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Necl-4/Cadm4 recruits Par-3 to the Schwann cell adaxonal membrane*

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Abstract

Interactions between axons and Schwann cells are essential for the acquisition of Schwann cell radial and longitudinal polarity and myelin sheath assembly. In the internode, the largest of these longitudinal domains, axon-Schwann cell interactions are mediated by the Nectin-like (Necl) cell adhesion proteins, also known as SynCAMs or Cadms. In particular, Necl-1/Cadm3 expressed on the axon surface binds to Necl-4/Cadm4 expressed along the adaxonal membrane of myelinating Schwann cells. Necl-4 promotes myelination *in vitro* and is required for the timely onset of myelination and the fidelity of the organization of the myelin sheath and the internode *in vivo*. A key question is the identity of the downstream effectors of Necl-4 that mediate its effects. The cytoplasmic terminal region (CTR) of Necl-4 contains a PDZ-domain binding motif. Accordingly, we used the CTR of Necl-4 in an unbiased proteomic screen of PDZ-domain proteins. We identify Par-3, a multi-PDZ domain containing protein of the Par-aPKC polarity complex previously implicated in myelination, as an interacting protein. Necl-4 and Par-3 are colocalized along the inner Schwann cell membrane and coprecipitate from Schwann cell lysates. The CTR of Necl-4 binds to the first PDZ domain of Par-3 thereby recruiting Par-3 to sites of Necl-4/Necl-1 interaction. Knockdown of Necl-4 perturbs Par-3 localization to the inner membrane of Schwann cells in myelinating co-cultures. These findings implicate interactions of Necl-1/Necl-4 in the

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Main Points: We identify Par3, a major component of a key polarity complex, as an interacting partner of Necl-4/Cadm4. Interactions of Necl-4 on the Schwann cell with Necl-1 on the axons recruit Par3 to the inner Schwann cell membrane, promoting radial polarity.

recruitment of Par-3 to the Schwann cell adaxonal membrane and the establishment of Schwann cell radial polarity.

Keywords

Schwann cell; axon; polarity; cell adhesion; myelin

Introduction

Myelinated fibers form as the result of reciprocal interactions between axons and myelinating glial cells, i.e. Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system (CNS). In the PNS, axons promote Schwann cell proliferation and differentiation, most strikingly the formation of the compact myelin sheath that enwraps axons (Monk et al. 2015; Salzer 2015). Schwann cells, in turn, organize the axon into a series of specialized domains, each with a distinct molecular composition and physiological function (Salzer et al. 2008; Zollinger et al. 2015a). Among these domains are the node of Ranvier and the flanking paranodes and juxtaparanodes. These domains contain different sets of protein complexes composed of cell adhesion molecules (CAMs) and ion channels on the axon that interact with their cognate CAMs on the apposed glial processes. These complexes on both the axon and glial cell bind to adapter proteins that stabilize and link these complexes to cytoskeletal proteins just under the plasma membrane (Zhang and Rasband 2016). Although critical to the function of myelinated axons in saltatory conduction, these nodal region domains only account for a few percent of the longitudinal length of myelinated axons.

The largest domain of myelinated axons by far is the internode, the portion of the axon and glial membrane located under the compact myelin sheath. The plasma membranes of the axon and myelinating glia are closely apposed and uniformly separated in this region. Among the proteins that mediate axon-glial interactions along the internode are members of the Necl cell adhesion proteins (Maurel et al. 2007; Spiegel et al. 2007). The Necl proteins are single pass transmembrane proteins that contain three Ig-like domains in their extracellular segment and a short cytoplasmic tail. They have been implicated in a variety of biological activities, including cell adhesion, regulation of cell growth, synaptic function and cell polarity (Biederer et al. 2002; Miyoshi and Takai 2007; Ogita and Takai 2006; Sakisaka and Takai 2004). The Necls are thought to function as homodimers (Miyoshi and Takai 2007; Ogita and Takai 2006), with the two N-terminal Ig-like domains mediating heterophilic interactions (Fogel et al. 2007). The intracellular domain (ICD) of the Necl proteins contains a protein 4.1-ezrin-radixin-moesin (FERM)-binding domain in their juxtamembrane region, and a class II PSD-95/Dlg/ZO-1 (PDZ)-binding sequence at their C terminus.

