The tyrosine phosphatase Shp2 (PTPN11) directs Neuregulin-1/ErbB signaling throughout Schwann cell development

Katja S. Grossmann[a,b], Hagen Wende[a], Florian E. Paul[c], Cyril Cheret[a], Alistair N. Garratt[a], Sandra Zurborg[d], Konstantin Feinberg[e], Daniel Besser[b], Herbert Schulz[c], Elior Peles[e], Matthias Selbach[c], Walter Birchmeier[b,1], and Carmen Birchmeier[a,1,2]

[a]Neuroscience Department, [b]Department of Cancer Research, [c]Department of Cardiovascular Research, Max-Delbrück-Centrum for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany; [d]EMBL Monterotondo, Adriano Buzzati-Traverso Campus, Via Ramarini 32, 00016 Monterotondo, Italy; and [e]Weizmann Institute of Science, PO Box 26, Rehovot 76100, Israel

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The nonreceptor tyrosine phosphatase Shp2 (PTPN11) has been implicated in tyrosine kinase, cytokine, and integrin receptor signaling. We show here that conditional mutation of Shp2 in neural crest cells and in myelinating Schwann cells resulted in deficits in glial development that are remarkably similar to those observed in mice mutant for Neuregulin-1 (Nrg1) or the Nrg1 receptors, ErbB2 and ErbB3. In cultured Shp2 mutant Schwann cells, Nrg1-evoked cellular responses like proliferation and migration were virtually abolished, and Nrg1-dependent intracellular signaling was altered. Pharmacological inhibition of Src family kinases mimicked all cellular and biochemical effects of the Shp2 mutation, implicating Src as a primary Shp2 target during Nrg1 signaling. Together, our genetic and biochemical analyses demonstrate that Shp2 is an essential component in the transduction of Nrg1/ErbB signals.

ErbB2/3 | myelination | tyrosine kinase receptors

Neural crest cells constitute a transient population of multipotent stem cells that generate many different cell and tissue types, like glial and neuronal cells of the peripheral nervous system, melanocytes, or connective tissue of the head (1). Much progress has been made in defining the regulatory networks that allow neural crest cell migration and differentiation (2, 3). In particular, Schwann cell precursors that associate with peripheral axons express the tyrosine kinase receptors ErbB2/3 and require these receptors as well as the neuronally produced ligand, Neuregulin-1 (Nrg1), for migration, proliferation, and differentiation (4–9; see also references 10–12 for recent reviews). In the ErbB2/ErbB3 heterodimer, ErbB3 and ErbB2 provide ligand binding and tyrosine kinase activity, respectively, and both receptors are essential for Nrg1 signaling (13). A wealth of information about downstream signaling molecules of ErbB receptors has been obtained in cultured cells (14, 15), whereas genetic analysis requires further attention.

The vertebrate nonreceptor tyrosine phosphatase Shp2 (also known as PTPN11) has been implicated in signaling of tyrosine kinase, cytokine, and integrin receptors (16). Recent genetic analyses in mice demonstrated that Shp2 functions in development and maintenance of various organs like the central nervous system, the heart, and the mammary gland (17–20). The *Drosophila* homolog of Shp2, Corkscrew (Csw), transduces signals provided by tyrosine kinase receptors like Torso (a PDGF-like receptor), Sevenless (a homolog of the c-ros receptor), Breathless (a FGF receptor), and the dEGF receptor (21–24). Dominant negative mutants of Shp2 inhibit the Ras/Erk pathway, immediate early gene activation, cell migration, and cell proliferation (16, 25, 26). The sustained activation of the Ras/Erk pathway represents the best-described event regulated by Shp2 during tyrosine kinase receptor signaling. Accordingly, Noonan Syndrome, a complex human developmental disorder with variable penetrance affecting many organs, is caused by gain-of-function mutations of genes that act in Ras/Erk signaling, i.e., Sos1, Shp2, Kras, or Raf1 (27). Targets and downstream regulators of Shp2 have recently been identified, like PDGF and EGF receptors, the adapters Gab1, FRS2α, and IRS, the enzymes Ras-Gap, and the Src-inhibitory kinase Csk, or the Ras/Erk inhibitor Sprouty (28–35). It should be noted that different receptors and/or cell types may use distinct Shp2 targets. In addition, Shp2 has receptor-dependent and/or cell context-dependent effects on PI3 kinase/Akt, focal adhesion kinase (Fak), and small G-proteins of the Rho family (36–42).

