Peripheral nervous system plasmalogens regulate Schwann cell differentiation and myelination

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Rhizomelic chondrodysplasia punctata (RCDP) is a developmental disorder characterized by hypotonia, cataracts, abnormal ossification, impaired motor development, and intellectual disability. The underlying etiology of RCDP is a deficiency in the biosynthesis of ether phospholipids, of which plasmalogens are the most abundant form in nervous tissue and myelin; however, the role of plasmalogens in the peripheral nervous system is poorly defined. Here, we used mouse models of RCDP and analyzed the consequence of plasmalogen deficiency in peripheral nerves. We determined that plasmalogens are crucial for Schwann cell development and differentiation and that plasmalogen defects impaired radial sorting, myelination, and myelin structure. Plasmalogen insufficiency resulted in defective protein kinase B (AKT) phosphorylation and subsequent signaling, causing overt activation of glycogen synthase kinase 3β (GSK3β) in nerves of mutant mice. Treatment with GSK3β inhibitors, lithium, or 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) restored Schwann cell defects, effectively bypassing plasmalogen deficiency. Our results demonstrate the requirement of plasmalogens for the correct and timely differentiation of Schwann cells and for the process of myelination. In addition, these studies identify a mechanism by which the lack of a membrane phospholipid causes neuro-pathology, implicating plasmalogens as regulators of membrane and cell signaling.
regated from axon bundles by Schwann cells in a process called radial sorting (19). Differentiated, promyelinating Schwann cells then undergo transcriptional, biochemical, and morphologic changes as they initiate axonal wrapping with a myelin sheath (20, 21). In the PNS, the extent of myelin deposition around individual axons is driven by the axonal expression of neuregulin 1 (NRG1) and by the signaling cascade initiated in Schwann cells expressing the ErbB receptors (22). In the adult PNS, Remak bundles are composed of immature, nonmyelinating Schwann cells that continue to associate with bundles of small-caliber axons that did not undergo radial sorting (23).

Here, we investigated the role of plasmalogens in myelination of the PNS. Our results demonstrate that plasmalogens are crucial for Schwann cell differentiation, as their absence impairs axonal sorting and myelination. Moreover, we found that plasmalogens and myelin basic protein (MBP) are two crucial players that coordinate myelination in the PNS. Mechanistically, we demonstrate that plasmalogens are essential for the correct phosphorylation and activation of protein kinase B (AKT). In mutant sciatic nerves, reduced AKT activity resulted in overt activation of glycogen synthase kinase 3β (GSK3β). Finally, we found that the exogenous inhibition of GSK3β with lithium or

Figure 1
Plasmalogen deficiency causes defects in axonal sorting and myelination. (A) Electron microscopic analysis of sciatic nerves from P5 and P15 WT and Gnpat-KO mice. Bundles in P5 Gnpat-KO nerves contained large axons (asterisks), whereas in W nerves these axons had been sorted (arrowhead), and the bundles contained very small-caliber axons (arrow). At P15, sciatic nerves from Gnpat-KO mice had Remak bundles with only 1 axon (arrowheads). Scale bars: 2 μm. (B) Composition of axon bundles in sciatic nerves from P5 WT, Pex7-KO, and Gnpat-KO mice. *P = 0.031; **P = 0.011. (C) Density of sorted axons in sciatic nerves from P5 WT, Pex7-KO, and Gnpat-KO mice. *P = 0.003. (D) Composition of Remak bundles in nerves from adult WT and Pex7-KO mice. *P = 0.013. (E) Density of unmyelinated fibers (UMF) in Remak bundles of nerves from adult WT and Pex7-KO mice. (F) Quantification of myelin thickness by g ratio in sciatic nerves at P15. Results are graphed as boxes with a line at the mean and whiskers from the minimal to maximal values. *P = 0.005. (G) DRG cocultures of neurons and Schwann cells from WT and Gnpat-KO mice stained for neuronal βII-tubulin (green) and for the myelin protein MBP (red). Scale bars: 200 μm. (H) Density of myelin segments in DRG cocultures from WT and Gnpat-KO mice. *P = 0.001. (I) Length of individual myelin segments in myelinating cocultures. *P = 0.001.
4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) rescued the impairment in Schwann cell differentiation and axonal sorting of plasmalogen-deficient mice. Together, our novel findings reveal the neuropathology behind a deficiency in plasmalogens and elucidate the mechanism by which a membrane phospholipid is essential for Schwann cell differentiation and function, implicating plasmalogens as regulators of membrane and cell signaling.

