Profilin 1 is required for peripheral nervous system myelination

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ABSTRACT

Myelination allows rapid saltatory propagation of action potentials along the axon and is an essential prerequisite for the normal functioning of the nervous system. During peripheral nervous system (PNS) development, myelin-forming Schwann cells (SCs) generate radial lamellae to sort and ensheath axons. This process requires controlled cytoskeletal remodeling, and we show that SC lamellipodia formation depends on the function of profilin 1 (Pfn1), an actin-binding protein involved in microfilament polymerization. Pfn1 is inhibited upon phosphorylation by ROCK, a downstream effector of the integrin linked kinase pathway. Thus, a dramatic reduction of radial lamellipodia formation is observed in SCs lacking integrin-linked kinase or treated with the Rho/ROCK activator lysophosphatidic acid. Knocking down Pfn1 expression by lentiviral-mediated shRNA delivery impairs SC lamellipodia formation in vitro, suggesting a direct role for this protein in PNS myelination. Indeed, SC-specific gene ablation of Pfn1 in mice led to profound radial sorting and myelination defects, confirming a central role for this protein in PNS development. Our data identify Pfn1 as a key effector of the integrin linked kinase/Rho/ROCK pathway. This pathway, acting in parallel with integrin β1/LCK/Rac1 and their effectors critically regulates SC lamellipodia formation, radial sorting and myelination during peripheral nervous system maturation.

KEY WORDS: Profilin, Myelination, Schwann cell, Mouse

INTRODUCTION

Schwann cells (SCs) are the myelin-forming cells of the peripheral nervous system (PNS). During development, SC precursors populate outgrowing axon bundles, where they proliferate and differentiate into immature SCs (Jessen and Mirsky, 2005). Possibly as a result of increasing cell density (Webster et al., 1973), these cells extend radial lamellae into axon bundles. SCs segregate and establish 1:1 relationships with individual large caliber (>1 μm diameter) axons. Upon maturing into the promyelinating stage, the SCs start to segregate and later myelinate such axons, leaving behind small axons that will remain unmyelinated. This process is collectively referred to as radial sorting (Webster et al., 1973), and requires the formation of a cytoplasmic protrusion radially in relation to the main SC axis, similar to a giant lamellipodia in form and function (Nodari et al., 2007; Feltri et al., 2008). Such radial lamellipodia are likely to be controlled by the same molecular machinery regulating actin polymerization at the leading edge of lamellipodia in cultured cells (Feltri et al., 2008). It is therefore important to understand which molecular players orchestrate the functional cytoskeletal remodeling that are necessary for these cellular structures to form. The small Rho GTPases Cdc42 and Rac1 are known to modulate actin cytoskeletal dynamics in SCs and regulate radial sorting. Whereas Cdc42 is mainly involved in the control of SC proliferation (Benninger et al., 2007), Rac1 promotes SC lamellipodia formation (Benninger et al., 2007; Nodari et al., 2007) downstream of laminin/integrin-β1/lamellipod cell kinase (LCK) (Feltri et al., 2002; Ness et al., 2013). Rac1 downstream targets N-WASP (Jin et al., 2011; Novak et al., 2011) and MLCK/myosin II (Wang et al., 2008; Leitman et al., 2011) are key regulators of radial sorting and PNS myelination. Negative regulation of Rho/ROCK signaling by integrin-linked kinase (Ilk) (Pereira et al., 2009) is also strictly required for SC cytoskeleton rearrangements leading to efficient radial sorting. However, the downstream mediators of Rho/ROCK have not yet been clearly identified.