Our analysis of Shp2 mutant mice that carry conditional mutations in neural crest cells or in myelinating Schwann cells indicate a role of Shp2 in early and late Schwann cell development. Analysis of primary mutant Schwann cells revealed that Shp2 is required in a cell-autonomous manner for Nrg1 signaling in Schwann cells and allowed us to define the function of Shp2 in the Nrg1/ErbB signaling cascade. Together, our genetic and biochemical analyses demonstrated that Schwann cells require the tyrosine phosphatase Shp2 for the transduction of Nrg1/ErbB signals.

Results

Deficits in Development of Schwann Cells and Other Neural Crest Derivatives in Shp2 Mutant Mice. To analyze Shp2 functions genetically, a mouse strain that carries "floxed" exons 3 and 4 of Shp2 ($Shp2^{fl}$) was generated; cre-mediated recombination of $Shp2^{fl}$ introduces a deletion and frameshift mutation ($Shp2^{\Delta}$ allele) that interferes with the production of functional Shp2 (Fig. S1). We first introduced a Shp2 mutation using Wnt1-cre, which introduces recombination in neural crest cells (43). Generation of neural crest cells occurred correctly in these mutants, as assessed by in situ hybridization using a Sox10-specific probe at E9.5 (Fig. S2 *A–D*). Pronounced changes in Schwann cell development were apparent at E11.5 and subsequent developmental stages: The numbers of BFABP+ Schwann cell precursors that line peripheral nerves were significantly reduced in the conditional Shp2 mutant mice at E11.5 (Fig. 1 *A* and *B*; see also insets). We did not observe significant differences in the numbers of apoptotic cells associated with peripheral nerves of

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[1]W.B. and C.B. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: cbirch@mdc-berlin.de.

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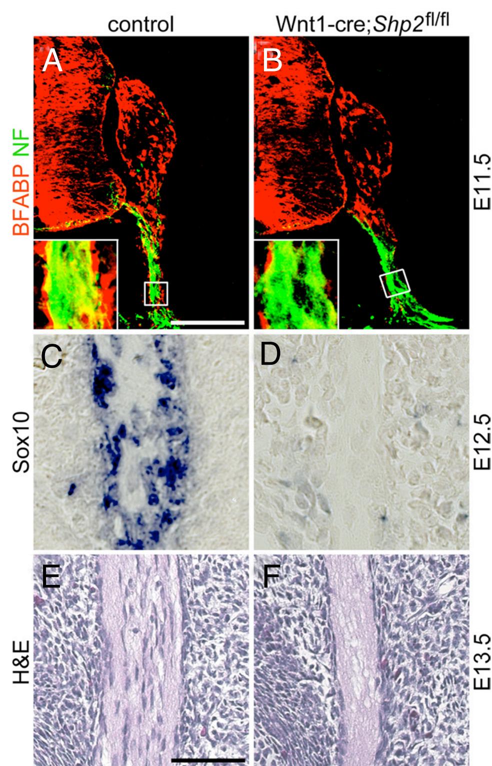

Fig. 1. Severe deficits in Schwann cell development in Wnt1-cre conditional Shp2 mutant mice. Analysis of Schwann cells associated with peripheral nerves in control and Wnt1-cre Shp2$^{fl/fl}$ mice at the indicated developmental stages using (*A* and *B*) immunohistochemistry for BFABP (red) and neurofilament (NF, green), (*C* and *D*) in situ hybridization with a Sox10-specific probe, and (*E* and *F*) histological sections stained with hematoxylin and eosin. [Scale bars, (*A* and *B*) 150 μm; (*C*–*F*) 50 μm.]

control and mutant mice at this stage, as analyzed by TUNEL staining. Sox10+ Schwann cell precursors (44) were not detectable in peripheral nerves at E12.5 (Fig. 1 *C* and *D*), and on semithin sections, Schwann cell nuclei in peripheral nerves were virtually absent at E13.5 (Fig. 1 *E* and *F*). In contrast, satellite cells, the glial cells associated with dorsal root ganglia, were present and detectable by anti-BFABP staining (Fig. 1 *A* and *B*). Furthermore, craniofacial structures were altered in conditional Shp2 mutants, the numbers of Ret+ enteric neural crest cells were reduced in a pronounced manner, and the numbers of melanocytes were reduced (Fig. S2 *E*–*L*). We also noted that the Wnt1-cre Shp2$^{fl/fl}$ mutation interfered with embryonic survival: The majority of the mutant embryos died around E13.5, and live embryos were only very rarely recovered at subsequent stages.

