The Polarity Protein Pals1 Regulates Radial Sorting of Axons

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Myelin is essential for rapid and efficient action potential propagation in vertebrates. However, the molecular mechanisms regulating myelination remain incompletely characterized. For example, even before myelination begins in the PNS, Schwann cells must radially sort axons to form 1:1 associations. Schwann cells then ensheath and wrap axons, and establish polarized, subcellular domains, including apical and basolateral domains, paranodes, and Schmidt-Lanterman incisures. Intriguingly, polarity proteins, such as Pals1/Mpp5, are highly enriched in some of these domains, suggesting that they may regulate the polarity of Schwann cells and myelination. To test this, we generated mice with Schwann cells and oligodendrocytes that lack Pals1. During early development of the PNS, Pals1-deficient mice had impaired radial sorting of axons, delayed myelination, and reduced nerve conduction velocities. Although myelination and conduction velocities eventually recovered, polyaxonal myelination remained a prominent feature of adult Pals1-deficient nerves. Despite the enrichment of Pals1 at paranodes and incisures of control mice, nodes of Ranvier and paranodes were unaffected in Pals1-deficient mice, although we measured a significant increase in the number of incisures. As in other polarized cells, we found that Pals1 interacts with Par3 and loss of Pals1 reduced levels of Par3 in Schwann cells. In the CNS, loss of Pals1 affected neither myelination nor the establishment of polarized membrane domains. These results demonstrate that Schwann cells and oligodendrocytes use distinct mechanisms to control their polarity, and that radial sorting in the PNS is a key polarization event that requires Pals1.

Key words: axon; myelin; polarity; Schwann cell

Significance Statement

This paper reveals the role of the canonical polarity protein Pals1 in radial sorting of axons by Schwann cells. Radial sorting is essential for efficient and proper myelination and is disrupted in some types of congenital muscular dystrophy.

Introduction

During development of the PNS, myelinating Schwann cells sort and wrap axons in a myelin sheath to enable fast, efficient, and reliable action potential propagation. Although some of the factors that control myelination have been described (Michailov et al., 2004; Taveggia et al., 2005), the mechanisms regulating many other Schwann cell functions remain poorly understood. For example, how Schwann cells sort axons, initiate myelination, ex-

ecute the dramatic cytoskeletal rearrangements necessary for axon wrapping and myelin extension, and form distinct subcellular membrane domains remains poorly understood.

Many cell types with remarkable morphologies are polarized, with subcellular domains that are structurally, molecularly, and functionally unique. Recently, insights into the mechanisms of myelination have come from viewing it as yet another example of cellular polarization (Salzer, 2003; Masaki, 2012; Simons et al., 2012). In epithelial cells, a well-characterized model of polarity, three main complexes regulate polarization and junction formation: (1) the Par complex, consisting of Par3, Par6, and atypical protein kinase C (aPKC), provides the signaling complex that regulates formation of the apical domain. (2) The Scribble complex, consisting of Scribble, discs large1 (DLG1), and lethal giant larva (LGL), signals the formation of the basolateral domain. The Par and Scribble complexes locally inhibit each other, giving rise to cell polarity. (3) The Crumbs complex, consisting of Crumbs, protein associated with Lin-7 (Pals1/Mpp5), and Pals1 associated tight junction protein (PatJ), acts to anchor the Par complex to the membrane and to aid in junction formation (Straight et al., 2004; Assémat et al., 2008; Mellman and Nelson, 2008; Kim et al., 2010; Park et al., 2011). Although many canonical polarity pro-

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teins localize to an apical-like domain in Schwann cells (including Schmidt-Lanterman incisures (SLIs), paranodal loops, and adaxonal junctions) (Poliak et al., 2002; Ozçelik et al., 2010) and loss of Par3 inhibits myelination *in vitro* and *in vivo* (Chan et al., 2006; Tep et al., 2012), interactions among polarity proteins and the signaling pathways they regulate can vary depending on cell context and even species (McCaffrey and Macara, 2009). Thus, mechanisms of polarization must be identified and confirmed for each unique cell type, including myelinating glia.

Previously, efforts to define the role of polarity in myelination included silencing of Pals1 using lentiviral vectors to deliver Pals1 shRNA into postnatal sciatic nerve. These experiments suggested that Pals1 plays essential roles in regulating myelin sheath thickness and length (Ozçelik et al., 2010). However, postnatal viral transduction of Schwann cells and the use of shRNA limits the temporal precision and efficiency of Pals1 deletion. To circumvent these limitations and to determine the role of polarity and of Pals1 throughout PNS and CNS myelin development, we genetically disrupted Pals1 in myelinating glia. We found that Pals1-dependent polarization events play important roles in radial sorting of axons and myelination by Schwann cells, but that Pals1 is dispensable for CNS myelination by oligodendrocytes.

Materials and Methods

Animals. *Mpp5^{fl/fl}* mice, hereafter referred to as *Pals1^{fl/fl}*, were previously described (Kim et al., 2010). The *CNP-cre* mice were a generous gift from Dr. Klaus Nave (Lappe-Siefke et al., 2003). All experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. A minimum of three mice of either sex were used per experiment.