Profilins (Pfn1s) are small, ubiquitous, 12-16 kDa actin-binding proteins. Mammals encode four different Pfn proteins, Pfn1 being the major isoform found in nearly all tissues (Witke et al., 1998). In the nervous system, Pfn1 might play a role in Huntington’s disease (Shao et al., 2008), and mutations in the PFN1 gene were recently linked to familial amyotrophic lateral sclerosis (Wu et al., 2012). Pfn2a, the only other isoform expressed in the developing nervous system (Lambrechts et al., 2000; Witke et al., 2001), has been implicated in the regulation of neuritogenesis (Da Silva et al., 2003). Pfn1s are pivotal in promoting actin dynamics at the plasma membrane to drive actin-linked processes. They form 1:1 complexes with monomeric actin, regulating its availability by sequestration (Carlsson et al., 1977), and catalyze the exchange of actin-bound ADP to ATP to enable rapid microfilament polymerization (Finkel et al., 1994; Witke et al., 1998; Witke et al., 2001; Witke, 2004). Pfn1 is also a ROCK substrate. ROCK-mediated Pfn1 phosphorylation on Ser137 leads to its inactivation in both HEK293 cells and primary neurons (Shao et al., 2008). In light of the central role of Pfn1 in the regulation of actin dynamics and lamellipodia formation (Cao et al., 1992; Syriani et al., 2008), Pfn1 is poised as an excellent candidate to regulate myelination downstream of Rho/ROCK by potentially modulating SC radial lamellipodia formation. Using mouse genetics and in vitro experiments, we demonstrate a novel crucial function for Pfn1 in radial sorting and ensheathment during PNS development. Moreover, we show that Ilk/Rho/ROCK negatively controls Pfn1 function and that this signaling acts in parallel with the integrin β1/LCK/Rac1 pathway to regulate SC lamellipodia formation. Furthermore, we found that Igf1, which is produced by SCs, can inhibit Rho/ROCK signaling thereby enhancing Pfn1 function and driving radial lamellipodia formation.

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RESULTS

Rho/ROCK signaling regulates radial lamellipodia formation in SCs

Ilk-negative regulation of Rho/ROCK signaling is crucial for radial sorting and PNS myelination (Pereira et al., 2009). Such processes are thought to depend on the capacity of SCs to extend radial lamellipodia mediated by Rac1 activation downstream of integrin β1 (supplementary material Fig. S1) (Nodari et al., 2007; Feltri et al., 2008). However, Ilk mutant mice displayed impaired radial sorting and myelination, despite Rac1 hyperactivation (Pereira et al., 2009). We found that the numbers of radial lamellipodia in Dhh-Cre Ilkfl/fl (mutant) SCs were significantly lower compared with those in Dhh-Cre Ilkfl/wt (control) SCs (Fig. 1Aa,b). These data further support the crucial role of radial lamellipodia formation in efficient radial sorting and myelination. Furthermore, they suggest the existence of a negative signaling pathway that is able to counteract Rac1 activation of lamellipodia formation in Ilk mutant mice. The Rho/ROCK pathway is hyperactivated in Ilk mutant mice and responsible for the observed radial sorting phenotype (Pereira et al., 2009). Thus, we tested whether it could be responsible for the failure in radial lamellipodia formation. Pharmacological inhibition of ROCK by Y27632 (10 μM) or fasudil (40 μM) restored formation of radial lamellipodia in Ilk mutant SCs (Fig. 1Ac-f), suggesting that Rho/ROCK signaling is capable of inhibiting radial lamellipodia formation even in the presence of Rac1 hyperactivation.

Rho/ROCK regulates Pfn1 activity independently of integrin β1 and Rac1 signaling

Pfn1 is an actin remodeling protein that is capable of regulating lamellipodia formation in different cell types (Cao et al., 1992; Syriani et al., 2008), and is a ROCK substrate (Shao et al., 2008). Therefore, it is a strong candidate in mediating lamellipodia formation downstream of Rho/ROCK in SCs. To address whether Rho/ROCK signaling regulates Pfn1 in SCs, we analyzed phosphoS137-Pfn1 levels (Shao et al., 2008) in lysates obtained from rat SC cultures exposed to lysophosphatidic acid (LPA), a known SC activator of Rho signaling. Although 3 hours of LPA treatment of SCs increased phospho-Pfn1 without affecting Pfn1 total levels (Fig. 1B), exposure of LPA-treated SC to the ROCK inhibitor Y27632 (10 μM) prevented the increase in Pfn1-P levels (Fig. 1B). Very short-term exposure to LPA (<3 minutes) has been reported to transiently increase Rac1 activation in the SCL4.1/F7 SC line (Barber et al., 2004). Thus, we tested whether LPA also increased Rac1 activation in our experimental conditions, in which primary SCs were exposed to LPA for 3 hours. Although the analysis of Rac1 expression and activity by pull-down assay and

Fig. 1. Rho/ROCK regulates radial lamellipodia formation and Pfn1 phosphorylation in SCs.