**Results**

Plasmalogen deficiency causes defects in radial sorting and myelination. To assess whether *Pex7-* and *Gnpat-KO* mice had defects in PNS development, sciatic nerves from 5- and 15-day-old (P5 and P15, respectively) mice were analyzed by electron microscopy. In nerves from P5 WT mice, we observed axon bundles composed of small-caliber axons (Figure 1A, arrow) and sorted axons (Figure 1A, arrowhead) that displayed a 1:1 relationship with Schwann cells. However, in sciatic nerves from P5 *Gnpat-KO* mice, the bundles contained small- and large-caliber axons (Figure 1A, asterisks). Consistent with a failure in the process of radial sorting, we observed an increased number of axons per bundle (Figure 1B) and a decreased number of sorted, promyelinating fibers (Figure 1C) in nerves from P5 *Pex7-* and *Gnpat-KO* mice. Two main consequences of impaired radial sorting were observed in nerves from *Gnpat-KO* mice. In what seemed to be an attempt by Schwann cells to rescue the impaired radial sorting, we observed an increase in Schwann cells engulfing single, small-caliber axons in nerves from P15 *Gnpat-KO* mice (Figure 1A, white arrowheads). Ultrastructural analysis of sciatic nerves from adult *Pex7-KO* mice revealed a reorganization of Remak bundles, since we observed a reduction in the number of axons in these bundles (Figure 1D), without changes in the density of these unmyelinated fibers (Figure 1E). Additionally, the presence of large-caliber axons in Remak bundles caused Schwann cells to myelinate these structures (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI72063DS1). Surprisingly, this abnormal myelination of Remak bundles was not temporary. In sciatic nerves from 1.5-year-old *Gnpat-KO* mice, we continued to observe myelina-
Plasmalogens are important players during remyelination of the PNS. (A) PPD-stained cross sections of the distal segment of sciatic nerves 15 days after nerve crush. Scale bars: 10 μm. (B) Degree of regeneration as measured by the density of myelinated axons in the distal segment 15 days after sciatic nerve crush. *P = 0.014. (C) Extent of impaired regeneration as measured by g ratio determination. Results are graphed as boxes with a line at the mean and whiskers from the minimal to maximal values. *P = 0.029. (D) Electron microscopic analysis of the distal segment of crushed sciatic nerves from WT and Pex7-KO mice. In WT nerves, remyelination of regenerating axons was evident (arrowheads), whereas Pex7-KO axons of a similar caliber were devoid of myelin (asterisks). Scale bars: 5 μm. (E) Density of axons lacking myelin (demyelinated axons) in the distal segment following sciatic nerve crush. Error bars represent SEM. *P = 0.012.

Figure 3
Plasmalogens are important players during remyelination of the PNS. (A) PPD-stained cross sections of the distal segment of sciatic nerves 15 days after nerve crush. Scale bars: 10 μm. (B) Degree of regeneration as measured by the density of myelinated axons in the distal segment 15 days after sciatic nerve crush. *P = 0.014. (C) Extent of impaired regeneration as measured by g ratio determination. Results are graphed as boxes with a line at the mean and whiskers from the minimal to maximal values. *P = 0.029. (D) Electron microscopic analysis of the distal segment of crushed sciatic nerves from WT and Pex7-KO mice. In WT nerves, remyelination of regenerating axons was evident (arrowheads), whereas Pex7-KO axons of a similar caliber were devoid of myelin (asterisks). Scale bars: 5 μm. (E) Density of axons lacking myelin (demyelinated axons) in the distal segment following sciatic nerve crush. Error bars represent SEM. *P = 0.012.