The initial differentiation of peripheral sensory neurons occurred correctly in Wnt1-cre Shp2 mutant mice, as assessed by the expression of Brn3a, Islet1/2, TrkA, and TrkC in lumbar dorsal root ganglia at E11.5 (Fig. 2 *A*–*D*; see *E* for quantification). To assess sensory projections, we performed whole mount immunohistochemistry using antibodies directed against the cell adhesion molecule L1. We observed defasciculation and reduced axonal arborization of cutaneous projections in the Shp2 mutant mice at E12.5 (Fig. 2 *F* and *G*; see *H* for quantification). Changes in arborization and fasciculation also occurred in intercostal and limb nerves (Fig. S3 *A*–*D*). Numbers of cells in dorsal root ganglia were similar at E11.5 in control and Shp2 mutant mice, but a pronounced cell death of sensory neurons was apparent in the mutants starting in lumbar dorsal root ganglia around E12.5 (Fig. 2 *I* and *J*). At E13.5, numbers of cells in dorsal root ganglia were strongly reduced (Fig. 2 *K* and *L* and Fig. S2 *M*–*P*, see Fig. 2*M* for quantification).

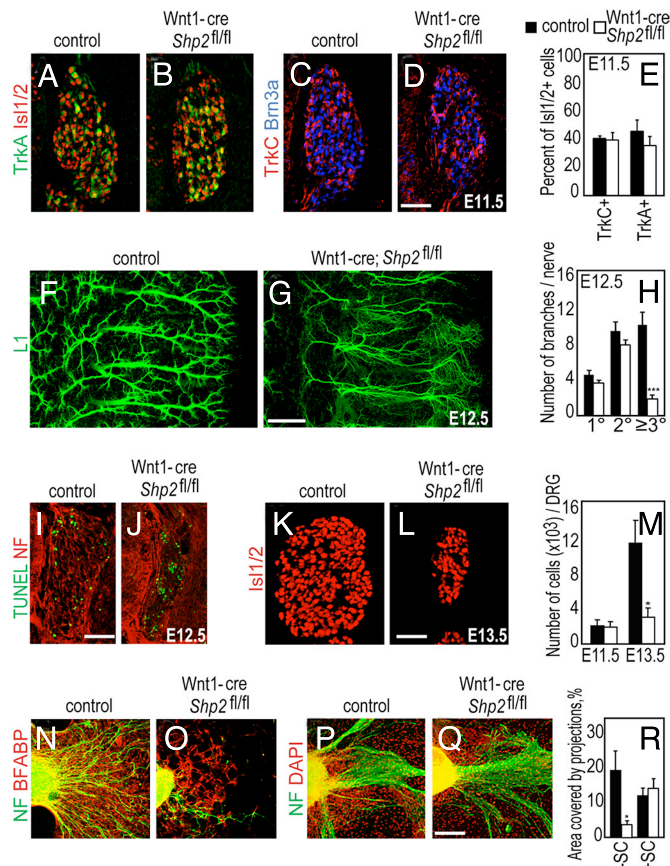

Fig. 2. In vivo and in vitro development of sensory neurons from Wnt1-cre conditional Shp2 mutant mice. Differentiation of sensory neurons in lumbar dorsal root ganglia was analyzed by immunohistochemistry using antibodies directed against (*A* and *B*) TrkA and Isl1/2, (*C* and *D*) TrkC and Brn3a in control and Wnt1-cre Shp2$^{fl/fl}$ mice. (*E*) Ratios of TrkA+/Isl1/2+ and TrkC+/Isl1/2+ neurons at E11.5; the absolute number of Isl1/2+ neurons were similar in lumbar dorsal root ganglia at this stage (2537 ± 297 and 2101 ± 327 Isl1/2+ cells in control and Shp2 mutant ganglia, respectively; $n = 4$ and $P = 0.074$). (*F* and *G*) Cutaneous axonal projections of control and Wnt1-cre Shp2$^{fl/fl}$ mice at E12.5, as revealed by whole mount immunohistochemistry using anti-L1 antibodies. (*H*) Quantification of the branching of cutanous axonal projections of control and Wnt1-cre Shp2$^{fl/fl}$ mice. (*I* and *J*) Apoptosis of cells in lumbar dorsal root ganglia at E12.5 in control and Wnt1-cre Shp2$^{fl/fl}$ mice, as assessed by TUNEL staining. (*K* and *L*) Appearance of lumbar dorsal root ganglia at E13.5 in control and Wnt1-cre Shp2$^{fl/fl}$ mice, as assessed by immunohistochemistry using antibodies directed against Isl1/2. (*M*) Number of cells in dorsal root ganglia of control and Wnt1-cre Shp2$^{fl/fl}$ mice at E11.5 and E13.5. (*N* and *O*) Dorsal root ganglia from E11.5 control and Wnt1-cre Shp2$^{fl/fl}$ mice were cultured in the presence of Nrg1, NGF, and NT3 for 2 days; depicted are cultures stained with antibodies directed against NF160 and BFABP. (*P* and *Q*) Cultured dorsal root ganglia from control and Wnt1-cre Shp2$^{fl/fl}$ mice; the ganglia were cultured for 2 days on a layer of Schwann cells obtained from wild-type mice in the presence of Nrg1, NGF, and NT3. (*R*) Quantification of axonal outgrowth observed in dorsal root ganglia cultures. [Scale bar, (*A*–*D*, *I*–*L*) 50 μm; (*F* and *G*) 150 μm; (*N*–*Q*) 200 μm.] *, $P < 0.05$; ***, $P < 0.001$.