Antibodies. The primary antibodies used were as follows: chicken anti-βIV spectrin SD (Chang et al., 2010), rabbit anti-βIV spectrin SD (Yang et al., 2004), rabbit anti-Caspr (Schafer et al., 2004), rabbit anti-gliomedin (provided by Dr. Elmor Peles) (Eshed et al., 2005), rabbit anti-Nav1.6 (Schafer et al., 2006), rabbit anti-Kv1.2 (Zhang et al., 2013), chicken anti-pan neurofascin (AF3235, R&D Systems), sheep anti-Pals1 (AF7979, R&D Systems), mouse anti-actin (MAB1501, Millipore), mouse anti-Caspr (K65/35, NeuroMab), rabbit anti-ZO-1 (40-2200, Invitrogen), rabbit anti-Par3 (07-330, Millipore), and rabbit anti-P75-NTR (G323A, Promega). Secondary antibodies were purchased from Invitrogen or Jackson ImmunoResearch Laboratories.

Immunoblotting and immunoprecipitation. Sciatic nerves from P21 mice were homogenized with sonication in homogenization buffer (320 mM sucrose, 5 mM sodium phosphate, pH 7.2, 0.2 mM NaF, 0.2 mM Na₃VO₄, 1× PhosSTOP, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidin, and 0.5 mM PMSF). Homogenates were centrifuged at 700 × g for 10 min at 4°C; 20 μg of supernatant was boiled in reducing sample buffer and run on a 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membrane. Membranes were blocked with 20 mM Tris, pH 8.0, containing 4% (w/v) milk and 0.05% (v/v) Tween 20. Primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated for 1 h at room temperature. For immunoprecipitation, rabbit anti-Par3 and sheep anti-Pals1 were conjugated to protein-A agarose (Thermo Scientific) and protein-G Sepharose beads (GE Healthcare), respectively. Sciatic nerve homogenates were diluted to a final protein concentration of 1 mg/ml with lysis buffer (0.5% (final v/v) Triton X-100, 20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 10 mM Na₃N₃, 1× PhosSTOP, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidin, and 0.5 mM PMSF) and rotated for 1 h at 4°C. Lysates were spun at 13,000 × g at 4°C for 30 min. Supernatants were loaded on the beads and rotated overnight at 4°C. Beads were boiled in reducing sample buffer after 4 washes with lysis buffer to elute bound proteins.

Immunostaining. Immunostaining was performed as described previously (Ogawa et al., 2006). In brief, tissues were dissected, fixed in 4% PFA, cryoprotected in 20% sucrose, cryosectioned, and mounted on cov-

erslips. In some cases, nerves were fixed as above and individual axons were gently teased apart on coverslips with fine-tipped forceps. Tissues were blocked in 0.1 M sodium phosphate, pH 7.4, containing either 10% goat serum (Invitrogen) or 5% donkey serum (Equitech-Bio) and 0.3% Triton X-100. Primary antibodies were added overnight at 4°C. Tissues were washed, and secondary antibodies were added for ≥1 h. The coverslips were then washed, air dried, and mounted. All images were acquired using a Zeiss Imager Z1 fluorescence microscope fitted with a Zeiss AxioCam Mrm CCD camera (Carl Zeiss). Microscope objectives used in this study included 20× (0.8 NA) air, 40× (0.75 NA) air, and 63× (1.4 NA) oil objectives. Image analysis was performed using Zen software from Carl Zeiss or ImageJ (National Institutes of Health), and figures were cropped and assembled using Adobe Photoshop and Adobe Illustrator. For measurement of internodal lengths, multiple fields were merged in Adobe Photoshop.

Explant cultures. Coverslips were washed with 1N NaOH, 1N HCl, then 1N H₂SO₄ for 1 h each followed by three washes with milliQ water. Coverslips were autoclaved, cooled, dried, and coated with Matrigel (Corning) for 1 h. Coverslips were washed and stored in Minimal Essential Media containing 4 mg/ml glucose, 10% FBS, 1× Glutamax, 50 ng/ml NGF, and 1× penicillin/streptomycin. Postnatal day 0 pups were anesthetized on ice and decapitated. DRGs were removed and placed on coverslips. Cultures were then incubated at 37°C, 5% CO₂ for 24 h. Media was replaced with Neurobasal media containing 1× B27 supplement, 4 mg/ml glucose, 2 mM L-glutamine, 50 ng/ml NGF, and 1× penicillin/streptomycin. Neurobasal media was changed 2 d later. At 1–2 d later, cells were fixed in 4% PFA for 20 min on ice. All reagents were purchased from Invitrogen or Thermo Scientific unless otherwise indicated.