The Necls are strikingly localized to and highly expressed along the internode. Necl-1 and Necl-2 are expressed by the axon whereas Necl-4 and Necl-2 are expressed by Schwann cells on the periaxonal membrane, i.e. the inner Schwann cell membrane that apposes the axon. All three Necl proteins are also expressed in the Schmidt-Lanterman incisures (SLI),

periodic sites of non-compacted membrane distributed along the myelin sheath. Axonal Necl-1 and Schwann cell Necl-4 mediate robust heterophilic binding along the internode and promote myelination *in vitro* (Fogel et al. 2007; Maurel et al. 2007; Spiegel et al. 2007). While Necl-4 is not essential for myelination based on knockout mouse models (Golan et al. 2013; Zhu et al. 2013), it is required for proper axon-Schwann cell adhesion, myelin thickness and the organization of the internodal domains (Golan et al. 2013).

The mechanisms by which Necl-4 in Schwann cells mediates these effects are not understood. The IDC domain of Necl-4 has a key role in its functions: over-expression of the IDC in Schwann cells inhibits myelination *in vitro* (Spiegel et al. 2007) and selective loss of the IDC domain *in vivo* recapitulates the phenotype of Necl-4 knockout (Golan et al. 2013). These results suggest that interactions mediated by the FERM- and/or PDZ-binding domains of the Necl-4 IDC mediate important functions of Necl-4 in axon-myelinating Schwann cell interactions (Spiegel et al. 2007). The FERM binding domain is considered to interact with 4.1G, the major 4.1 protein expressed by Schwann cells, which co-localizes and functionally interacts with Necl-4 in both the incisures and along the internode (Einheber et al. 2013; Ivanovic et al. 2012). The protein(s) that interact with the Necl-4 PDZ-binding domain, and how they may promote myelination, however have not been known.

Accordingly, we carried out a solid phase binding study to identify PDZ domain proteins that interact with the C-terminus of Necl-4. We identified a prospective interaction between Necl-4 and Partitioning-defective 3 (Par-3), a multi-PDZ domain containing protein of the Par- atypical protein kinase C (aPKC) polarity complex. Prior studies had shown that Par-3 localizes to the adaxonal Schwann cell membrane upon axonal contact and implicated it promoting myelination (Chan et al. 2006; Tep et al. 2012), notably in the timing of its onset (Blasky et al. 2014). We now show that Par-3 is co-expressed with Necl-4 at the adaxonal Schwann cell membrane along the internode (Chan et al. 2006) and in the Schmidt-Lanterman incisures (SLI) (Ozcelik et al. 2010; Poliak et al. 2002). We further report that Necl-4 recruits Par-3 to the membrane upon heterophilic binding to Necl-1; this results in the accumulation of Par-3 to the adaxonal membrane of the Schwann cell. This recruitment is mediated by the PDZ-binding domain of Necl-4 interacting with the PDZ1 domain of Par-3. Taken together, these results suggest that upon binding to axonal Necl-1, Necl-4 helps establish Schwann cell polarity by localizing Par-3 to the inner membrane of myelinating Schwann cells.

EXPERIMENTAL PROCEDURES

Screening of PDZ domain interactions-

A fluorescently labeled peptide corresponding to the C-terminal 10 amino acids of Necl-4, e.g. GHKRKEEFFI, was used to probe microarrays containing 289 PDZ domains. As previously described (Stiffler et al. 2007), PDZ domains were prepared within individual wells of microtiter plates and probed, in triplicate, with a 1 μ M solution of the Necl-4 probe. A 3-fold increase in the median fluorescence on the array was scored as an array positive and further analyzed by a solution-phase, fluorescence polarization (FP) assay at varying concentrations of each PDZ domain; dissociation constants (K_d) of < 100 μ M were considered as as FP positives.

Preparation of teased sciatic nerves-

Sciatic nerves were obtained from freshly sacrificed 8-month-old WT mice and fixed with ice cold 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in 0.1M phosphate buffer pH 7.4 (PB) and dH₂O for 30 minutes, washed 3 times with 0.1M PB, and stored at 4°C until teased. Teased sciatic nerve fibers were mounted on Super Frost Plus slides (Fisher) and dried overnight at room temperature, and stored at -80°C until used for immunofluorescence staining. Slides were permeabilized with 100% acetone for 10 min at RT before staining.