We explanted and cultured E11.5 dorsal root ganglia of control and mutant mice in the presence of NGF, NT3, and Nrg1 and observed a severe reduction of Schwann cell numbers and impaired axonal outgrowth in Shp2 mutant explants (Fig. 2 *N* and *O*). Interestingly, axonal outgrowth was rescued when the mutant explants were cultured on a layer of Schwann cells obtained from control mice (Fig. 2 *P* and *Q*; see *R* for quantification). Furthermore, in the presence of NGF and Nrg1, NT3 was able to induce ER81 expression in such co-cultured dorsal root ganglia, albeit at reduced efficacy (Fig. S3 *E*–*H*).

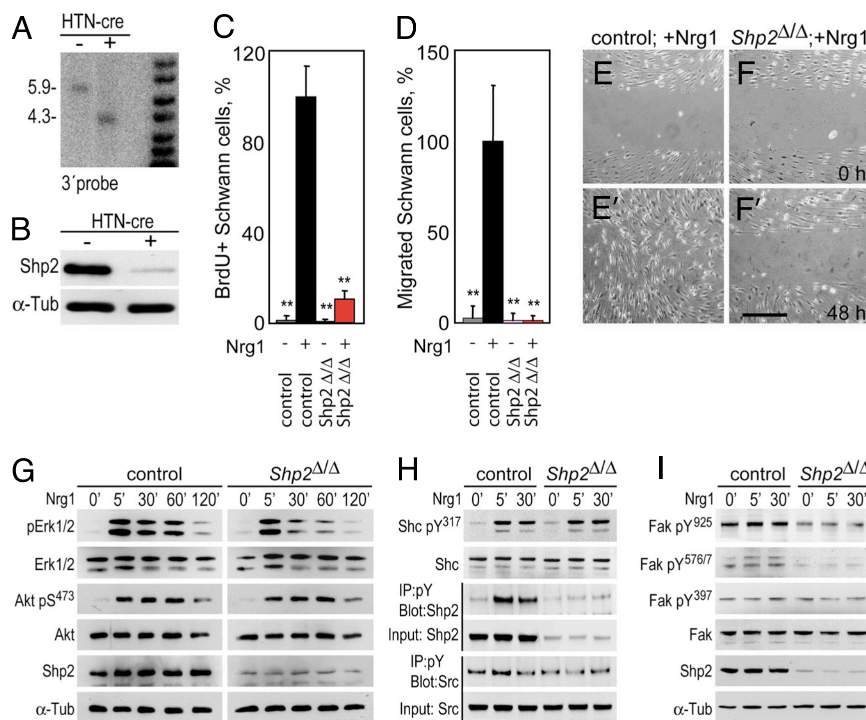

Fig. 3. Nrg1-evoked cellular and biochemical responses in Shp2 mutant Schwann cells. Southern blot (*A*) and western blot (*B*) analyses after HTN-cre induced recombination in cultured $Shp2^{fl/fl}$ Schwann cells. (*C* and *D*) Quantification of the Nrg1-induced proliferation and migration. Shown are the percentage of Schwann cells that incorporated BrdU or the percentage of Schwann cells that migrated into the scratched area, using control and $Shp2^{\Delta/\Delta}$ Schwann cells (HTN-cre-treated $Shp2^{fl/fl}$ Schwann cells) in the presence and absence of Nrg1. (*E–F′*) Migration of Schwann cells into a scratched area. (*G*, *H*, and *I*) Nrg1-induced phosphorylation of Erk1/2, Akt, Shc, Shp2, Src, and Fak in control and $Shp2^{\Delta/\Delta}$ Schwann cells analyzed by western blot analysis. Depletion of Shp2 after HTN-cre-induced mutation was assessed in *B* and *H*; α-tubulin served as a loading control. [Scale bar, (*E–F′*) 50 μm.] **, $P < 0.01$.