Transmission electron microscopy. Electron microscopy was performed as previously described (Chang et al., 2010). In brief, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) by intraperitoneal injection. Mice were then perfused with 2% PFA/2.5% glutaraldehyde in cacodylate, pH 7.4, then immersion fixed overnight at 4°C. Tissues were postfixed with 4% osmium tetroxide, dehydrated, and then embedded in Spurr's resin. Thick sections were cut at 1 μm on an RMC Ultramicrotome 6000×L and stained with toluidine blue. Thin sections were cut at <65 nm. Sections were stained with lead citrate followed by uranyl acetate. Images were acquired on a Hitachi H7500 or JOEL 1230 with 80 kV accelerating voltage and a Gatan US1000 high-resolution 4MP digital camera. Images were analyzed using ImageJ. All reagents were purchased from Electron Microscopy Sciences.

Compound action potential (CAP) recordings. CAP recordings were performed as described previously (Zhang et al., 2013). In brief, nerves were dissected and placed in oxygenated Locke's solution containing 1 mg/ml glucose. Nerves were drawn into suction electrodes, and increasing current was applied (Digitimer, model DS3) until a supramaximal threshold was reached. CAPs were amplified with a Warner DR-311 differential amplifier and converted using a Digidata 1440A and Clampex 10.2 software (Molecular Devices). Data were analyzed using Clampfit (Molecular Devices). CAP latency was the difference between stimulus onset and the time of maximal peak. Nerve length was measured using 20 mm × 20 mm microscope slide grids (Electron Microscopy Sciences). Conduction velocities were calculated by dividing nerve length by CAP latency.

Behavioral analysis. Mice were acclimated on the rotating rod (Ugo Basile) for 5 min at 4 rpm then allowed to rest for 1 h. Mice were then tested for latency to fall on the accelerating rod (4–40 rpm) for 5 min over three different trials with a minimum of 30 min breaks between trials. Latencies were averaged across trials.

qRT-PCR. qRT-PCR was performed as previously described (Chang et al., 2014). The primers used are as follows (from 5' to 3'): Pals1 (*Mpp5*) ex4-6 (forward: ACAGTACACATGAGCAAGG; reverse: GCTCTAGCTGCATTTCCTG), Pals1 (*Mpp5*) ex1-2 (forward: GAGGAATCGGGAGTTTCTG; reverse: CCTCTCCCAATATTACAGTTAG), Pals2 (*Mpp6*) (forward: GTGTGGATGAAATGTGGC; reverse: CGAGAATACGGATGGCATC), Pals3 (*Cask*) (forward: TGCGAAGGAATAAGCG; reverse: CGAAC TCTGGTCACATTCTC), and Polr2a (forward: CATCAAGAGAGTGCAGTTTC; reverse: CCATTAGTCCCCCAAGTTTG). Polr2a was used as

Figure 1. Pals1 is found at the axon–Schwann cell interface, paranodes, and SLI throughout development. **A**, Immunostaining of DRG explant cultures at 5 DIV using antibodies against Pals1 (green), neurofilament-M (NF-M, red), and Hoechst to label Schwann cell nuclei (blue). Arrowheads indicate the asymmetric position of the axon–Schwann cell interface. **B**, Immunostaining of DRG explant cultures at 5 DIV for Pals1 (green), Par3 (red), and nuclei are labeled using Hoechst (blue). Arrowheads indicate the asymmetric position of the axon/Schwann cell interface. **C–E**, Immunostaining of teased sciatic nerves from control *Pals1^{fl/fl}* mice. Myelinated axons were immunostained at P7 (**C**), P21 (**D**), and P120 (**E**) with antibodies against Pals1 (green), β IV spectrin (β IV, red), and pan-neurofascin (Nfasc, blue). Paranodes and SLIs are indicated by arrows and arrowheads, respectively. **F**, **G**, Immunostaining of teased sciatic nerves from control (**F**) and cKO (**G**) mice at P21 with antibodies against Pals1 (green), β IV spectrin (red), and pan-neurofascin (blue). **H**, Immunoblots of P21 sciatic nerves from control and cKO mice using antibodies against Pals1 and actin as a loading control. **I**, Rotarod analysis performed on control and cKO mice at P30 (mean $\pm$ SEM, Student's *t* test, $p = 0.23$, $n = 7$ mice). **J**, Average conduction velocities calculated from CAPs in P7, P21, and P160 sciatic nerves (mean $\pm$ SEM, one-way ANOVA followed by Bonferroni correction). *** $p = 5.5 \times 10^{-8}$. $n \geq 10$ nerves. **K**, Representative CAPs from P7 sciatic nerves of equal length show reduced conduction velocity and attenuation of the CAP in cKO mice. Scale bars, 10 μ m.

the internal control (Radonić et al., 2004). One-sample two-tailed *t* tests were used for statistical comparison of mRNA levels in WT and cKO tissues.

Statistical analysis. Statistical analyses were performed using Student's *t* test or one-way ANOVA followed by Bonferroni correction using OriginPro 9.1 software. Graphs were generated in OriginPro, and figures were assembled in Adobe Illustrator.

