment. (*H*) Increased number of unmyelinated axons in Remak bundles in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Remak bundles analyzed were 359 for *erbin*$^{+/+}$ and 272 for *erbin*$^{\Delta C/\Delta C}$. (*I*) Increased number of unmyelinated axons in SC pockets in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Pockets analyzed were 2852 for *erbin*$^{+/+}$ and 1988 for *erbin*$^{\Delta C/\Delta C}$. (*J*) Reduced transverse surface areas of unmyelinated axons in *erbin*$^{\Delta C/\Delta C}$ mice. $n = 97$ for *erbin*$^{+/+}$, $n = 102$ for *erbin*$^{\Delta C/\Delta C}$. ***, $P < 0.001$. The age of mice was P30 in *A*, *B*, and *G-J* and P60 in *E* and *F*.

shRNA, NRG1 elicited rapid activation of Akt, which remained at high levels 60 min after stimulation. In contrast, suppression of Erbin expression inhibited Akt activation by NRG1, which was delayed and more transient, returning to basal level within 60 min of stimulation. The altered kinetics of Akt activity and ErbB2 endocytosis suggest that Erbin controls the time and amplitude of NRG1 signaling. To eliminate the possibility of off-target effect of 4049-shRNA, we overexpressed the PDZ domain of Erbin (myc-Erbin/PDZ), which functions in a dominant negative manner to prevent ErbB2 from interacting with endogenous Erbin. As shown in revised Fig. 3 *I* and *J*, expression of the PDZ domain had similar effect on Akt activation to that by shRNA. The similar effects by these 2 different approaches (dominant negative inhibition and shRNA knockdown) provide strong evidence that Erbin regulates NRG1 signaling in SCs. Note that expression of 4049-shRNA and PDZ did not appear to alter NRG1-induced ErbB phosphorylation (Fig. S3 *D–F*), suggesting that Erbin acts by stabilizing surface ErbB proteins. To determine whether Akt activation is altered in *erbin*$^{-/-}$ mice, we measured active Akt in sciatic nerves of *erbin*$^{-/-}$ and control mice. Significantly, phospho-Akt was consistently lower in *erbin*$^{-/-}$ sciatic nerves than that in control littermates during development (Fig. 3 *K* and *L*). Together, these observations indicate a critical role of Erbin in NRG1 activation of PI3K/Akt in SCs.

If Erbin regulation of myelination depends on maintaining ErbB2 stability and NRG1 signaling, in vivo deletion of the PDZ domain that interacts with ErbB2 should duplicate the phenotypes of *erbin*$^{-/-}$ mice. To this end, we characterized *erbin*$^{\Delta C/\Delta C}$ mice that were generated by gene trapping (Fig. S4 *A–F*). Erbin in *erbin*$^{\Delta C/\Delta C}$ mice was replaced by a mutant protein with C-terminal truncation ($Erbin_{1-693}\beta gal$). Remarkably, *erbin*$^{\Delta C/\Delta C}$ mice showed similar myelin deficits of *erbin*$^{-/-}$ mice. First, myelinated axons had thinner myelin without apparent changes in ultrastructure and periodicity (Fig. 4*A*). Averaged *g*-ratio of myelinated axons increased from 0.644 ± 0.0196 ($n = 169$) in wild-type to 0.738 ± 0.0134 ($n = 277$, $P < 0.001$) in *erbin*$^{\Delta C/\Delta C}$ mice, regardless of axonal size (Fig. 4*B*). P0 protein levels were lower in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves than those in littermates (Fig. 4 *C* and *D*). Functionally, *erbin*$^{\Delta C/\Delta C}$ mice had reduced nerve conduction velocity (Fig. 4*E*) and elevated mechanical sensory threshold (Fig. 4*F*). Second, similar deficits were observed in unmyelinated fibers. Remak bundles in *erbin*$^{\Delta C/\Delta C}$ mice contained more unmyelinated axons (Fig. 4*G*). Quantitative analyses indicate a substantial increase in the number of bundles containing 20 or more axons (Fig. 4 *G* and *H*). Axons in Remak bundles were segregated completely (Fig. 4*G*) and smaller in size (Fig. 4 *G* and *J*), resulting increased number of axons in SC pockets (Fig. 4 *G* and *I*). These observations demonstrate similar deficits in myelination and ensheathment of sciatic nerves in *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ mice. The phenotypic similarity provides strong genetic evidence that the null mutation and C-terminal truncation share mechanism of action and indicates a critical role of the PDZ domain of Erbin in regulation of myelination. This idea is supported by biochemical studies that ErbB receptors were reduced at protein (Fig. S5 *A* and *B*), but not mRNA, levels (Fig. S5 *C* and *D*) in *erbin*$^{\Delta C/\Delta C}$ sciatic nerves. Note that NRG1 levels were similar in mutant mice (Fig. S5 *E* and *F*).