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Defects in plasmalogens impair regeneration and preservation of axons and myelin. To further investigate the role of plasmalogens in Schwann cells and myelin, we performed sciatic nerve crush in adult mice. Histological and morphometric analyses performed 15 days after crush in the distal segment of crushed nerves from WT and Pex7-KO mice revealed reduced density of remyelinated axons in mutant nerves (Figure 3, A and B). In addition, the extent of remyelination was reduced, showing a higher g ratio in mutant nerves (Figure 3C). Our ultrastructural analysis highlighted that the defect in remyelination was not due to impaired axonal regeneration, as crushed nerves from Pex7-KO mice had a 3-fold increase in the density of demyelinated fibers (Figures 3, D and E), i.e., large-caliber axons surrounded by a Schwann cell but lacking myelin sheaths (Figure 3D, asterisks). These results indicate that plasmalogen deficiency primarily affects the ability of Schwann cells to remyelinate regenerating axons.

Analysis of sciatic nerves from aged Pex7- and Gnpat-KO mice (mean age 17 ± 3.3 months) revealed axonal loss and demyelination (Figure 4A), with decreased numbers of myelinated axons which lack MBP, a major constituent of PNS myelin, showed that other myelin components, namely, P0 and P2, are compensatory, allowing shi mice to attain normal myelination and compaction (24). To further investigate whether plasmalogens were crucial for myelination, we generated Pex7-shi and Gnpat-shi double-mutant (DM) mice. Phenotypically, the DMs shared the features of Pex7- and Gnpat-KO mice and the characteristic shivering caused by MBP deficiency (data not shown). When compared with single mutants and WT mice, nerves from Pex7-shi DM mice were characterized by a severe hypomyelination (Figure 2E) without axonal loss (WT 248,704 ± 15,639 axons/mm²; DM 243,884 ± 15,851 axons/mm²; P = 0.434). Myelin thickness was reduced in Pex7-KO nerves, but the double deficiency of plasmalogens and MBP in Pex7-shi DM mice caused a pronounced defect in myelination as judged by the high g ratio values (Figure 2F). At the functional level, the single mutants had defects in nerve conduction, but in DM mice, the combined deficiency of MBP and plasmalogens affected nerve conduction by less than half the normal values (Figure 2G). These findings indicate that in the absence of plasmalogens, the presence of normal amounts of MBP (Supplemental Figure 2B) is sufficient to achieve normal amounts of myelin. Our results highlight the possible coordination between membrane phospholipids and myelin components to attain normal myelination and show that plasmalogen deficiency impairs the organization of myelin and myelinating Schwann cells.
Deficiency in plasmalogens impairs the ability of Schwann cells to sustain myelinated axons. (A) PPD-stained cross sections of sciatic nerves from 1.5-year-old WT and Pex7-KO mice revealing demyelination and loss of axons in mutant nerves. Scale bars: 10 μm. (B) Density of myelinated fibers (%/mm) in sciatic nerves from 1.5-year-old WT and Pex7-KO mice. *P = 0.026. Error bars represent SEM. (C) Quantification of the degree of myelination by g ratio in sciatic nerves from 1.5-year-old WT and Pex7-KO mice. *P = 0.012. (D) Electron microscopic analysis of sciatic nerves from representative 1.5-year-old WT and Pex7-KO mice showing an axon devoid of myelin (asterisk), severely demyelinated axons (arrows), and the presence of extended Schwann cell processes (arrowheads) throughout the perineurium. Scale bars: 2 μm. (E) Schwann cell from an aged Gnpat-KO mouse undergoing remyelination of a demyelinated axon. Scale bar: 2 μm. (F) Engulfment of a demyelinated axon by 2 Schwann cells, generating concentric deposition of membrane processes, similar to the formation of onion bulbs. Scale bar: 2 μm.

Phosphorylation of AKT was also impaired in serum-starved mouse embryonic fibroblasts (MEFs) from Gnpat-KO mice after stimulation with 10% FBS (Figure 5G and Supplemental Figure 4D). However, FBS stimulation was able to normally activate ERK1/2 in serum-starved MEFs from KO mice (Figure 5G and Supplemental Figure 4E). We next investigated whether the NRG1/ErbB signaling pathway would also be affected by the plasmalogen deficiency. We measured the levels of AKT phosphorylation in primary Schwann cells from WT and Gnpat-KO mice after stimulation with NRG1, the endogenous axonal signal that regulates Schwann cell differentiation and myelination (18). We detected decreased levels of phosphorylation, indicative of impaired AKT activation in Schwann cells from Gnpat-KO mice (Figure 5, H and I). The recruitment of AKT to the plasma membrane plays a critical role in its activation by PDK1 and by the mammalian TOR complex 2 (25, 26). Compared with WT MEFs, the plasmalogen deficiency in Gnpat-KO MEFs impaired the FBS-induced phosphorylation of AKT at the membrane (Figure 5J and Supplemental Figure 4F), despite normal levels and localization of PDK1 (Supplemental Figure 4G). Failure to activate AKT at the membrane was also evident by the decreased levels of phosphorylated AKT in the cytosolic fraction (Figure 5J). To
Figure 5