(A) Immunocytochemistry reveals the cytoskeleton of Ilk control and mutant SCs (a and b, c and d, e and f). Lamellipodia formed along SC processes (radial lamellipodia, arrows and magnified panels) or formed at the tip of the processes (axial lamellipodia, arrowheads) are depicted. Ilk mutant SCs form significant fewer radial lamellipodia (arrows) compared with controls (P=0.0003). No significant differences were seen in axial lamellipodia (arrowheads) (P=0.1955) (a,b), or in axial or radial lamellipodia between mutants and controls treated with ROCK inhibitor Y27632 (10 μM) (c,d) (P=0.1313 and P=0.5392, respectively) or fasudil (40 μM) (e,f) (P=0.3654 and P=0.2302, respectively). n=3 independent cell culture experiments. (B) Increased Pfn1 phosphorylation on Ser-137 in rat SCs treated with LPA (1 μM; 3 hours) (P=0.0192) compared with non-treated controls or SCs treated for 3 hours with Y27632 (2 μM) and LPA. Total levels of Pfn1 were not significantly changed (P=0.7964). n=3 independent cell culture experiments. Error bars indicate ±s.e.m. *P<0.05; ***P<0.001. Scale bar: 50 μm.
Ilk levels of phospho-Pfn1 and total Pfn1 in lysates obtained from P5 lamellipodia formation via Pfn1 activation. Thus, we analyzed the Rho/ROCK signaling (Pereira et al., 2009) could promote SC LPA were not significantly altered (Fig. 1C).

We hypothesized that Ilk-mediated negative regulation of Rho/ROCK axis regulates Pfn1 phosphorylation, which affects lamellipodia formation. (A) Increased phospho-Pfn1 in protein lysates from P5 sciatic nerves of Ilk mutant mice (n=3 CT and MU mice, P=0.0024) compared with those of controls. Total levels of Pfn1 are not significantly changed (n=3 CT and MU mice, P=0.9750). Phospho-Pfn1 (n=3 CT and MU mice, P=0.2546) and Pfn1 total levels (n=3 CT and MU mice, P=0.6600) are not significantly changed in lysates from integrin β1 mutant SNs compared with those from controls. (B) Lentivirus-mediated knock-down of Rac1 in rat SCs significantly reduces Rac1 total levels (n=3 independent cell culture experiments; P=0.0345) compared with controls, but not Pfn1 phosphorylation (n=3 independent cell culture experiments; P=0.5574) or Pfn1 total levels (n=3 independent cell culture experiments; P=0.3169). (C) Igf1 treatment (150 ng/ml) decreases activation of RhoA, RhoB and Rhoc (termed Rho) (n=3 independent cell culture experiments; P=0.0106), whereas their total levels (n=3 independent cell culture experiments; P=0.1075), the levels of activated Rac1 (n=3 independent cell culture experiments; P=0.7227) and Rac1 total levels (n=3 independent cell culture experiments; P=0.1075) are not significantly changed. The Rho antibody used is not specific for individual isoforms. (D) Pfn1 phosphorylation is significantly reduced in Igf1-treated SCSs (n=3 independent cell culture experiments; P=0.0209) but not its total levels (n=3 independent cell culture experiments; P=0.2016). (E) Immunocytochemistry reveals the cytoskeleton of SCs infected with control or Pfn1-shRNA lentivirus. Pfn1-shRNA infection significantly decreases radial (arrows) (n=3 independent cell culture experiments; P=0.006) and axial (arrowheads) (n=3 independent cell culture experiments; P=0.0013) lamellipodia. Fibroblasts, when present, were excluded from the quantification (asterisks). Error bars indicate ±s.e.m. *P<0.05; **P<0.005. Scale bar: 50 μm.

Rac1 activity was not significantly altered by LPA treatment (Fig. 1C), and we had previously reported that high endogenous activation of Rac1 (Pereira et al., 2009) in Ilk mutant sciatic nerves did not counteract Rho/ROCK inhibition of lamellipodia formation. Thus, we postulated that Rho/ROCK and Rac1 could be working through independent downstream targets towards cytoskeleton remodeling and lamellipodia formation. To test this, we first analyzed phospho-Pfn1 and total Pfn1 levels in lysates obtained from P5 control and integrin β1 mutant sciatic nerves, in which Rac1 activation is decreased (Benninger et al., 2007; Nodari et al., 2007). We found that the levels of phospho-Pfn1 and total Pfn1 were not significantly changed in protein lysates from mutants (Fig. 2A). Then, to further confirm that Pfn1 phosphorylation is not Rac1 dependent, we knocked down Rac1 in SC cultures by lentiviral shRNA-mediated gene silencing (Fig. 2B) and analyzed the levels of phospho-Pfn1 and total Pfn1. We found no significant changes of either in lysates obtained from SC cultures after Rac1 knock down (Fig. 2B). Collectively, these results indicated that Rho/ROCK regulation of Pfn1 phosphorylation and function is indeed independent of Rac1 activation.