Nrg1 Signaling in Shp2 Mutant Schwann Cell Precursors. To assess if Shp2 has a cell-autonomous function in developing Schwann cells, we first cultured primary Schwann cells of the conditional mutant mice, but this was hindered by very low cell yields (see Fig. 2 *M* and *O*). Subsequently, primary Schwann cells from $Shp2^{fl/fl}$ mice were cultured and expanded, and the Shp2 mutation was introduced by the addition of His-TAT-NLS-cre (HTN-cre), a cre-fusion protein able to cross the membrane (45). HTN-cre induced efficient recombination in the $Shp2^{fl}$ locus of cultured Schwann cells, resulting in strongly reduced amounts of Shp2 protein (Fig. 3 *A* and *B*). The pronounced proliferative response evoked by Nrg1 in control Schwann cells, as assessed by BrdU incorporation, was severely blunted in Shp2 mutant cells (Fig. 3*C*). Similarly, Nrg1-induced migration was also blocked in Shp2 mutant Schwann cells as determined in a scratch-wound assay (Fig. 3 *D* and *E–F′*). Impaired Schwann cell proliferation and migration was also observed in the presence of the Shp2 inhibitor PHPS1 (Fig. S4 *D–H*). Thus, primary Schwann cells require Shp2 in a cell autonomous manner for Nrg1-induced proliferation and migration.

To assess Nrg1/ErbB signaling systematically in Schwann cells, we quantitatively compared proteins precipitated by anti-phosphotyrosine antibodies in the absence or presence of Nrg1 using SILAC and mass spectrometry (reference 46; cf. *Methods* for further details). We observed marked changes in the tyrosine phosphorylation or in the association with tyrosine phosphorylated proteins: The ErbB2/3 receptors, the adaptor proteins Shc1–4 and Dok1, the tyrosine phosphatase Shp2, and protein kinases like Erk, PI3 kinase, Fak, and Src (Table S1 and *SI Methods*). To assess the effects of Shp2 on the Nrg1-evoked responses, we also compared the phosphorylation of protein kinases in control and Shp2 mutant Schwann cells by western blot analyses. In Shp2 mutant Schwann cells, transient Erk1/2 phosphorylation was unchanged, but we observed a block in the sustained Erk1/2 phosphorylation (Fig. 3*G*). Akt phosphorylation was unaffected. Shc phosphorylation was similar in control and Shp2 mutant cells (Fig. 3*H*). Differential phosphorylation of Src was observable in the presence and absence of Nrg1 (Fig. 3*H* and Table S1 and *SI Methods*). Phosphorylation of Fak on tyrosine residues 925 and 576/577, but not on tyrosine residue 397, was severely down-regulated in Shp2 mutant cells (Fig. 3*I*). Tyrosine residues 925 and 576/577 of Fak are well-known sites of Src phosphorylation (47). We also used pharmacological inhibitors of Src, Fak, and Mek, the regulator of Erk1/2, to study the role of the downstream kinases further (48, 49). Remarkably, the Src family kinase inhibitor PP2 blocked Nrg1-evoked proliferation and migration, decreased Nrg1-evoked Fak phosphorylation, and interfered with Nrg1-evoked sustained Erk1/2 activation (Fig. 4 and Fig. S4*C*). Fak inhibition by TAE226, a generous gift of Novartis Pharma AG, affected Nrg1-induced proliferation and migration, but not Nrg1-evoked Erk1/2 phosphorylation; Mek inhibition by U0126 impaired Nrg1-induced proliferation, but not migration (Fig. 4 *A* and *B* and Fig. S4 *A* and *B*). Thus, the Src inhibitor PP2, but not the Fak or Mek inhibitors, mimicked all cellular and biochemical changes observed in Shp2 mutant Schwann cells.

Shp2 and Myelination. We also introduced Shp2 mutations late in Schwann cell development using the Krox20-cre allele that induces recombination in peripheral nerves at the onset of myelination; in addition, recombination is induced in dorsal and ventral roots before the onset of myelination (cf. 50). The adult Krox20-cre Shp2 mutant mice displayed marked hypomyelination of peripheral nerves (Fig. 5 *A* and *B*). The axon diameter and the thickness of myelin was determined from electron micrographs and used to calculate g ratios (ratio between the diameter

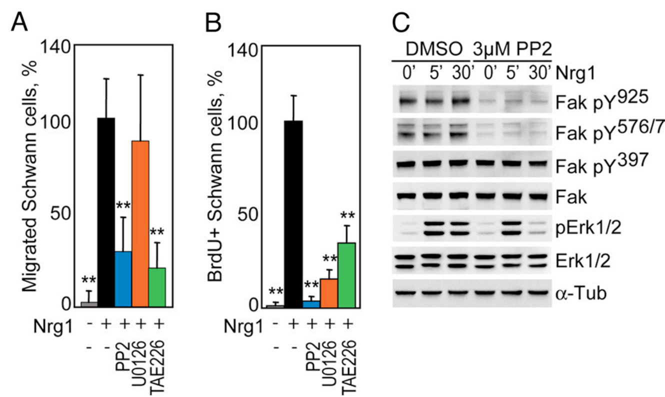

Fig. 4. Nrg1-evoked cellular and biochemical responses in Schwann cells treated with pharmacological inhibitors of Src, Mek, and Fak. (*A* and *B*) Quantification of Nrg1-induced migration and proliferation of control Schwann cells and Schwann cells treated with the Src inhibitor PP2, the Mek inhibitor U0126, and the Fak inhibitor TAE226. (*C*) Phosphorylation of Fak and Erk1/2 in the presence or absence of the Src inhibitor PP2. **, $P < 0.01$.