Defects in plasmalogens result in impaired AKT activation and signaling. (A) Western blot analysis and quantification of AKT phosphorylation (p-AKT) in sciatic nerve lysates of P15 WT and Gnpat-KO mice. *P = 0.018; **P = 0.006. (B–E) Quantification of phosphorylated forms of GSK3β at Ser9 (B), c-Raf at Ser259 (C), PDK1 at Ser241 (D), and PTEN at Ser380 (E) in sciatic nerves from WT and Gnpat-KO mice. *P < 0.02. (F) Density of BrdU-positive cells in nerves from P4 WT and Gnpat-KO mice. *P = 0.020. (G) Western blot analyses of p-AKT and p-ERK1/2 in serum-starved MEFs from WT and Gnpat-KO mice stimulated with 10% FBS. (H and I) Quantification of p-AKT at Ser473 (H) and Thr308 (I) in primary WT and Gnpat-KO Schwann cells after stimulation with NRG1. (J) Western blot analysis of total and p-AKT in cytosolic and membrane fractions of serum-starved MEFs from WT and Gnpat-KO mice stimulated with 10% FBS. Western blot analysis of caveolin 1 (CAV1), GAPDH, and peroxisomal thiolase (ACAA1) used to control membrane fractions and cytosolic fractions and to monitor lack of solubilized organelles in cytosolic fractions, respectively. (K) DRG cocultures from WT and Gnpat-KO mice treated with DMSO (control) or with SC79, stained for neuronal βIII-tubulin (green) and MBP (red). Scale bars: 100 μm. (L) Density of myelin segments in DRG cocultures from WT and Gnpat-KO mice after DMSO and SC79 treatment. *P < 0.002. (M) Length of individual myelin segments in myelinating cocultures. *P < 0.01. Error bars represent SEM in all graphs.
determine whether the defect in AKT activation plays a critical role in the impaired myelination observed in DRG cocultures, we performed in vitro myelination assays in the presence of SC79. This small compound was shown to allow AKT activation in the cytosol, bypassing the need for AKT to be targeted to the membrane (27). Treatment of DRG cocultures from Gnpat-KO mice with SC79 (Figure 5K) increased the number and length of myelin segments (Figure 5, L and M), indicating the rescue in myelination. These results highlight that plasmalogens are important for the correct recruitment and activation of AKT at the membrane and that their absence causes a signaling defect independently of ligand-receptor activation.

Defective differentiation of Schwann cells in plasmalogen-deficient mice is rescued by treatment with GSK3β inhibitors. AKT promotes Schwann cell differentiation and myelination in part through the inhibitory phosphorylation of GSK3β at Ser9 (28). We there-
fore tested whether treatment with LiCl, a GSK3β inhibitor (29), would rescue the Schwann cell defects observed in Gnpat-KO mice. The addition of LiCl to the culture medium rescued the myelination defect of DRG mixed cultures from Gnpat-KO mice (Figure 6A). Therefore, we treated WT and Gnpat-KO mice with LiCl or NaCl in two treatment schemes (Figure 6B). Following LiCl treatment for 1 week from P7, analysis of nerves from LiCl-treated Gnpat-KO mice showed improved myelination (Figure 6I) and rescue in axonal sorting, with normal numbers of axons in bundles (Figure 6J) and normal density of sorted promyelinating Schwann cells containing a single axon (Figure 6K). Similar results were obtained when newborn WT and Gnpat-KO mice were treated for 4 days with TDZD-8, a highly specific inhibitor of GSK3β (31). In sciatic nerves from P4 Gnpat-KO mice treated with TDZD-8, we observed a decrease in the number of axons per bundle (Figure 6L) and an increase in the number of sorted axons (Figure 6M). Taken together, our results indicate that plasmalogen deficiency affects Schwann cell differentiation and function, causing defects in axonal sorting, myelination, assembly of myelin, and nerve conduction through the impairment of AKT activation at the plasma membrane and via GSK3β.