Discussion

This article presents evidence for a critical role of Erbin in regulating NRG1 signaling and in peripheral myelination. First, both

myelinated and unmyelinated axons of sciatic nerves became abnormal in *erbin*$^{-/-}$ mice. Myelinated axons were hypomyelinated with reduced expression of P0, a marker of mature myelinating SCs. Deficits were also observed in ensheathment of unmyelinated axons and formation of Remak bundles. Second, associated with morphological deficits were compromised nerve conduction and increased sensory threshold to mechanistic stimulation. These results corroborate to underscore an important role of Erbin in myelination. Third, the similarity of phenotypes in both null and *erbin*$^{\Delta C/\Delta C}$ mice indicate a necessary role of the PDZ domain in Erbin regulation of myelination in vivo and suggest that a target protein of Erbin may be the substrates of the PDZ domain. Indeed, fourth, we showed that ErbB2 was reduced at protein, but not mRNA, levels in *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ mice. ErbB2 stability and internalization were altered when Erbin levels were decreased and consequently, Akt activation was reduced and/or delayed. These observations indicate a critical role of Erbin in myelination and identify a novel regulatory mechanism of NRG1 signaling. Our results suggest that Erbin, via the PDZ domain, binds to and stabilizes ErbB2, which is necessary for NRG1 signaling.

NRG1 is also thought to be an axon-derived signal for oligodendrocyte development. Disruption of NRG1 signaling by DN-ErbB4 or kinase dead ErbB1 impairs oligodendrocyte differentiation in transgenic mice (32, 33). However, myelination deficits in the central nervous system (CNS) are relatively minor and limited to frontal brain regions in hypomorphic mutants of type III NRG1 (34). These observations suggest unique NRG1 signaling mechanisms in oligodendrocyte-dependent myelination. However, no consistent changes were observed in optical nerve myelination and levels of MBP, a key CNS myelin protein, in *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ mice (Fig. S6), suggesting that Erbin is specifically involved in PNS, but not CNS, myelination. The specificity may be due to enriched expression of ErbB4, but not ErbB2 and ErbB3, in CNS myelin. However, peripheral myelin expressed mostly ErbB2 and ErbB3, but not ErbB4 (*10*). Erbin does not interact with ErbB4 (18); therefore, it may be unable to regulate NRG1 signaling in oligodendrocytes. In agreement with this idea, ErbB3 is dispensable for CNS myelination (35). Alternatively, mild CNS myelin deficits could be due to functional redundancy of Densin-180, a protein with similar domain structure that is expressed in the brain (18).

ErbB2 stability is regulated by complex mechanisms. We show that ErbB2 levels were lower in both *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ mice, suggesting that the maintenance of ErbB2 levels require the interaction with Erbin. Deletion of key residues in the C-terminal PDZ-binding motif accelerates degradation of surface ErbB2 (36), whereas Erbin expression increases surface ErbB2 (18). However, Erbin regulation of ErbB2 stability appeared to be specific because levels of other Erbin-interacting proteins including integrinβ4, δ-catenin and EBP50 (19, 20, 24) were not altered in sciatic nerves of both mutant mice (Fig. 2*A*). These observations could suggest the involvement of additional mechanisms.

In summary, this article provides evidence for a key role of Erbin in PNS myelination. Our results are consistent with a model that Erbin gates the intensity of NRG1 signaling by regulating the stability of ErbB2 and ErbB3. In light of NRG1/ErbBs' roles in tumorigenesis, heart development, neural development and synaptic transmission (1, 27), the observations identify a potential target of therapeutic interventions of related disorders.

Materials and Methods

Reagents, generation of *erbin*$^{-/-}$ and *erbin*$^{\Delta C/\Delta C}$ mice, EM stuides, Schwann cell culture, nucleofection, RT-PCR, immunostaining, immunoblotting, immunoprecipitation, conduction velocity and Von Frey fiber/sensory test, endocytosis assays, and statistic analysis are described in *SI Materials and Methods*.