of the axon and the outer diameter of the myelinated fiber). In wild-type mice, the average g ratio was 0.69 ± 0.06. In contrast, myelin of conditional Krox20-cre Shp2 mutants was considerably thinner (reflected by a higher g ratio of 0.78 ± 0.05), similar to the myelin of Krox20-cre ErbB2 mutant mice (g ratio of 0.80 ± 0.06). Binning of the axon diameter versus the thickness of the myelin sheath revealed that the extent of hypomyelination was dependent on the axon diameter in Shp2 and ErbB2 mutant strains: Thus, the thickness of the myelin sheath of small diameter axons was little affected, but pronounced changes were apparent in large diameter axons (Fig. 5*C*). In accordance with early recombination induced by Krox20-cre in glial cells of the ventral roots, we observed a more severe phenotype at this site, i.e., pronounced hypomyelination, a large proportion of amyelinated axons, groups of axons that lack Schwann cells, as well as aberrant and ectopically located perineuria (Fig. S5). These alterations in myelination were accompanied by a reduction in motoneuron numbers in adult mutant animals, as assessed by counting axons in the ventral roots (980 ± 53 and 766 ± 101 in control and mutant L5 ventral roots; $n = 6$, $P < 0.001$). In contrast, neuron numbers in the L5 dorsal root ganglia were unchanged. Furthermore, compared to control mice, the conduction velocity of the saphenous nerve of the mutants was reduced by 36% ($n = 4$, $P < 0.01$).

We also determined changes in gene expression in conditional Krox20-cre Shp2 and ErbB2 mutant peripheral nerves by Affymetrix microarray analyses. A large fraction of the genes that were deregulated in Shp2 mutant nerves (64% and 83%, using false discovery rates of 10^{-3} and 10^{-4} as cut-offs, respectively) were also deregulated in ErbB2 mutant nerves (Fig. 5*D*). Finally, we assessed steady state phosphorylation of Erk1/2, Akt, and Fak kinases using peripheral nerve extracts from control and conditional mutant mice (Fig. 5*E*). Both, the Shp2 and the ErbB2 mutation affected phosphorylation of Erk1/2 and Fak, whereas Akt phosphorylation was affected by the ErbB2 but not by the Shp2 mutation.