**Discussion**

In this study, we report that a plasmalogen defect in the PNS primarily affects Schwann cells and that plasmalogen s are crucial for Schwann cells at two developmental time points. During the early postnatal period, plasmalogens are important for axon-glia interaction and the myelination process. The recognition of axons by Schwann cells or the ability to actively segregate axons destined for myelination is impaired by the deficiency in plasmalogens. The presence of unsorted axons caused abnormal myelination of the Remak bundles and reorganization of these structures. Additionally, impaired myelination, increased regions of noncompact myelin, and defective compartmentalization of Schwann cells in mutant nerves highlight the role of plasmalogens in the correct assembly of myelin and the organization of Schwann cells. Surprisingly, the extent of myelin thickness was normal in adult mutant mice, which led us to hypothesize that like a deficiency in MBP, which has minimal effects in the PNS despite the complete lack of myelin in the CNS, other myelin components may contribute to achieving normal myelination (24, 32). MBP, known to modulate the apposition of the cytoplasmic leaflets of myelin membranes through electrostatic interactions, is also involved in the partition of myelin into compact and noncompact myelin (33). To further investigate the role of plasmalogens during myelination, we asked whether depleting myelin of plasmalogens and MBP, two major constituents of myelin at the cytoplasmic apposition, would impair myelination. In DM mice, we found a major impairment in myelination and nerve conduction, indicating that plasmalogens are important regulators of myelination. In addition, we found that upon Wallerian degeneration, injury-induced remyelination was also affected by the deficiency in plasmalogens, although axonal regeneration seemed to be unaffected. Failure to maintain myelin levels was also evident in sciatic nerves from aged mutant mice, in which we observed extensive demyelination, axonal loss, and failed attempts to remyelinate the PNS.

Our results highlight that in mice, a deficiency in plasmalogens is sufficient to impair PNS myelination and to cause age-dependent peripheral neuropathy. As such, the evaluation of the PNS...
in RCDP type 2 and type 3 patients should be informative. In Refsum’s disease, the single defect in α-oxidation of phytanic acid is known to cause peripheral neuropathy (34). Previously, we identified a group of atypical RCDP type 1 patients with mild mutations in PEX7 and a Refsum-like presentation, which included retinitis pigmentosa and nerve hypertrophy (17). In addition to the accumulation of phytanic acid, these patients also had a defect in plasmalogen synthesis. Based on our current findings, we propose that RCDP type 1 patients should also be investigated for defects in nerve conduction and pathology. In these patients, the peripheral neuropathy can be aggravated by the combined defect in plasmalogen synthesis and phytanic acid accumulation.

In the present study, we analyzed the phosphorylation levels of several kinases involved in PNS development to understand how a defect in plasmalogens impairs Schwann cell differentiation and Schwann cell–mediated myelination. The results obtained in nerves of Gnpat-KO mice during the active period of myelination in the PNS revealed an impairment of AKT activation. Our observations of impaired activation of the AKT pathway following induction with FBS or NRG1, which was not mediated by defects in upstream regulators, suggested a more direct role of plasmalogens in AKT activation rather than that of multiple defects in several ligand-receptor interactions and activations. AKT activation by phosphorylation at the plasma membrane was impaired in plasmalogen-deficient cells. In mutant cells, AKT was found in membrane fractions, but upon FBS stimulation, AKT was not phosphorylated despite the correct localization and phosphorylation of PDK1. The requirement of AKT to associate with raft microdomains for efficient activation and signal transduction, combined with the enrichment of plasmalogens in lipid rafts, suggests that the defect in plasmalogens affects the correct compartmentalization of AKT, and thus its activation by phosphorylation (25, 34). The AKT signaling pathway is important for lens fiber differentiation, chondrocyte differentiation and maturation, adipogenesis, and spermatogenesis (35–38).

Based on our results showing that a plasmalogen defect impairs AKT activation in Schwann cells, we hypothesized that in tissues affected by a plasmalogen defect (e.g., lens, cartilage, adipocytes, and testis), impaired AKT signaling modulates the observed pathology, namely, cataracts, impaired ossification, lipodystrophy, and loss of spermatocytes (39).