Results

Pals1 is highly polarized in Schwann cells

During early development, premyelinating Schwann cells associate with axons. The polarity protein Par3 and cytoskeletal scaffolding proteins α II and β II spectrin establish an asymmetric, or polarized, distribution at the interface of Schwann cell–axon contact (Chan et al., 2006; Susuki et al., 2011). To determine whether Pals1 also occupies a polarized distribution in immature Schwann cells, we immunostained DRG explant cultures using antibodies against Pals1. We found that Pals1 is asymmetrically localized to the initial contact site between the premyelinating Schwann cell and the axon (arrowheads) and that it colocalizes with Par3 (Fig. 1A,B). To determine whether Pals1 is also found in polarized domains of myelinating Schwann cells, we examined

Pals1 localization in sciatic nerve at postnatal day 7 (P7), P21, and P120. Consistent with previous reports of polarity protein distribution in PNS myelin (Poliak et al., 2002; Özçelik et al., 2010), we found that Pals1 is enriched at paranodes (arrows) and SLIs (arrowheads) at P7 and P21 (Fig. 1C,D). However, Pals1 immunoreactivity decreased after completion of myelination such that, in the adult sciatic nerve, Pals1 was no longer detected at paranodes or SLIs (Fig. 1E). Thus, Pals1 is highly polarized in premyelinating and actively myelinating Schwann cells, suggesting that it may play important roles during early development.

Loss of Pals1 causes radial sorting defects

To determine the function of Pals1 in myelination, we generated mice lacking Pals1 protein in Schwann cells and oligodendrocytes. To this end, we crossed mice with a floxed allele of *Mpp5*, the gene encoding Pals1 (referred to as *Pals1^{fl/fl}*) (Kim et al., 2010), with mice expressing Cre recombinase under the control of the 2', 3'-cyclic nucleotide phosphodiesterase (CNP) promoter (*CNP^{cre/+}*) (Lappe-Siefke et al., 2003); hereafter the conditional knock-out mice lacking Pals1 in myelinating glia will be

referred to as $CNP^{cre/+};Pals1^{fl/fl}$ or cKO mice. Immunostaining and Western blotting of sciatic nerves confirmed the loss of Pals1 from cKO mice (Fig. 1*F–H*). Specifically, cKO mice lacked Pals1 immunoreactivity in paranodes and SLIs (Fig. 1*G*). To determine whether cKO mice have gross motor dysfunction, we performed rotarod analysis on 1-month-old mice. cKO mice performed as well as littermate controls (Fig. 1*I*), suggesting little or no impairment in nervous system function at this age. Nevertheless, because neonatal mice cannot be tested for motor function on the rotarod, we also measured the CAP and calculated CAP conduction velocities in sciatic nerves of cKO and control mice at P7, P21, and P160. We measured a significantly attenuated CAP and slower conduction velocity in P7 cKO sciatic nerves (Fig. 1*J,K*). However, the CAP conduction velocities were indistinguishable by P21 (Fig. 1*J*).

What explains the reduced conduction velocity in the very young cKO mice? Although many things can cause a reduction in CAP amplitude and conduction velocity, the temporary nature of the reduction suggests a simple delay in myelination due to either delay in the number of axons myelinated and/or the rate at which a given axon is myelinated. To test these possibilities, we stained cross sections of resin-embedded sciatic nerves at different developmental time points with toluidine blue to visualize myelinated axons. Consistent with the notion that loss of Pals1 results in a delay in myelination, we found significantly fewer myelinated axons per field of view at P4, P7, and P21, but adult animals (e.g., P160) showed comparable myelination (Fig. 2*A,B*). If wrapping is delayed or impaired by loss of Pals1, myelin sheath thickness should be reduced. To test this possibility, we performed transmission electron microscopy of developing sciatic nerves (Fig. 3*A*). We then measured myelin thickness relative to axon caliber as G-ratios and found no significant differences in myelin thickness at any time point during development (Fig. 3*B*). Thus, Pals1 regulates events preceding myelination but is not necessary for the process of myelination itself.

In addition to the delay in myelination, we also observed large regions of developing nerve with bundles of axons devoid of myelin (Fig. 2*A*, asterisks). During early development, promyelinating Schwann cells surround and interact with bundles of axons; the largest of these axons are then selected from this bundle for myelination through a process termed radial sorting. Axons that are not myelinated (predominantly small-diameter axons) remain associated with nonmyelinating Schwann cells in Remak bundles (Salzer, 2012; Feltri et al., 2015). Although these nonmyelinating Schwann cells do not wrap small-diameter axons with myelin, they still wrap axons with a single cytoplasm-filled process (Fig. 4*A*; P21 and P160). To determine whether Pals1 regulates radial sorting, we used TEM to examine the bundles of unmyelinated axons in developing peripheral nerves of control and cKO mice (Fig. 4*A,B*). In cKO mice, we found the number of axons per bundle was significantly greater than littermate controls up to P21, but this difference resolved in adult animals (Fig. 4*A–C*). Furthermore, cKO bundles frequently contained unsorted large diameter axons ($>1\ \mu\text{m}$ diameter; Fig. 4*B*, asterisks). We also noted a clear deficit in the ability of nonmyelinating Schwann cells to segregate and ensheath axons compared with control mice (Fig. 4*A,B*, P21).