Igf1 modulates Rho/ROCK regulation of Pfn1
Igf1 is produced by SCs and promotes SC cytoskeleton remodeling (Cheng et al., 2000) and myelination (Ogata et al., 2006; Liang et al., 2007). Igf1 treatment of SCs resulted in a reduction in the levels of activated Rho and of phospho-Pfn1 compared with controls (Fig. 2C,D). Rac1 activity and total Rho, Rac1 and Pfn1 protein levels were not significantly changed (Fig. 2C,D). These data suggest that SC physiological stimuli, such as Igf1, can regulate lamellipodia formation by modulating ROK/Pfn1 phosphorylation and, as a consequence, Pfn1 activity.

Pfn1 is required for SC lamellipodia formation, radial sorting and myelination
Lentiviral-mediated shRNA knockdown of Pfn1 in SCs reduced the formation of axial and radial lamellipodia compared with controls.
(Fig. 2E). To examine its functions in vivo, we conditionally ablated Pfn1 in SCs by expressing Cre recombinase under the control of the desert hedgehog (Dhh) gene regulatory sequences (Fig. 3A) (Jaegle et al., 2003). Although recombination of the conditional Pfn1 allele (Fig. 3B) reduced Pfn1 protein levels in lysates obtained from P1 mutant SNs (Fig. 3C), Pfn2 levels remained unchanged (Fig. 3C). In vitro, Pfn1 knockout SCs displayed reduced numbers of axial and radial lamellipodia, and these also appeared to be smaller and to contain less filamentous polymerized actin (Fig. 3D). Pfn1 mutant mice were born at a sub-Mendelian frequency (<10%), and died of unknown causes at around P15. As a result, experiments were performed at P14 or earlier. P14 mutant sciatic nerves were thinner than those of controls (Fig. 3E). During postnatal development, immature SCs progressively segregate axons with a diameter over 1 μm. After having established a 1:1 relationship with such axons, pro-myelinating SCs will then myelinate them. This initial process, referred to as radial sorting, is usually complete by P14. Accordingly, at P0, SCs were present between bundles of tightly apposed axons in the sciatic nerves of control mice (Fig. 4Aa) and at P5 only few bundles still contained large caliber unsorted axons (Fig. 4Ac). Most of the large caliber axons were engaged in a 1:1 relationship with SCs (Fig. 4Ac). By P14, in control nerves all large caliber axons were sorted and myelinated (Fig. 4Ac). Only small caliber axons (less than 1 μm diameter) remained in so-called Remak bundles, surrounded by non-myelinating SCs. By contrast, in mutant sciatic nerves radial sorting and myelination were impaired. Bundles containing unsorted large caliber axons were still present in P14 mutant nerves (Fig. 4Af,C). More promyelinating SCs, meaning SCs in a 1:1 relationship with an axon without having produced myelin (Fig. 4Af,D), were also present. In addition, myelinated fibers that did form in mutants were thinner than those in control sciatic nerves (Fig. 4Ad,f, yellow arrowheads). Overall, the differentiation of SCs was severely impaired in the mutants. This conclusion was also supported by the findings that in P2 mutant sciatic nerves Oct6 expression was increased and Krox20 expression was reduced (Fig. 3F), compared with controls. The expression of Oct6 peaks at promyelinating and early myelinating stage, and is downregulated at later stages of myelination (Jaegle et al., 1996). Krox20 expression is required for downregulation of Oct6 and for the activation of myelin genes (Topilko et al., 1994). An insufficient number of SCs could also explain the persistence of axon bundles (Benninger et al., 2007; Grove et al., 2007). However, despite a slight increase in the percentage of apoptotic cells at P0 and P5 in mutant nerves (Fig. 3G), the number of proliferating Ki67-positive cells at different stages of development (Fig. 3G) and the total number of cells at P5 (Fig. 3G) were not significantly different from those in nerves from control mice.