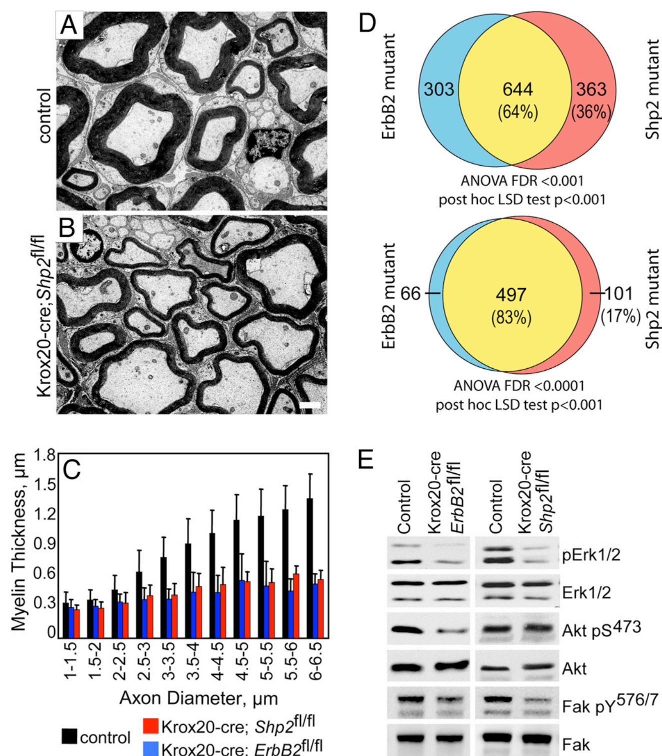

Fig. 5. Hypomyelination of peripheral nerves in Krox20-cre Shp2 mutant mice. (*A* and *B*) Electron microscopic analysis of control and Krox20-cre $Shp2^{fl/fl}$ mice show a marked hypomyelination in the adult. (*C*) Quantification of myelin thickness in adult control, Krox20-cre $Shp2^{fl/fl}$, and Krox20-cre $ErbB2^{fl/fl}$ mice. Displayed is a plot of the myelin thickness versus axon diameter. In each animal analyzed, 100 axons were counted. (*D*) Comparison of genes whose expression is deregulated in peripheral nerves of Krox20-cre $Shp2^{fl/fl}$ and Krox20-cre $ErbB2^{fl/fl}$ mutant mice at P8. The percentages of coregulated genes were determined using false discovery rates of 10^{-3} or 10^{-4} as cut-offs (see *SI Methods*). (*E*) Phosphorylation of Erk1/2, Akt, and Fak in peripheral nerves of control, Krox20-cre $Shp2^{fl/fl}$, and Krox20-cre $ErbB2^{fl/fl}$ mutant mice at P8. [Scale bar, (*A* and *B*) 1 μm.]

Discussion

We show here that conditional mutation of Shp2 in neural crest cells and in myelinating Schwann cells result in deficits in glial development that are remarkably similar to those observed in mice mutant for Nrg1 or the Nrg1 receptors, ErbB2 and ErbB3 (5–7, 51, 52). Cultured primary Schwann cell precursors require Shp2 in a cell-autonomous manner to respond to Nrg1 by proliferation and migration. Thus, our genetic and biochemical data indicate that Schwann cells depend on the tyrosine phosphatase Shp2 to respond to neuronally derived Nrg1.

The cultured Schwann cells allowed analysis of the biochemical consequences of a loss of Shp2. Several aspects of the Nrg1-dependent signaling were affected in mutant Schwann cells, like sustained Erk1/2, Src, and Fak activation. Similar observations in trophoblast and in carcinoma cells had led previously to the proposal that Shp2 mediates its function by activation of Src family kinases and Src substrates like Fak (35, 42). In accordance with this model, we observed that Src inhibition by a small molecular weight compound sufficed to mimic all cellular and biochemical changes caused by Shp2 mutation in Schwann cells like cell proliferation, migration, and phosphorylation of Erk1/2 and Fak. In contrast, pharmacological inhibiton of Mek or Fak blocked only particular responses of ErbB/Shp2 signaling. It has been shown that glial-specific mutation of Fak affects Schwann cell proliferation and radial sorting of axons; aberrant myelination that is also observable in the mutant mice was attributed to a reduction in Schwann cell numbers (53). However, the phenotype observed in these Fak mutants is considerably milder than the one we report here, indicating that the deficit in Fak activation cannot or only partly account for the changed Schwann cell development observed in Shp2 mutants. Pharmacological inhibition of PI3 kinase affected Nrg1-evoked proliferation and migration responses of cultured rat Schwann cells (54). In our Shp2 mutant Schwann cells, Nrg1-evoked proliferation and migration was severely impaired,

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while Akt activation and thus PI3 kinase signaling was intact, indicating that the PI3 kinase pathway does not suffice to elicit the cellular responses to Nrg1. Pharmacological inhibition of Erk1/2 signaling affected Nrg1-evoked migration (55). As yet, the contribution of Erk1/2 to Schwann cell development has not been assessed genetically, but mutant mice are now available that will allow this in the future (56). The conditional Shp2 mutation in neural crest cells affects not only development of Schwann cells, but also that of sensory neurons, which might indirectly impair Schwann cells. However, we show that cultured Shp2 mutant Schwann cells do not respond to Nrg1 by proliferation and migration, demonstrating that Shp2 is required in a cell-autonomous manner in these cells. The mechanisms used to mediate signals between ErbB receptors and Shp2 was not studied here. In the case of other tyrosine kinase receptors, Shp2 binds directly to the receptors, e.g., the PDGFR, or indirectly via adaptor proteins, for instance FRS2α or Gab1 that are recruited to the FGF or Met receptors, respectively (28, 30, 57, 58).

Krox20/Egr2 is essential for myelination and myelin gene expression (59). Several lines of evidence indicate that Nrg1/ErbB provides a signal that controls the onset of myelination by controlling the onset of the expression of the transcription factor Krox20/Egr2 (8, 9, 60, 61). It was thus suggested that Nrg1 elicits proliferation and migration in early Schwann cell development and is important for Krox20/Egr2 expression at later stages. We analyzed here the role of the Shp2 signal in myelination by the use of conditional mutagenesis using Krox20-cre. This precludes an analysis of Shp2 function in the initiation of myelination in peripheral nerves, but allows analysis of Shp2 functions during myelination. Comparison of nerves in the Krox20-cre-induced conditional Shp2 and ErbB2 mutant mice revealed strikingly similar hypomyelination, supporting the notion that Shp2 also acts downstream of Nrg1/ErbB in differentiating Schwann cells (this study and reference 62). It should be noted that Krox20 expression is not affected in the peripheral nerves of these mutants (Fig. S5); thus, initiation and maintenance of Krox20 expression might be controlled by separate mechanisms.