Tissue Culture Methods-

Establishment of primary rat Schwann cell and dorsal root ganglion (DRG) neuron cultures has been described previously (Einheber et al. 1997). To summarize, neurons were isolated from embryonic day 16 DRGs by trypsinization and plated on Matrigel (BD Biosciences) coated 12mm glass coverslips in neurobasal medium (Invitrogen) supplemented with 2% B27 supplement (Invitrogen), and 50ng/ml NGF (Harlan). Nonneuronal cells were removed by alternating between standard neuronal medium supplemented or not supplemented with 5-fluorodeoxyuridine and uridine (FdUR/U) (Sigma-Aldrich) every 2 days for 10 days, and then maintained another 6 days in absence of FdUR/U until DRG axons filled the coverslips. Schwann cells prepared from postnatal day 2 sciatic nerves were expanded in DMEM (Bio-Whittaker), 10% FBS (Invitrogen), 2mM L-glutamine (Invitrogen), 4μM forskolin (Sigma-Aldrich) and 5 ng/ml EGF domain of rhNRG-1-β1 (R&D Systems), until used. Myelinating Schwann cell-neuron co-cultures are established by seeding purified DRG neuron cultures with 200,000 Schwann cells in Minimal Essential Medium (MEM) (Invitrogen) supplemented with 10% FBS, 2mM L-glutamine, 0.4% glucose and 50 ng/ml NGF. After 2 days, myelination was initiated by supplementing the media with 50 μg/ml ascorbic acid (Sigma-Aldrich), and cultures were maintained for 1 week.

Antibodies and immunofluorescence-

Guinea pig polyclonal antibodies included the anti-Necl-1 gp1733 and anti-Necl-4 gp1734 (Maurel et al. 2007). Rabbit polyclonal antibodies included anti-Par-3 (Millipore) and anti-GFP (Invitrogen). Mouse monoclonals included antibodies against anti-HA (Covance), anti-V5 (Invitrogen) and anti-FLAG M2 (Sigma-Aldrich). Secondary antibodies include IRDye-680CW-conjugated goat anti-rabbit and goat anti-mouse (LI-COR Biosciences), IRDye-800CW-conjugated goat anti-rabbit and goat anti-mouse (LI-COR Biosciences), rhodamine-X-conjugated donkey anti-rabbit, donkey anti-guinea pig and donkey anti-mouse, FITC-conjugated donkey anti-rabbit, donkey anti-guinea pig and donkey anti-mouse, Cy5-conjugated donkey anti-guinea pig, goat anti-mouse IgG Fc_γ Subclass 2a specific, FITC-conjugated goat anti-mouse IgG Fc_γ Subclass 1 specific (Jackson ImmunoResearch).

Cultures were fixed with 4% PFA for 10 min, washed with dPBS, permeabilized in either 100% methanol or 100% acetone, washed with dPBS, and incubated for at least 1 hr at RT in blocking solution consisting of dPBS, 5% BSA, 1% normal donkey serum, and 0.2% Triton X-100 (Sigma-Aldrich). Coverslips were incubated overnight at 4°C in a humidifying chamber with primary antibodies diluted in the blocking solution, washed with dPBS, and incubated with corresponding secondary antibodies diluted in blocking solution for 1 hr at RT. Coverslips were washed twice with dPBS and once with dH₂O, and mounted with

Fluoromount-G (Southern Biotechnology Associates) on Super Frost Plus slides (Fisher) and sealed with clear nail polish.

Immunofluorescence preparations were examined by epifluorescence on a LSM510 confocal microscope (Carl Zeiss MicroImaging, Inc.) or Eclipse Ti scope (Nikon Instruments, Inc.). Images were acquired with Neofluor 40× NA 1.3 oil or Apochromat 63× NA 1.4 oil objectives (Carl Zeiss MicroImaging, Inc.) on an 8-bit photomultiplier tube (Carl Zeiss MicroImaging, Inc.) using LSM software (Carl Zeiss MicroImaging, Inc.). Images were also acquired with Plan Aproximat Objective 20X or 40X CFI60 objectives (Nikon Instruments, Inc.) on Clara Interline CCD camera (Andor Technology) using NIS elements software (Nikon Instruments, Inc.). In some cases, brightness and contrast were adjusted with Photoshop CS4 (Adobe).