To better understand why SC differentiation was impaired, we carried out a more detailed EM analysis of P5 and P14 control and mutant sciatic nerves (Fig. 4B). At P5, control SCs were at different stages of differentiation: immature SCs associated with axon bundles, promyelinating SCs in one-to-one relationship with axons, and a few myelinating SCs. In Pfn1 mutant sciatic nerves, cytoplasmic processes of immature SCs enveloped the majority of axon bundles (Fig. 4Ba), and targeted and sorted axons (Fig. 4Ba). At the promyelinating stage, SC-axon profiles in control and mutant nerves were surrounded by an apposed basal lamina (Fig. 4Bb,c,e, black arrows). In P14 control SCs, radial sorting of large caliber fibers was virtually complete. Mature SCs formed a compact multilayered sheath of myelin around single large caliber axons. In P14 mutant nerves, hypomyelination was pronounced (Fig. 4Af,b,d, yellow arrowheads) and axon bundles enveloped by immature SCs still persisted. However, immature SCs were capable of enveloping axon bundles and only rarely were small sections of the axon bundle left naked (Fig. 4Bc, black arrowhead). The distinctive feature of these mutant nerves was the high number of SC-axon promyelinating profiles that did not progress to the mature

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**Fig. 3. Analysis of SC-specific Pfn1 mutant.** (A) Upon Dhh-driven Cre recombination, the conditional Pfn1 allele is inactivated. (B) PCR shows the recombination of the conditional Pfn1 allele in genomic DNA isolated from P1 sciatic nerve, lung and brain of control and mutant mice. The fragment sizes for wild-type (wt), Pfn1 floxed (flox) and Pfn1 mutant (KO) alleles are indicated by black arrows. (C) Western blot analysis of protein lysates from P2 control and mutant SNs reveals that Pfn1 levels are reduced compared with controls (n=3 CT and MU mice; P=0.0001). Pfn2 expression was not significantly changed. (D) Immunocytochemistry reveals the cytoskeleton of Pfn1 mutant and control SCs. Pfn1 mutant SCs form reduced numbers of radial (n=3 independent cell culture experiments, P=0.0074) (arrows) and axial (n=3 independent cell culture experiments, P=0.0108) lamellipodia compared with controls. Scale bar: 50 μm. (E) SNs from P14 mutants are thinner and more transparent than those of control litters. Scale bar: 1 mm. (F) Western blot analysis of protein lysates from P2 control and mutant SNs reveals that Oct6 levels are increased compared with controls (n=3 CT and MU mice, P=0.0216) and those of Krox20 are reduced (n=3 CT and MU mice, P=0.0024). (G) Pfn1 mutant nerves contain significantly more TUNEL-positive, apoptotic cells at P0 (n=3 CT and MU sciatic nerves from 3 CT and MU mice, P=0.0011) and P5 (n=3 CT and MU sciatic nerves from 3 CT and MU mice, P=0.0006). However, the total number of cells in P5 control and mutant nerves was not changed. The number of Ki67-positive proliferating cells in E18, P0 and P5 control and mutant nerves were not significantly changed. Error bars indicate ±s.e.m. *P<0.05; **P=0.005; ***P<0.001.
myelinated form (Fig. 4Bb,d,e, asterisks). Mutant promyelinating SC-axon profiles were often irregular in shape and displayed abnormal cytoplasmic protrusions (Fig. 4Bb,d, white arrowheads). The Pfn1 mutant phenotype seemed to resemble that of the SC-specific N-Wasp (Wasl – Mouse Genome Informatics) mutant mouse (Jin et al., 2011; Novak et al., 2011), in which the diminished capacity of SCs to form lamellipodia at later stages affected mainly the ability to enwrap axons. This differed from the integrin β1 (Feltri et al., 2002), Rac1 (Benninger et al., 2007; Nobardi et al., 2007) or Ilk (Pereira et al., 2009) SC-conditional mutant mice, in which defects in radial lamellipodia formation affected mainly the capacity of SCs to sort axons, resulting in persistent sorting defects. In an attempt to study the effects of the loss of Pfn1 beyond P14, we conditionally ablated Pfn1 by expressing Cre recombinase under the control of the 2,3'-cyclic nucleotide 3'-phosphodiesterase (CNP) gene regulatory sequences (Lappe-Siefke et al., 2003). The SC phenotype of CNP-Cre Pfn1fl/fl mutant mice (Fig. 5), which were also born at a sub-Mendelian frequency, was similar to and at least as severe as that observed in the Dhh-Cre mutant mice. Nevertheless, a few CNP-Cre Pfn1fl/fl mutant mice survived beyond 3 weeks. The analysis of their phenotype at P24 was consistent with Pfn1 being required for efficient radial sorting and for promyelinating SCs to myelinate axons (Fig. 5). Similarly, in Dhh-Cre Pfn1fl/fl mutants, the nerves of P24 CNP-Cre Pfn1fl/fl mutant mice contained a large number of SC-axon promyelinating profiles that did not progress to myelination (Fig. 5Af,h, arrows; 5Be,f, #). The few of those that myelinated had thinner myelin sheaths (Fig. 5Ad,f,Be, yellow arrowheads), resulting in reduced fiber diameter versus axon diameter ratio in mutant sciatic nerves at P24 compared with controls (Fig. 5Ag). The myelin in mutant nerves was also significantly thinner compared with that of controls (Fig. 5H). Occasionally, some axon/SC profiles contained empty loops of redundant basal lamina (Fig. 5Bc, white arrows), interpreted before (Benninger et al., 2007; Pereira et al., 2009) as a sign of SC process instability.