In sciatic nerves from Gnpat-KO mice, we found that impaired AKT activation resulted in dysregulated phosphorylation of GSK3β. GSK3β activity and its role in the regulation of transcription are known to modulate Schwann cell differentiation and myelination (28, 40). Active GSK3β, phosphorylated at Tyr216, is inhibited by AKT phosphorylation at Ser9 (41). In nerves from Gnpat-KO mice, we observed increased levels of Tyr216 phosphorylation and decreased levels of Ser9 phosphorylation, which, combined, indicate overtly active GSK3β (41). Lithium, a known inhibitor of GSK3β, can directly inhibit the kinase through Mg²⁺ competition and can indirectly inhibit GSK3β by promoting further inhibition through Ser9 phosphorylation (42, 43). Lithium administration to Gnpat-KO mice was able to normalize GSK3β phosphorylation status, induced the stimulation of Schwann cell differentiation, rescued the radial sorting defect, and improved myelination independently of the plasmalogen defect. Similar findings were obtained when Gnpat-KO mice were treated with TDZD-8, a highly specific inhibitor of GSK3β. Treatment of plasmalogen-deficient mice with alkyl-glycerol rescues the biochemical defect and pathology in several tissues, but has minimal effects on restoring plasmalogen levels in nervous tissue (39). As such, improving the defective AKT/GSK3β signaling pathway may have clinical potential in combined therapeutic interventions with alkyl-glycerol during the active period of PNS myelination and in mildly affected patients displaying signs of peripheral neuropathy.

In summary, our data reveal the role of plasmalogens in the neuropathic course of two models of RCDP (see proposed model in Figure 7), showing that plasmalogen deficiency severely impairs the ability of Schwann cells to differentiate and mature, thus causing defects in myelination and nerve conduction. Through the identification of the mechanism by which a defect in plasmalogens mediates the pathology, we unraveled that ether phospholipids are crucial for the activation of the AKT pathways and identified a candidate therapeutic intervention that overcomes the plasmalogen deficiency and rescues the signaling pathway and Schwann cell differentiation.

**Methods**

Mouse strains, procedures, and treatments. WT, Pex7-KO, and Gnpat mice have been previously described (9, 10). KO and WT littermates of both sexes were obtained from mating of heterozygous mice. Shiverer mice (shi; Mbp shi/J) were obtained from The Jackson Laboratory. Pex7-shi and Gnpat-shi DM mice were obtained from F2 mice after crossing heterozygous Pex7 or Gnpat mice with homozygous shi mice.

For nerve crush, 2-month-old WT (n = 4) and Pex7-KO (n = 6) mice were anesthetized (i.p. 100 mg/kg ketamine and 1 mg/kg medetomidine), and the exposed right sciatic nerve was injured (15-second crush 2 times) at the level of the notch with a fine hemostat. The contralateral nerve was used as a control. Posturgical analgesia (1 mg/kg butorphanol s.c.) was performed twice a day for 3 days. Nerve conduction (WT, n = 6; shi, n = 4; Pex7-KO, n = 3; Pex7-shi DM, n = 4; Gnpat-KO, n = 5; Gnpat-shi DM, n = 3) was determined as previously described (44).

For the BrdU incorporation assay, BrdU in 0.9% NaCl and 7 mM NaOH were injected i.p. at a dosage of 50 mg/kg in WT and Gnpat-KO mice (n = 4) for each genotype 4 and 20 hours before the collection of sciatic nerves. LiCl or NaCl at a dosage of 50 mg/kg was injected s.c. daily from P0.5 to P6 (control WT, n = 6; Gnpat-KO, n = 6; lithium WT, n = 7; Gnpat-KO, n = 6) or on alternating days from P7 to P15 (control WT, n = 7; Gnpat-KO, n = 7; lithium WT, n = 7; Gnpat-KO, n = 7). TDZD-8 (Sigma-Aldrich) at 5 mg/kg or DMSO at 20% (v/v) was injected s.c. daily from P0.5 to P4 (control WT, n = 4; Gnpat-KO, n = 4; TDZD-8 WT, n = 5; Gnpat-KO, n = 5). Mice were used under standard conditions and had free access to food and water. Experiments and mouse manipulations were performed in compliance with the institutional guidelines and recommendations of the Federation for Laboratory Animal Science Association (FELASA) and were approved by the National Authority for Animal Health (DGAV; Lisbon, Portugal).