Co-Immunoprecipitation (coIP) –

V5 tagged-full length Necl-4 or V5 tagged-Necl-4 PDZbd were expressed via lentivirus in 10 cm Schwann cell monocultures for 3 days. Cell cultures were washed 3 times in ice-cold PBS then lysed in 1 mL of Lysis Buffer (20mM Tris/HCl pH7.4, 2.5mM EDTA, 2.5mM EGTA, 1% NP40, 10% Glycerol with a cocktail of protease inhibitors [Roche]). Nucleic acids and cell debris were pelleted by centrifugation (16,000×g; 10min) and the supernatant collected for pre-clearing for 3 hours (room temperature) with 50 µL agarose-Protein G beads (Thermo-Pierce). 1/10th volume (100µL) of pre-cleared supernatant was collected as 'Input' lysate (Fig. 4), and the remaining lysate was incubated overnight with 5 µg V5 antibody (GenScript) and 50µL agarose-Protein G beads. The following day, agarose-Protein G beads were pelleted by centrifugation (3mins, 2500×g) and washed 5 times in 1 mL chilled Lysis Buffer. Laemmli buffer + dithiothreitol (DTT) were added to 'Input' and 'IP' samples before heating (70° C for 10 mins). Beads were pelleted and beads and supernatant were collected and subjected to SDS-PAGE separation followed by Western blotting with V5 (1:1000) and Par3 (Clontech; 1:500) antibodies.

Immunoblotting-

Transfected CHO cells were lysed in 95mM NaCl, 25mM Tris buffer pH 7.5, 10mM EDTA, 2% SDS, supplemented with a protease inhibitor cocktail (Roche). Lysates were vortexed, boiled for 10 minutes at 100°C and cleared by centrifugation at 18,000× g for 10 min. Protein concentrations were determined by the BCA method (Thermo Scientific). To facilitate detection of Necl proteins, protein lysates were deglycosylated with PNGase F (New England BioLabs). Twenty micrograms of proteins were fractionated by SDS-PAGE (7.5%, 10%, 15%), and transferred onto PROTRAN BA85 Nitrocellulose (Whatman). The nitrocellulose was stained with Ponceau S Solution (Sigma) and the appropriate regions of the blots were cut and probed using antibodies mentioned above. Proteins were visualized with IRDye secondary antibodies and the intensity of the detected bands was quantified by measuring the integrated intensity using the Odyssey Imaging System (LI-COR Biosciences).

Generation of Domain Deletion Constructs –

The PDZ-binding domain of Necl-4 (Necl-4- PDZ) was deleted from the codon-modified pLenti6/V5-Necl-4 construct (Maurel et al. 2007) using the QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies) with the following primers: sense (CGGCGGCGATGGACACAAACGGAAATGAAAGGGCTCGAG) and antisense (CTCGAGCCCTTTCATTTCCGTTTGTGTCCATCGCCGCCG). Each of the three PDZ domains of Par-3 was deleted from pEGFP-C2-mPar3 (Shi et al. 2003) using the Phusion Site-Directed Mutagenesis Kit (Finnzymes). The mPar-3- PDZ1 construct has deletion of amino acids (AA) 271–359 of mPar-3 using sense (GCAGCGAACAAGGAGCAGTATGAA) and antisense (ATCATCGAGGGAAAAGTTGGGCATA) primers. The mPar-3- PDZ2 construct has deletion of AA 461–546 of mPar-3 using sense (CAAGAAGAGGCTTTCACCCAAGG) and antisense (GAGCCTCTTGCTACTCTCTTCGTG) primers. The mPar-3- PDZ3 construct has deletion of AA 590–673 of mPar-3 using sense (ATCAGCAGATGTAACGAGTTGCGGT) and antisense (GAAAGTCAGAACTCCCTGGTACCGTC) primers.

Chinese Hamster Ovary (CHO) Cell Mixing Experiments-

CHO cells expressing HA-tagged Necl-1 cells were obtained from expanding frozen stock previously generated in the lab (Maurel et al. 2007). CHO cells were transfected with the V5-Necl-4 or V5-Necl-4 PDZ construct using LipoD293 (SigmaGen Laboratories). CHO/HA-Necl-1 and CHO/V5-Necl-4 or CHO/V5-Necl-4 PDZ cells were counted, mixed and plated on Poly-L-lysine (Sigma-Aldrich) coated 12mm glass coverslips (Carolina) in 4-well Nunc plates (Thermo Scientific). The mixture of CHO cells was allowed to grow until cell-cell contact was established, then fixed with 4% PFA and imaged by immunofluorescence staining.