**DISCUSSION**

Radial sorting and axon ensheathment by SCs are key events in the development of the PNS. They depend on laminin/integrin β1 interactions (Feltri et al., 2002), downstream activation of the small RhoGTPase Rac1 (Benninger et al., 2007; Nobardi et al., 2007) via lymphoid cell kinase phosphorylation (Ness et al., 2013), and regulation of its targets N-WASP (Jin et al., 2011; Novak et al., 2011) and MLCK/myosin II (Wang et al., 2008; Leitman et al., 2011). Together with the negative regulation of Rho/ROCK signaling by Ilk (Pereira et al., 2009), they control SC cytoskeleton reorganization and the formation of SC lamellipodia largely through not yet fully characterized downstream targets and functional mechanisms.

In this study, using SC-specific gene ablation and other appropriate methodologies, we identify Pfn1, a G-actin-binding protein and ROCK substrate, as a novel key regulator of SCs lamellipodia formation, radial sorting and, of major impact, axon ensheathment during PNS development.

**Pfn1 regulates SC radial lamellipodia independently of Rac1**

Rac1 activity can directly regulate the number, extension and localization of lamellipodia in several cell types (Pankov et al., 2005). Although low levels of active Rac1 stimulate migratory behavior by inducing the formation of axial lamellipodia at the front and rear of fibroblasts and epithelial cells, higher levels of Rac1 stimulate cell adhesion and spreading by inducing the formation of radial lamellipodia around the whole perimeter of the cell (Pankov et al., 2005). Accordingly, Rac1 is essential for the formation of lamellipodia in SCs (Nobardi et al., 2007). Its activation is mediated by ECM/integrin β1 interactions, mainly through LCK (Ness et al., 2013). Impairment of such interactions in SCs resulted in diminished Rac1 activation (Nobardi et al., 2007) and consequently reduced radial lamellipodia formation and radial sorting (Feltri et al., 2002; Nobardi et al., 2007). Positive modulation of lamellipodia formation by Rac1 is likely to be mediated, at least partially, through regulation of N-Wasp (Tomasevic et al., 2007; Jin et al., 2011; Novak et al., 2011).
Radial sorting was also defective in SC-specific Ilk mutant mice (Pereira et al., 2009). Ilk is a negative regulator of Rho/ROCK signaling, and injections of the ROCK inhibitor fasudil in Ilk mutant mice were able to re-establish radial sorting to control levels (Pereira et al., 2009). In line with this, we show that Ilk-deficient SCs form fewer radial lamellipodia compared with controls, and that pharmacological inhibition of ROCK by Y27632 or fasudil is sufficient to restore lamellipodia formation. In Ilk mutant sciatic nerves, Rac1 activity is significantly increased in relation to controls (Pereira et al., 2009). As this is not sufficient to counteract Rho/ROCK activation and resulting Pfn1 phosphorylation observed in this study. Such conclusion is also supported by the fact that the Rac1 hyperactivation seen in Ilk mutant mice could not counteract the defects on SC lamellipodia formation, radial sorting and myelination present on those mice (Pereira et al., 2009). Overall, our data suggest that Pfn1 is a Rho/ROCK-specific downstream target in the regulation of SC lamellipodia formation. In point of fact, exposure of SCs to Igf1, which is known to promote SC lamellipodia formation (Cheng et al., 2000), can reduce the activation levels of Rho and phospho-Pfn1 without significantly changing Rac1 activation. Using lentiviral-mediated shRNA gene silencing, and conditional gene ablation in SCs, we demonstrate that Pfn1 is indeed essential for SC radial lamellipodia formation.