In adult cKO mice, approximately two-thirds of Remak bundles appeared normal with appropriate ensheathment of axons by nonmyelinating Schwann cells (Fig. 4*B*, P160). However, the remaining approximately one-third of bundles lacked Schwann cell processes surrounding each axon (Fig. 4*D*). Remarkably, some axon bundles were even myelinated (Fig. 4*E*). Indeed, these myelinated

Figure 2. PNS myelination is delayed. *A*, Cross sections of sciatic nerves were stained with toluidine blue. Asterisks indicate large areas devoid of myelinated axons. *B*, Quantification of myelinated axons per field of view (mean ± SEM, one-way ANOVA followed by Bonferroni correction). * $p = 0.014$. ** $p = 0.007$. $n \geq 3$ mice. Scale bars, 10 μm .

bundles, termed polyaxonal myelination, were a hallmark of the cKO nerves, and we found examples in every cKO older than P21, but never in control animals. Conditional heterozygotes had an intermediate phenotype with some bundles of unsorted axons and occasional instances of polyaxonal myelination at P21 (data not

shown), which is consistent with previous studies showing the gene-dosage dependence of Pals1 function (Kim et al., 2010). Interestingly, polyaxonal myelination has also been reported in other mouse models with disrupted radial sorting (Saito et al., 2003). Together, these results suggest that Pals1 regulates proper and efficient radial sorting of axons and development of Remak bundles.

Loss of Pals1 increases SLI frequency but does not affect paranodes

After myelination has begun, Pals1 is highly enriched at developing paranodes and SLIs (Fig. 1), suggesting that it may have other roles in addition to regulating radial sorting. Pals1-enriched structures include both axoglial and autotypic junctions. Because Pals1 facilitates junction formation in mammalian epithelial cells, we determined whether loss of Pals1 disrupts paranodes or SLIs by immunostaining sciatic nerve with anti-neurofascin (Nfasc) antibodies that robustly label these structures (Fig. 5*A,B*, arrows and arrowheads, respectively). We found that Pals1-deficient mice had a significant increase in the number of SLIs, and this difference persisted in adults (Fig. 5*B,C*). Consistent with a delay in myelination, internodal length was reduced at P21 but recovered in adults (Fig. 5*D*). We observed no change in the clustering of paranodal Nfasc (Fig. 5*A,B*, arrows). Nevertheless, to more carefully determine whether loss of Pals1 affects development of the axoglial or autotypic junctions found at paranodes, we immunostained sciatic nerves using antibodies against Caspr, an essential component of the paranodal junction (Bhat et al., 2001), and ZO-1, a component of autotypic junctions (Poliak et al., 2002). Immunostaining at P4, P21, and P120 showed no defects in either paranodal axoglial or autotypic junctions (Fig. 5*E,F*). Furthermore, TEM analysis of paranodes and SLIs showed no disruption in cKO mice (Fig. 5*G*). Immunostaining juxtaparanodal Kv1.2 channels showed that these channels were excluded from paranodal regions, indicating that the paranodal barrier is intact in cKO mice (Fig. 5*H*). Thus, loss of Pals1 affects the number of SLIs in the myelin sheath, but not paranode function or assembly. Last, we determined whether loss of Pals1 affects the localization of the canonical polarity protein, Par3. Intriguingly, immunostaining of teased sciatic nerve showed that Par3 localizes to both paranodes and SLI in control and cKO mice (Fig. 5*I*).

While examining node of Ranvier organization by immunostaining with antibodies against β IV spectrin (Fig. 5*E,F*), a key nodal cytoskeletal protein, we noticed frequent instances of β IV spectrin clusters that were elongated and had abnormal morphologies. Furthermore, these clusters were found only in regions that were labeled with anti-p75 neurotrophin receptor (p75-NTR) antibodies, a marker of the nonmyelinating Schwann cells that ensheath axon bundles (Fig. 6*A*) (Cosgaya

Figure 3. PNS myelin thickness is unaffected in Pals1 cKO. *A*, Electron microscopy of myelinated axons in the sciatic nerve. *B*, Scatter plots depicting G-ratios of control (black circles) and cKO mice (red dots). No significant differences in mean g-ratio were observed (one-way ANOVA followed by Bonferroni correction, $n = 3$ mice). Scale bars, 2 μ m.

et al., 2002). The aberrant clusters were observed at all ages in cKO mice but were never observed in control mice (Fig. 6*A*). Remarkably, other nodal proteins, including gliomedin, Nav 1.6, Nfasc, and ankG (Fig. 6*B*; ankG not shown), colocalized with β IV spectrin in these aberrant node-like clusters. Paranodal proteins frequently flanked the clusters on one edge, forming a heminode (Fig. 6*B*, arrows). These observations suggest that ectopic node-like clusters may form on axons within perturbed Remak structures.