Antibody Clustering Experiments-

CHO cells or Schwann cells on 12mm glass coverslips (Carolina) were washed with Leibovitz's L-15 (Gibco) for 5 min, incubated with primary antibody (mouse anti-V5) diluted in 10% Bovine Serum Albumin (BSA) IgG-Free, Protease-Free (Jackson ImmunoResearch) in L-15, for 45 min at RT. Cells are then washed with L-15 and incubated with second antibody (anti-mouse) diluted in 10% BSA in L-15 for 2.5hr at 37°C in a humidified chamber. Cells are then washed with L-15, PBS and fixed with 4% PFA and imaged by immunofluorescence staining.

Quantification of V5/GFP Co-Clusters-

20X images were taken at regular intervals (0.5mm) across each coverslip using the Eclipse Ti (Nikon Instruments, Inc.). After the pictures were captured, the images were randomized and renamed so the quantification was done in a blinded fashion. Each cell was accessed for the presence or absence of GFP clusters co-localizing with V5 clusters.

Statistical Methods-

All studies were done a minimum of three separate iterations and typically more. The Student's t-tests performed were two-tailed (non-directional) and computed using Microsoft Excel.

RESULTS

Identification of Par-3 as a candidate to interact with Necl-4 at the internode and Schmidt-Lanterman incisures

To identify proteins that interact with Necl-4 via the class II PDZ-binding sequence at its C-terminus, we used quantitative protein microarray methods to interrogate a genome-wide collection of PDZ protein domains. A similar strategy was used previously to establish networks of protein-protein interactions (Stiffler et al. 2007). We used the fluorescently labeled PDZ-binding domain of Necl-4 in a solid phase assay to arrays of PDZ domains; positive hits were reprobed in a solution-phase fluorescence polarization assay. We identified a number of PDZ domain-containing proteins as potential interactors with Necl-4 based on both solid and solution phase assays (Table 1). Among these was Par-3, a multi-PDZ domain containing protein of the Par-aPKC polarity complex (Suzuki and Ohno 2006).

We focused on Par-3 as a potential interactor due to its proposed role in promoting myelination. In addition, Par-3 is concentrated along the periaxonal membrane at the onset of myelination (Chan et al. 2006; Lewallen et al. 2011; Susuki et al. 2011) and, subsequently, stably expressed in clefts (Poliak et al. 2002), which are sites also highly enriched in Necl-4 (Maurel et al. 2007; Ozcelik et al. 2010; Spiegel et al. 2007). Consistent with these reports, Necl-4 and Par-3 co-localize in the periaxonal glial membrane along the internode (arrow) and in the SLIs (arrowhead) of adult teased sciatic nerve fibers (Fig. 1).

Necl-1/Necl-4 interactions recruit Par-3 to the membrane

The solid-phase binding studies and co-localization in the same membrane compartments suggest that Necl-4 directly interacts with and may recruit Par-3 to these Schwann cell sites, notably to the adaxonal membrane. To examine this possibility further, we carried out mixing experiments in which Chinese hamster ovary (CHO) cells expressing Necl-1 were cocultured with CHO cells expressing full-length Necl-4; cultures were then stained and assessed for recruitment of Necls and endogenous Par-3 to sites of cell contact. Par-3 is present at low levels at the membrane of CHO cells (data not shown). However, when Necl-4 expressing cells (red, Fig. 2A) contacted Necl-1 expressors (unstained, Fig. 2A), there was a dramatic recruitment of endogenous Par-3 to sites of heterophilic Necl interaction (orange in merged images indicated by arrows, Fig. 2A). There was also a corresponding depletion of Par-3 from the cytoplasm of the Necl-4 expressing cells, several of which are indicated by asterisks (Fig. 2A), but not from the cytoplasm of Necl-1 expressors. These results strongly suggest that Par-3 specifically interacts with the Necl-4 cytoplasmic domain and that this interaction is dependent on binding of Necl-4 to Necl-1. No significant accumulation of Necl proteins or of Par-3 was observed at sites of homophilic contact between Necl-1 cells or between Necl-4 expressing cells (Fig. 2A). To visualize both Necl-1 and Necl-4 cells directly, we added distinct N-terminal epitope tags, expressed these

constructs in CHO cells and performed similar mixing experiments (Fig. 2B); these results again demonstrate an exquisite recruitment of Par-3 just to sites of heterophilic interactions.