Pfn1 regulates radial sorting and axon ensheathment
SC-specific Pfn1 gene ablation profoundly impaired radial sorting and axon ensheathment in sciatic nerves of mutant mice. Although we also observed a strong delay in radial sorting, the main feature of both Dhh-Cre and CNP-Cre Pfn1<sup>fl/fl</sup> mutant SNs was the presence...
It is thought that a minimum threshold number of SCs is required to initiate radial sorting (Martin and Webster, 1973; Webster, 1971). Ablation of genes involved in SC proliferation, such as Cdc42 (Benninger et al., 2007) or Fak (Grove et al., 2007) results in radial sorting impairment. In Pfn1 mutant nerves, SC proliferation was not affected, and despite a slight increase in SC death the total number of SCs was normal. Therefore, similar to integrin β1 (Feltri et al., 2002), Rac1 (Benninger et al., 2007; Nodari et al., 2007) and Ilk (Pereira et al., 2009) mutant nerves, we consider it likely that the radial sorting and axon ensheathing defects observed are a direct consequence of deficits in radial lamellipodia formation.

The complex nature of radial sorting and axon ensheathment is likely to depend on the strict control exerted by multiple crosstalk signaling pathways, including those modulating Ilk/Rho/ROCK or integrin β1/LCK/Rac1 activation (Fig. 6). Such pathways control several cytoskeleton modulators, including N-WASP (Tomasevic et al., 2007; Jin et al., 2011; Novak et al., 2011) and MLCK/myosin II (Wang et al., 2008; Leitman et al., 2011), that are key in regulating radial lamellipodia formation, an essential requirement for radial sorting and axon ensheathing. Although Rac1 is a positive regulator, promoting radial lamellipodia and radial sorting, Rho/ROCK can counteract it as a negative regulator, repressing their formation. Our data show for the first time that the G-actin binding protein Pfn1, a ROCK substrate, is essential for SC radial lamellipodia formation and consequently for radial sorting and axon ensheathing. The fact that somehow unexpectedly one single ROCK effector can have such a profound impact on SC development and myelination justifies the need for a better characterization of other molecular players participating in these and other related pathways. This knowledge is crucial for a better understanding of PNS development in health and disease.

**MATERIALS AND METHODS**

**Animals**

All animal experiments were performed with the approval and in strict accordance with the guidelines of the Swiss Cantonal Veterinary Office, the Portuguese Veterinary Office and EU directives. All efforts were made to minimize animal suffering, reduce the number of required animals and replace with *in vitro* experiments whenever possible.

**Generation of conditional knockout mice**

Mice homozygous for the integrin-β1 floxed allele were crossed with mice expressing Cre recombinase under the control of the 2′,3′-cyclic nucleotide 3′-phosphodiesterase (*CNPase*) promoter (‘knock-in’) (Lappe-Siefke et al., 2003; Saher et al., 2005; Grove et al., 2007) and which were heterozygous for the floxed allele, as previously described (Benninger et al., 2006; Thurnherr et al., 2006). Mice homozygous for the Rac1 or the Ilk floxed allele were crossed with mice expressing Cre recombinase under the control of the desert hedgehog (*Dhh*) promoter (Jaegle et al., 2003) and which were heterozygous for the floxed allele, as previously described (Benninger et al., 2007; Pereira et al., 2009).

Mice homozygous for the *Pfn1* floxed allele were crossed with mice expressing the Cre recombinase under the control of either the *Dhh* or *CNPase* promoter and heterozygous for the *Pfn1* floxed allele (Böttcher et al., 2009). Progeny of interest were *Cre++; Pfn1* flox/flox mice (hereafter called mutant mice), and *Cre++; Pfn1* flox/Wt (hereafter called control mice). All genotypes were determined by PCR on genomic DNA. All experiments were performed on mice kept on a C57Bl/6 background.