Several cell types generated by the neural crest are affected in Wnt1-cre Shp2 mutant mice, demonstrating that peripheral glia are not the sole cell type that requires Shp2 for its development (reference 63 and this study). In particular, we observe a pronounced cell death of sensory neurons in Shp2 mutants, which initiates at earlier stages and is more severe than the ones observed in mice mutant for neurotrophin receptors (64). Instead, extent and onset of cell death in conditional Shp2 mutants are similar to that observed in mice mutant for the Nrg1 receptor ErbB2 and ErbB3 (6, 7). ErbB3 is not acting cell-autonomously in sensory neuronal survival, but cell death is caused by indirect mechanisms and was attributed to the severe Schwann cell deficit. We therefore suggest that the severe cell death of sensory neurons observed in Shp2 mutant mice is caused by the deficit in Schwann cells. Axonal outgrowth of cultured sensory ganglia of Shp2 mutant dorsal root ganglia was also impaired, but this was rescued by the presence of wild-type Schwann cells, highlighting the important role of such glia in neuronal development in vitro.

Genetic analyses combined with cell culture and biochemical experiments allow to assess the relative importance of downstream signaling molecules and branches of the cascades, which are used by receptor tyrosine kinases in tissue and organ development. Our genetic and biochemical analyses assign an essential role of Shp2 in orchestrating Nrg1/ErbB signaling in Schwann cells.

Methods

Schwann Cell and Dorsal Root Ganglia Culture, Microscopy, and Biochemical Experiments. Schwann cells were isolated from E13.5 dorsal root ganglia as described (65). For proliferation assays, cells were grown in the presence of 30 μg/mL BrdU for 20 h. For migration experiments, Schwann cells were treated with 10 μg/mL mitomycin and cultured with 10 μM AraC to prevent proliferation. Dorsal root ganglia from control and Wnt1-cre Shp2$^{fl/fl}$ E11.5 mice were cultured on coverslips in defined medium (65); growth factors were added as indicated (Nrg1, 10 ng/mL; NT3, 50 ng/mL; and NGF, 50 ng/mL). Recombinant HTN-cre protein was produced and used as described previously (45). For the inhibition of kinases, PP2 (Calbiochem), U0126 (Calbiochem), and TAE226 (Novartis Pharma AG) were used. Cells were pretreated for 20 h with the inhibitors, which were also present during the assay.

Immunofluorescence was performed on cryosections or cultured cells as described (6). For whole mount stainings, embryos were eviscerated, fixed in 4% PFA, bleached in 10% H_2O_2, and postfixed (20% DMSO, 80% methanol). Embryos were incubated for 5 days with anti-L1 antibody and for 2 days with secondary antibody. Before examination, embryos were dehydrated in methanol and bleached in benzylalcohol and benzyl benzoate. Immunofluorescence images were taken at a confocal microscope, LSM 510 META, with the software package LSM 5 Pascal version 3.2. Electron microscopy of sciatic nerves and (L4, L5) dorsal and ventral roots was performed as described (59). Axon diameters and g ratios were determined using EM photographs. For statistical analysis, Excel and SPSS16.0 were used. See also *SI Methods* for more details.

Dissected sciatic nerves and cultured Schwann cells were lysed in RIPA and HEPES/Triton X-100 lysis buffer, respectively, which contained protease inhibitor mixture (Roche) and 1 mM sodium orthovanadate. For immunoprecipitation experiments, Schwann cell lysates containing equal amounts of protein were incubated with pTyr-specific antibodies for 4 h. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). For detection of antibodies, an ECL kit (Amersham) was used.

Microarray Hybridization and Data Analysis. Total RNA was isolated from P8 sciatic nerves using TRIzol (Invitrogen) and the RNeasy kit (Qiagen). Total RNA (4 μg) was labeled and hybridized to Mouse Genome 430 2.0 chips (Affymetrix) as specified by the manufacturer. RNA obtained from the nerves of five animals were pooled to generate one probe set, and three independently generated probes were used for hybridization. Microarray data were analyzed using Partek Genomics Suite. Normalization was performed using the gcRMA method; probe sets with expression levels $<$64 were eliminated as not expressed. Genes showing high differential expression in ANOVA (Benjamini Hochberg false discovery rates of $<$0.001 or $<$0.0001) were selected.

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