We next addressed whether Necl-4 is necessary for Par-3 recruitment to the adaxonal membrane of Schwann cells. To this end, we examined the localization of Par-3 in Schwann cells cocultured with sensory neurons whose axons are known to express Necl-1 on their surface (Maurel et al. 2007; Spiegel et al. 2007). We examined the localization in non-infected Schwann cells and in Schwann cells in which Necl-4 was knocked down with an shRNA encoded by a lentivirus (Maurel et al. 2007); a scrambled shRNA served as a control for non-specific effects of lentiviral infection. Viral infection of the subset of Schwann cells was evidenced by GFP expression (Fig. 2C). As expected, Par-3 was concentrated in the periaxonal membrane of a subset of Schwann cells at the onset of myelination (asterisks, Fig 2C control). This asymmetric, periaxonal Par-3 localization was present in ~ 10% of Schwann cells infected with scrambled shRNA but in only ~ 2% of cells infected with a lentivirus expressing shRNA to Necl-4. Quantification of these results is shown in Fig. 2D. These results demonstrate that upon contact between Necl-1 on the axon and Necl-4 on the Schwann cell, Par-3 is localized to the sites of interaction, and that this recruitment depends in part, on the presence of Necl-4.

The PDZ-binding domain of Necl-4 is required for recruitment and binding to Par-3

The cytoplasmic segment of Necl-4 contains a C terminal PDZ-binding domain that likely mediates interactions with the PDZ domains of Par-3 (Table 1). To test whether the PDZ-binding domain of Necl-4 is indeed required for the recruitment of Par-3, we generated a V5-tagged construct (Maurel et al. 2007) lacking this C-terminal binding sequence, i.e. Necl-4- PDZ (Fig. 3, A and B). CHO cells expressing Necl-1 were mixed with CHO cells expressing either wild type Necl-4 or the Necl-4- PDZ construct and allowed to grow to confluency. Sites of heterophilic contact between Necl-1 and both of the Necl-4 constructs demonstrated a dramatic recruitment of the Necls (Fig. 3), indicating that the C-terminus of Necl-4 is not essential for its adhesive function. While the accumulation of the Necl-4- PDZ construct at heterophilic contact sites was comparable to wt Necl-4, recruitment of Par-3 to these sites was abolished (Fig. 3C). This result demonstrates that the PDZ-binding domain of Necl-4 is essential for the recruitment of Par-3 to the sites of interaction between Necl-4 and Necl-1.

To assess the requirement for the PDZ-binding domain further, we expressed V5-tagged wild type Necl-4 or Necl-4- PDZ constructs in Schwann cell cultures; such cultures express limited amounts of Necl-4 when grown without neurons (Maurel et al. 2007). We specifically pulled down these constructs using their V5-tag and asked if Par-3 was coprecipitated or not (Fig. 4A, quantified in Fig. 4B). Consistent with the requirement of the PDZ-binding domain, Necl-4 but not Necl-4- PDZ was able to much more efficiently co-immunoprecipitate endogenous Par-3 in Schwann cells.

The first PDZ domain of Par-3 mediates interactions with Necl-4

Results from the microarray analysis suggest that PDZ domains in Par-3 interact with the C-terminus of Necl-4. Thus, interactions were observed for protein constructs containing all

three domains expressed in tandem (PAR3 PDZ 1+2+3), and for the third domain expressed alone (PAR3 PDZ3) (Table 1). The interaction with the third domain was further confirmed by fluorescence polarization. Unfortunately, purified recombinant proteins corresponding to either the first or the second PDZ domains alone could not be purified to sufficient quality for use on the microarrays. Thus, to examine which of the three PDZ domain of Par-3 binds to Necl-4 under more physiological conditions, we analyzed interactions of Necl-4 with single PDZ domain constructs. We assessed interactions in CHO cells co-transfected with V5-Necl-4 and flag-tagged full length or individual FLAG-Par-3 PDZ domain constructs (Fig. 5A). Necl-4 was clustered with anti-V5 antibodies. Cultures were then fixed, stained, and scored for the extent of Necl-4 co-clustering with each PDZ domain, as well as the endogenous Par-3 (red; Fig. 5B). As expected, the full-length Par-3 construct and endogenous Par-3 robustly co-clustered with Necl-4. Both PDZ1 and PDZ3 also co-clustered with Necl-4, although PDZ1 clustering was more consistent than was PDZ3 (Fig. 5B, insets). PDZ2 did not co-cluster at all. These results indicate that the Par-3 PDZ1 domain is sufficient for recruitment by Necl-4.