Pals1 binds to Par3 in Schwann cells

Because Par3 localization did not change in the cKO sciatic nerve (Fig. 5*I*), we wondered whether Pals1 and Par3 interact in Schwann cells. To this end, we immunoprecipitated Par3 from P21 sciatic nerve lysates. We detected Pals1 in a protein complex with Par3 in control animals, but not cKO mice (Fig. 7*A*). Similarly, a 180 kDa isoform of Par3 could be detected by immunoblot after immunoprecipitation of Pals1 (Fig. 7*B*). Thus, Pals1 and Par3 interact in Schwann cells. Because Pals1 participates in the crumbs complex, which anchors Par3 to the membrane, we asked whether the loss of Pals1 affects the amount of Par3 protein. To test this, we performed immunoblots of lysates from P21 sciatic nerve. We found the amount of Par3 protein is reduced by $\sim 40\%$ in cKO mice (Fig. 7*C,D*).

Figure 4. Radial sorting is delayed in *Pals1* cKO. **A, B**, Electron microscopy of unmyelinated axons throughout development in both control and cKO mice. Unmyelinated axons $>1\ \mu\text{m}$ in diameter are marked with asterisks. High-magnification images of the boxed areas with axons and Schwann cell processes pseudocolored blue and red, respectively. **C**, Quantification of unmyelinated axons per bundle (mean $\pm$ SEM, one-way ANOVA followed by Bonferroni correction). ***p* = 0.0045 (P4); *p* = 0.0013 (P21). ****p* = 0.0009 (P7). *n* = 3 mice (minimum 10 bundles per animal). **D**, Some nonmyelinating Schwann cells do not ensheath each individual axon. **E**, Electron microscopy of cKO mice reveals polyaxonal myelination at various ages. Scale bars, $1\ \mu\text{m}$.

Thus, *Pals1* may regulate Schwann cell polarity through interactions with the Par complex.

Pals2, a *Pals1* paralog, is increased in cKO Schwann cells

The temporary delay in myelination, normal-appearing paranodes and SLIs, and the preservation of Par3 localization suggests that other, redundant mechanisms may contribute to polarity in Schwann cells and partially compensate for loss of *Pals1*. Consistent

with this idea, *Pals2* is expressed in Schwann cells and, like *Pals1*, localizes to SLIs and paranodes (Terada et al., 2012). To determine the expression level of *Pals2*, we performed qRT-PCR and found it modestly increased in the sciatic nerves of P21 cKO mice (Fig. 7E). Similarly, immunoblots of *Pals2* protein showed increased protein levels (Fig. 7F). Thus, elevated levels of *Pals2* may partially compensate for the loss of *Pals1*, especially at later developmental time points.

Figure 5. Loss of Pals1 increased SLI frequency, temporarily reduced internodal length, but did not affect paranodes. **A, B**, Immunostaining of teased sciatic nerves from control and cKO mice at P21 (**A**) and P120 (**B**) stained with antibodies against pan-neurofascin (green), β IV spectrin (red), and Hoechst (blue). Nodes and SLI are indicated by arrows and arrowheads, respectively. **C**, The number of SLI/mm in P21 and P120 mice (mean $\pm$ SEM, one-way ANOVA). ** $p = 0.0008$. * $p = 0.047$. $n = 3$ mice (50 cells per mouse). **D**, Quantification of internodal length (INL) in P21 and P120 mice (mean $\pm$ SEM, one-way ANOVA followed by Bonferroni correction). * $p = 0.0025$. $n = 3$ mice (50 cells per mouse). **E**, Immunostaining of longitudinal sections of sciatic nerves from control and cKO mice. Nerves stained with antibodies against Caspr (red) and β IV spectrin (green) at P4, P21, and P120. **F**, Immunostaining of teased sciatic nerves stained with antibodies against ZO-1 (red) and β IV spectrin (green) at P4, P21, and P120. **G**, Electron microscopy of longitudinal sections of P21 sciatic nerve showing paranodal loops (top) and SLIs (bottom) in cKO and control mice. **H**, Immunostaining of teased sciatic nerves from control and cKO mice at P21 stained with antibodies against β IV spectrin (green), Caspr (red), and Kv1.2 (blue). Dashed lines indicate the boundary between the juxtaparanode and paranode. **I**, Immunostaining of teased sciatic nerves from control and cKO mice at P21 stained with antibodies against Par3 (red) and either β IV spectrin or pan-neurofascin (green) to label nodes and SLI, respectively. Scale bars: **A, B**, 50 μ m; **E, F, H, I**, 10 μ m; **G**, 200 nm.

Pals1 is dispensable for CNS myelination

Given the clear role for Pals1 in the PNS, we next asked whether it is also important for CNS myelination. We performed TEM on optic nerves of cKO mice throughout development (Fig. 8A). We found that G-ratios and the percentage of myelinated axons for a given axon diameter were indistinguishable between cKO and control mice (Fig. 8B,C). Consistent with normal myelination, cKO mouse optic nerve CAP conduction velocities were indistinguishable from control mice (Fig. 8D). Furthermore, qRT-PCR analysis of P21 spinal cord showed no significant increase in the amount of either Pals2 or Pals3 transcripts (Fig. 8E). Like the PNS, paranodes were also normal. However, in contrast to the PNS, immunostaining of optic nerve for Pals1 showed no enrichment at paranodes (Fig. 8F). Thus, Pals1 is dispensable for CNS myelination.