**Histological and morphological analyses.** Sciatic nerves were dissected and fixed by immersion on 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 week. After postfixation with 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.4) for 2 hours, nerves were dehydrated and embedded in Epon (Electron Microscopy Sciences). Sections (1-μm-thick) were stained for 10 minutes with 1% p-phenylenediamine (PPD) in absolute methanol, dried, and mounted on a drop of DPX (Merk). The entire area of the nerve was photographed on an Olympus optical microscope equipped with an Olympus DP 25 camera and Cell B software, and images were imported into Photoshop (Adobe). For g ratio analyses, the Photoshop recording measurement tool was used to determine the area of the axon and the entire myelinated fiber (axon plus myelin). Derived diameters were used to calculate the g ratio in 200 to 300 individual fibers per genotype and age (P5 mice: WT, n = 7; Pex7-KO, n = 4; Gnpat-KO, n = 4; P15 mice: WT, n = 5; Pex7-KO, n = 4;
**Gnpat-KO, n = 4; P30 and P90, n = 4 for all genotypes; 1.5-year-old: WT, n = 6; Pex7-KO, n = 6; Gnpat-KO, n = 5.** For the determination of the density of myelinated fibers (MFs), the total number of MFs was divided by the area of the entire cross section of the sciatic nerve.

Ultrathin sections (60-nm) prepared on a Leica ultramicrotome were placed on 200-mesh copper grids (Electron Microscopy Sciences) and were counterstained, first with alcoholic uranyl acetate solution (2% w/v; 10 minutes), then by aqueous uranyl acetate solution (2% w/v, 10 minutes) and lead citrate (4% w/v; 10 minutes). Grids were observed on a JEOL, JEM-1400 transmission electron microscope equipped with an Orius Sc1000 digital camera. Ten to 15 nonoverlapping images were obtained and used for all determinations.

**Cell culture and in vitro myelination.** MEFs were prepared from E12 embryos and cultured in high-glucose DMEM with 10% FBS and antibiotics. Schwann cells were prepared from 12 hours before stimulation with 30 ng/ml recombinant human neuregulin 1 EGF-like domain (R&D Systems). The lysates were prepared after 0, 7, and 15 minutes at 37°C. Serum-starved MEFs were stimulated with 10% FBS for 0, 1, 3, and 5 minutes. The lysates were prepared in PBS containing 0.3% Triton X-100, protease inhibitors (Complete, Mini; Roche), and 2 mM orthovanadate.

In vitro myelination was performed as described (45). Mixed cultures of DRG neurons and Schwann cells from WT (n = 7) and Gnpat-KO (n = 4) embryos were maintained in high-glucose MEM with 2 mM L-glutamine, 10% FBS, 50 ng/ml nerve growth factor (NGF) (2.5S; Millipore), and 1% penicillin-streptomycin, and were then plated onto 13-mm-diameter Matrigel-coated coverslides (1:10 in DMEM). After 24 hours at 37°C, cells were cultured for 10 days in neurobasal medium supplemented with 4 g/l glucose, 2 mM L-glutamine, 50 ng/ml NGF, and 1x B27 (Gibco). Myelination was induced at 10 days in vitro (div) by the daily addition of 50 ng/ml ascorbic acid to high-glucose MEM medium supplemented with 2 mM L-glutamine, 10% FBS, and 50 ng/ml NGF. For lithium treatment in myelination assays, medium was supplemented with 351226099157; E-mail: pedro.brites@ibmc.up.pt.

**X-ray diffraction.** Sciatic nerves from WT (n = 6) and Pex7-KO (n = 7) mice were fixed by immersion in 2% glutaraldehyde in 0.12 M phosphate buffer (pH 7.4). Diffraction experiments and data analysis were carried out as detailed previously (47). For the determination of the relative amount of myelin by x-ray diffraction, the quotient M/M+B was used, in which the denominator includes the total x-ray scatter coming from the volume of nerve subtended by the x-ray beam (consisting of the multilamellar myelin, M, and background, B), and the numerator is the total intensity coming from the multilamellar myelin (the peak intensities above background), as previously described (47).

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