**Antibodies**

rabbit anti-β1-integrin (WB 1:500, Abcam, UK), mouse and rabbit anti-Pfn1 (WB 1:1000, Abcam, UK), rabbit anti-Pfn2 (WB 1:500, kindly provided by Prof. R. Fässler, Martinsried, Germany), rabbit anti-phosphoSer137 Pfn1 (WB 1:1000, kindly provided by Dr Marc Diamond, UCSF, San Francisco, USA), mouse anti-Rac1 (WB 1:1000, BD Biosciences, USA), goat anti-mouse IgG HRP coupled (WB 1:15,000, Jackson Laboratories, PA, USA), donkey anti-rabbit IgG HRP coupled (WB 1:10,000, Jackson Laboratories, PA, USA), goat anti-mouse IgG H-L Cy3 coupled (ICC 1:3000, Jackson Laboratories, PA, USA).

Electron microscopy
Mice were anesthetized and perfused with 0.1 M phosphate buffer (pH 7.4), followed by 3% glutaraldehyde and 4% paraformaldehyde. Fixed tissues were dehydrated through a graded acetone series, post-fixed in 2% osmium-tetroxide, and embedded in ‘Spurr’s resin’ (Electron Microscopy Sciences). Semi-thin sections were stained with 1% Toulidine Blue for light microscopy, and ultrathin sections on grids with 3% uranyl acetate, 1% lead citrate, before examination in a Hitachi H-600 and a Jeol 1400 transmission electron microscope.

Primary Schwann cell culture
Mouse SC cultures were obtained from P0-P2 sciatic nerves. Nerves were dehydrated through a graded series of alcohols, followed by drying in pentane, freeze-fracture in a Balzers BAF 80A freeze-etching apparatus, and critical point dried in a Balzers CPD 030 apparatus. Nerve sections were sputtered with gold in a Balzers SCD 005 sputter coater and examined in a Hitachi H-600 electron microscope. Secondary Schwann cells were isolated from P1 sciatic nerves and cultured for 7 days. After this time, cells were dehydrated and prepared for electron microscopy.

Lysophosphatidic acid (LPA)/ROCK and Igf1 activation assay
LPA assay was performed as previously described (Weiner et al., 2001). Rat SCs were serum starved overnight (16-18 hours) in modified SATO medium (DMEM+Glutamax (Gibco), human apo-transferrin (100 μg/ml), progesterone (60 ng/ml), insulin (5 μg/ml), putrescine (16 μg/ml), L-thyroxin (400 ng/ml), selenium (160 ng/ml), triiodothyronine (10 ng/ml) and BSA (300 μg/ml, Fluka). Rat SC cultures were obtained from P2-P3 Wistar rats sciatic nerves. Nerves were dehydrated and embedded in HM20 (Nanomelt). Tissues were cut in 0.5 mm thick sections and placed in a cryostat. Sections were examined in a Neophot 2 microscope (Zeiss) and images were acquired with a DP70 camera (Olympus) and a DP2-BSW software. For immunohistochemistry, sciatic nerve sections were blocked for 1 hour with 10% goat serum, 0.1% Triton X-100, in PBS and incubated with primary antibodies overnight (4°C). Tissue sections were washed (1× PBS) and incubated with secondary antibodies (1 hour, room temperature). Sections were mounted in Citifluor (Citifluor) containing 4’,6-diamidino-2-phenylindole (DAPI).

Immunohistochemistry
Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. Sections were dewaxed and rehydrated through a graded series of alcohols in water, then treated with 3% hydrogen peroxide to block endogenous peroxidase activity. After rinsing with water, sections were incubated with primary antibodies overnight at 4°C. Tissue sections were washed in PBS and incubated with secondary antibodies (1 hour, room temperature). Sections were mounted in Citifluor (Citifluor) containing 4’,6-diamidino-2-phenylidole (DAPI).

Immunoblotting
Tissues and cells were homogenized in RIPA lysis buffer [0.1% SDS, 10 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 1 mM NaVO₄, 1 mM EDTA, 0.5% sodium-deoxycholate, protease inhibitor cocktail (Sigma)]. Extracts were processed using standard SDS-PAGE and immunoblotting procedures. X-ray films were scanned on a GS-800 calibrated densitometer (Bio-Rad), using Quantity One software (Bio-Rad). Only images containing bands with no saturated pixels, confirmed by the software detection system color coding saturated pixels in red, were used for subsequent quantification. Densitometry and quantification of the relative levels were carried out with ImageJ software.

Statistical analysis
Data show mean±s.e.m. Statistical significance was determined using a two-tailed Student’s t-test, when two groups were compared, or one-/two-way analysis of variance when multiple groups were compared. Significance was set at *P<0.05, **P<0.01, ***P<0.001.

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Competing interests
The authors declare no competing financial interests.

Author contributions

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Supplementary material
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