Discussion

Myelinating glia are polarized and form specialized subcellular domains with specific functions. Here, we show that Pals1-dependent Schwann cell polarity regulates (1) efficient radial sorting of axons, (2) the frequency of SLIs, and (3) stability of Par3. Impaired radial sorting caused a delay in PNS myelination. However, Pals1 is not required for CNS myelination, suggesting that oligodendrocytes and Schwann cells have different mechanisms regulating their polarity. For example, whereas Schwann cells have a polarized abaxonal surface that participates in com-

Figure 6. Ectopic node-like structures form on unmyelinated axons. **A**, Immunostaining of longitudinal sections of sciatic nerve stained with antibodies against P75-Neurotrophin receptor (P75-NTR, red) and β IV spectrin (green). **B**, Immunostaining of longitudinal sections of sciatic nerve from cKO mice stained with antibodies against β IV spectrin (green), gliomedin, Nav1.6, pan-neurofascin, or Caspr (red). Arrows indicate heminodes. Scale bars, 10 μ m.

Figure 7. Pals1 interacts with Par3 in Schwann cells. **A**, Immunoblot for Pals1 following Par3 immunoprecipitation from P21 sciatic nerves. **B**, Immunoblot for Par3 following Pals1 immunoprecipitation from P21 sciatic nerves. **C**, Immunoblot for Par3 from P21 sciatic nerves. Actin was used as a loading control, and Pals1 is shown for comparison. **D**, Quantification of immunoblots from P21 sciatic nerve. Values were normalized to actin, then normalized to controls (mean $\pm$ SEM, Student's two-tailed t test, $n = 3$ mice, one pair of sciatic nerves each). * $p = 0.0165$. *** $p = 7.68 \times 10^{-4}$. **E**, qRT-PCR analysis of the genes encoding Pals1, Pals2, and Pals3 (mean $\pm$ SEM, Student's two-tailed t test). *** $p = 9.402 \times 10^{-6}$ (Pals1 exon 4); $p = 0.0003$ (Pals1 exon 1–2). * $p = 0.03$. $n = 4$ or 5 mice (one pair of sciatic nerves each). **F**, Pals2 immunoblot of sciatic nerves pooled from 3 mice. Actin was used as a loading control, and Pals1 is shown for comparison.

plex interactions with the basal lamina, oligodendrocytes do not. Remarkably, mice lacking laminin, or its receptors in Schwann cells (dystroglycan, $\alpha 6 \beta 1$ integrin, and $\alpha 7 \beta 1$ integrin), have defects in radial sorting and show polyaxonal myelination (Saito et al., 2003; Occhi et al., 2005; Wallquist et al., 2005; Pellegatta et al.,

2013). The similarities between the phenotypes of mice with impaired Schwann cell-basal lamina interactions and Pals1-cKO mice are striking and suggest they may participate in the same signaling pathways. Thus, we speculate that disruption of apical complex spatial regulation alters basolateral complex activity,

Figure 8. CNS myelination is unaffected by Pals1 cKO. **A**, Electron microscopy of myelinated axons in the optic nerve. **B**, Quantification of percentage myelinated axons by size (mean $\pm$ SEM, one-way ANOVA followed by Bonferroni correction, $n = 3$ mice). **C**, Scatter plots depicting G-ratios of control (black circles) and cKO (red dots) myelinated axons. No significant differences in average G-ratio were observed (one-way ANOVA followed by Bonferroni correction, $n = 3$ mice). **D**, Conduction velocities of optic nerves throughout development (mean $\pm$ SEM, one-way ANOVA followed by Bonferroni correction, $n \geq 8$ nerves). **E**, qRT-PCR analysis of the genes encoding Pals1, Pals2, and Pals3 from spinal cord (mean $\pm$ SEM, Student's two-tailed t test, $n = 4$ mice). ** $p = 0.0067$ (Pals1 exon 4). * $p = 0.025$ (Pals1 exon 1–2). **F**, Immunostaining of P21 optic nerve with antibodies against Caspr (red), Pals1 (green), and β IV spectrin (blue). Scale bars: **A**, 1 μ m; **F**, 10 μ m.

leading to perturbed Schwann cell-basal lamina interactions and disrupted radial sorting. Future experiments will be needed to define these connections.

Although Pals1 is enriched at paranodes and SLIs, we observed no disruption of these structures in the cKO mice. However, we did find more SLIs per internode. SLIs resemble the paranodal loops at the end of each myelinated segment and are thought to function as a pathway for trafficking of proteins across the myelin sheath and signaling between basolateral and apical compartments of the Schwann cell (Peters et al., 1976). Like Pals1, other polarity proteins, including Par3, are also found in SLIs (Poliak et al., 2002). However, the mechanisms controlling the number and location of SLIs along an internode remain

poorly characterized. Our observations suggest that Pals1 may directly regulate the number of SLIs per internode. Alternatively, the increased frequency of SLIs may simply be a secondary consequence of the transient delay in myelination. Determining the frequency of SLIs in mouse models with delayed myelination unrelated to loss of polarity proteins (e.g., *Lgi4* mutant mice) (Bermingham et al., 2006) may help distinguish among these possibilities. Dysregulation of basolateral elements in Pals1 cKO mice may also regulate SLI frequency. For example, *DLG1* interacts with *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) to act as a “brake” on myelination (Cotter et al., 2010). *PTEN* ablation leads to the formation of myelin outfoldings from SLIs and paranodes (Goebbels et al., 2012). Thus, de-

stabilization of the apical complex may promote a local increase in DLG1, leading to a local halt in myelin production and increased SLI formation.