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1. Mei L, Xiong WC (2008) Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nat Rev Neurosci* 9:437–452.
2. ShyME (2006) Peripheral neuropathies caused by mutations in the myelin protein zero. *J Neurol Sci* 242(1–2):55–66.
3. Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. *Nature* 378:386–390.
4. Wolpowitz D, et al. (2000) Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. *Neuron* 25:79–91.
5. Michailov GV, et al. (2004) Axonal neuregulin-1 regulates myelin sheath thickness. *Science* 304:700–703.
6. Taveggia C, et al. (2005) Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* 47:681–694.
7. Tzahar E, et al. (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* 16:5276–5287.
8. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL, III (1994) Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci USA* 91:8132–8136.
9. Adlkofer K, Lai C (2000) Role of neuregulins in glial cell development. *Glia* 29:104–111.
10. Garratt AN, Voiculescu O, Topilko P, Charnay P, Birchmeier C (2000) A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J Cell Biol* 148:1035–1046.
11. Lee KF, et al. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* 378:394–398.
12. Riethmacher D, et al. (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389:725–730.
13. Woldeyesus MT, et al. (1999) Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev* 13:2538–2548.
14. Chen S, et al. (2003) Disruption of ErbB receptor signaling in adult non-myelinating Schwann cells causes progressive sensory loss. *Nat Neurosci* 6:1186–1193.
15. Chen S, et al. (2006) Neuregulin 1-erbB signaling is necessary for normal myelination and sensory function. *J Neurosci* 26:3079–3086.
16. Flores AI, et al. (2008) Constitutively active Akt induces enhanced myelination in the CNS. *J Neurosci* 28:7174–7183.
17. Borg JP, et al. (2000) ERBIN: A basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. *Nat Cell Biol* 2:407–414.
18. Huang YZ, Wang Q, Xiong WC, Mei L (2001) Erbin is a protein concentrated at postsynaptic membranes that interacts with PSD-95. *J Biol Chem* 276:19318–19326.
19. Favre B, et al. (2001) The hemidesmosomal protein bullous pemphigoid antigen 1 and the integrin beta 4 subunit bind to ERBIN. Molecular cloning of multiple alternative splice variants of ERBIN and analysis of their tissue expression. *J Biol Chem* 276:32427–32436.
20. Laura RP, et al. (2002) The Erbin PDZ domain binds with high affinity and specificity to the carboxyl termini of delta-catenin and ARVCF. *J Biol Chem* 277:12906–12914.
21. Dai P, Xiong WC, Mei L (2006) Erbin inhibits RAF activation by disrupting the sur-8-Ras-Raf complex. *J Biol Chem* 281:927–933.
22. Huang YZ, Zang M, Xiong WC, Luo Z, Mei L (2003) Erbin suppresses the MAP kinase pathway. *J Biol Chem* 278:1108–1114.
23. McDonald C, et al. (2005) A role for Erbin in the regulation of Nod2-dependent NF-kappaB signaling. *J Biol Chem* 280:40301–40309.
24. Rangwala R, Banine F, Borg JP, Sherman LS (2005) Erbin regulates mitogen-activated protein (MAP) kinase activation and MAP kinase-dependent interactions between Merlin and adherens junction protein complexes in Schwann cells. *J Biol Chem* 280:11790–11797.
25. Dai F, et al. (2007) Erbin inhibits transforming growth factor beta signaling through a novel Smad-interacting domain. *Mol Cell Biol* 27:6183–6194.
26. Kolch W (2003) Erbin: Sorting out ErbB2 receptors or giving Ras a break? *Sci STKE* 2003(199):pe37.
27. Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nat Rev* Mol Cell Biol 2:127–137.
28. Perrin-Tricaud C, Rutishauser U, Tricaud N (2007) P120 catenin is required for thickening of Schwann cell myelin. *Mol Cell Neurosci* 35:120–129.
29. Van der Zee CE, Kreft M, Beckers G, Kuipers A, Sonnenberg A (2008) Conditional deletion of the Itgb4 integrin gene in Schwann cells leads to delayed peripheral nerve regeneration. *J Neurosci* 28:11292–11303.
30. Gatto CL, Walker BJ, Lambert S (2007) Asymmetric ERM activation at the Schwann cell process tip is required in axon-associated motility. *J Cell Physiol* 210:122–132.
31. Yang XL, Huang YZ, Xiong WC, Mei L (2005) Neuregulin-induced expression of the acetylcholine receptor requires endocytosis of ErbB receptors. *Mol Cell Neurosci* 28:335–346.
32. Kim JY, Sun Q, Oglesbee M, Yoon SO (2003) The role of ErbB2 signaling in the onset of terminal differentiation of oligodendrocytes in vivo. *J Neurosci* 23:5561–5571.
33. Roy K, et al. (2007) Loss of erbB signaling in oligodendrocytes alters myelin and dopaminergic function, a potential mechanism for neuropsychiatric disorders. *Proc Natl Acad Sci USA* 104:8131–8136.
34. Taveggia C, et al. (2008) Type III neuregulin-1 promotes oligodendrocyte myelination. *Glia* 56:284–293.
35. Schmucker J, et al. (2003) erbB3 is dispensable for oligodendrocyte development in vitro and in vivo. *Glia* 44:67–75.
36. Shelly M, et al. (2003) Polar expression of ErbB-2/HER2 in epithelia. Bimodal regulation by Lin-7. *Dev Cell* 5:475–486.