Despite the lack of paranode disruption in Pals1 cKO mice, we frequently observed aberrant clusters of nodal proteins. These sometimes included heminodes (nodal protein clusters flanked on one side by paranodal junctions), but most often consisted of irregularly shaped aggregates of β IV spectrin, gliomedin, ankG, Nfasc, and Na^+ channels. We speculate that the heminodes arise from discontinuously myelinated axons: axons that in some regions are correctly sorted and myelinated, but in other regions are incorrectly sorted and associated with a nonmyelinating Schwann cell. In contrast, the mechanisms responsible for the abnormal clusters not associated with a flanking paranodal junction are more enigmatic. Because the clusters contain gliomedin, they may reflect excess gliomedin in these incorrectly sorted bundles of axons, or secreted gliomedin may have access to naked axons not properly ensheathed by nonmyelinating Schwann cells (e.g., Fig. 4D). Remarkably, laminin-deficient mice show discontinuously myelinated axons (Stirling, 1975) and virtually identical clusters of node-associated proteins without any overlying myelin (Deerinck et al., 1997). We speculate that mutant mice lacking Schwann cell laminin receptors may also have similar clusters.

Using lentiviral delivery of shRNA to silence expression of Pals1 in postnatal, myelinating Schwann cells, Özçelik et al. (2010) previously reported severe thinning of the myelin sheath and a drastic reduction in internodal length. In contrast, we report that PNS myelin thickness was unaffected and the reduction in internodal length was temporary in Pals1 cKO mice. What accounts for these differences? Viral transduction of Schwann cells and the use of shRNA may reduce the temporal precision and efficiency of Pals1 deletion. The difference in measured myelin thickness may reflect limitations in experimental methods: Özçelik et al. (2010) used confocal imaging, whereas we used much more sensitive TEM. Alternatively, while the cKO guarantees loss of Pals1 at precisely defined developmental stages, we found that the amount of Pals2 increased after Pals1 deletion. Pals1 expression decreases with increasing age, suggesting reduced dependence on Pals1 with increasing Schwann cell maturity. Thus, the different Schwann cell phenotypes may reflect compensatory changes that occur specifically in the Pals1 cKO and not in Schwann cells with viral knockdown of Pals1.

In many polarized cell types, Pals1 is central to establish polarized domains. Why does loss of Pals1 not have a more significant effect on myelination or paranode formation? As in several other mutants with loss of polarity proteins in myelinating glia (Beirowski et al., 2014; Pooya et al., 2014; Shen et al., 2014; Jarjour et al., 2015) (e.g., Par4 and Scribble), we observed a delay in myelination rather than frank arrest or disruption of myelin and its associated domain; the delay likely reflects impaired radial sorting. These observations suggest that, in myelinating glia, compensation or redundancy may frequently occur among some polarity complexes. This is consistent with the increase in Pals2 protein expression levels after Pals1 deletion. Thus, double knock-outs lacking both Pals1 and Pals2 may be necessary to determine their involvement in myelination. Alternatively, inducible conditional knock-outs may reveal the role of polarity in mature Schwann cells and bypass developmental compensation. Finally, because polarity proteins function in large complexes, removing multiple polarity proteins may be required before Schwann cell polarity is completely disrupted. For example, we showed that Pals1 interacts with Par3 in the sciatic nerve, suggest-

ing that Schwann cells use evolutionary conserved mechanisms of polarity. However, reduction in the amount of Par3 protein after Pals1 deletion did not disrupt myelination.

Oligodendrocytes express canonical polarity proteins, some of which have even been implicated in oligodendrocyte development (Binamé et al., 2013). Recently, RNA sequencing indicated that Pals1 expression peaks in newly formed oligodendrocytes and then decreases in myelinating oligodendrocytes (Zhang et al., 2014). Despite its expression in oligodendrocytes, Pals1 is not found at paranodes and does not appear to regulate myelination. That some polarity proteins may play important roles in the PNS, but not CNS, is further underscored by the observation that mice lacking the polarity protein, Par4, have a profound delay in PNS myelination, but no defect in CNS myelination (Shen et al., 2014). In contrast, the polarity protein Scribble regulates CNS myelin initiation, longitudinal extension, thickness, and paranode formation (Jarjour et al., 2015).

In conclusion, we show here that the polarity protein Pals1 plays important roles in regulating Schwann cell functions during early development. However, because Pals1 is dispensable for CNS myelination, oligodendrocytes and Schwann cells must have distinct requirements for polarity. We propose that future studies to determine the unique polarity complexes in Schwann cells and oligodendrocytes may yield important insights into how these cells develop their complex morphologies and specialized signaling domains, including myelin and paranodal junctions.

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