To identify which PDZ domain(s) of Par-3 is necessary for recruitment by Necl-4, we deleted each of the Par-3 PDZ domains in turn (Fig. 6A and B). As in the experiments above, we performed antibody clustering experiments using CHO cells co-transfected with the V5-Necl-4 construct and either full length (FL) EGFP-mPar3 or EGFP-mPar3 PDZ1, PDZ2, PDZ3 deletion constructs (Fig. 6C). We observed that mPar3 FL and each of the single PDZ deletion constructs all displayed some degree of mutant Par-3 clustering. This is in potential agreement with the ability of Necl-4 to interact strongly with PDZ domain 1 and modestly with domain 3 (Fig. 5). Quantitation of the size of the clusters revealed that both the FL and PDZ2 constructs had comparably large V5-Necl-4/EGFP-Par3 clusters. In contrast, when PDZ1 was deleted, there was a large decrease in the ability of Necl-4 to recruit Par-3, with the majority of cells showing clusters of Necl-4 but no accompanying clusters of the mutant construct. Deleting the Par-3 PDZ3 domain had a more modest effect on Necl-4 clustering of Par-3, falling between the effects of the PDZ1 mutant and the FL constructs. Quantification of several co-clustering experiments, scored in a blinded fashion, is shown in Fig. 6D. These results indicate that the PDZ2 domain does not play a role in the interaction with Necl-4 and suggest that PDZ1 is the major, although not exclusive interacting domain. We also tested the EGFP-mPar3-delC construct (Shi et al. 2003), a C terminal deletion of mPar-3 that leaves the three PDZ domains intact but disrupts the aPKC-binding domain. Necl-4 still recruited this Par-3-delC mutant construct to the membrane demonstrating that the aPKC-binding domain and C-terminus do not mediate interactions with Necl-4 (Fig. 6C).

DISCUSSION

Reciprocal interactions between axons and Schwann cells are essential for driving myelination and the polarized organization of the axon/myelin unit. Necl-4, which is expressed by Schwann cells, is an important mediator of these interactions along the internode. Here, we have used an unbiased proteomic screen to identify proteins that bind to its cytoplasmic domain and are thus candidates to mediate its effects during myelination. We identified Par-3 in this screen and corroborate its interaction with Necl-4 via co-precipitation

and co-recruitment studies. These studies establish a mechanism by which axo-glial interactions recruit Par-3 to the adaxonal membrane to promote radial Schwann cell polarity. These findings, and their implications for myelination, are considered further below.

Necl-4 interactions contribute to localization of Par-3

Necl-4 and Par-3 are strikingly co-localized at the adaxonal membrane of myelinating Schwann cells, accumulating with the onset of myelination. Our findings suggest that Necl-4 is both necessary and sufficient for this recruitment of Par-3. Loss of Necl-4 in Schwann cells at the onset of myelination disrupts the asymmetrical localization of Par-3 to the adaxonal membrane (Fig. 2). In contrast, Par-3 strikingly accumulates at Necl-1/Necl-4 contact sites in CHO cells (Fig. 3C), which models the binding of Necl-1 on axons to Necl-4 in the adaxonal of Schwann cell membrane. Par-3 also accumulated when Necl-4 was clustered either by antibodies against its ectodomain (Figs. 4B, 5C) or upon binding to a pre-clustered Necl-1-Fc construct (data not shown). Potentially, Par-3 constitutively associates and redistributes with Necl-4 to sites of heterophilic interactions. Alternatively, and more likely, Par-3 recruitment is enhanced by binding of Necl-1 to Necl-4 potentially due to clustering of the latter protein. This possibility is consistent with the clearance of endogenous Par-3 from the cytoplasm of Necl-4 expressing cells (Fig. 2A, asterisks) and its accumulation at sites where Necl-4 is bound to Necl-1 (Fig. 2B) suggesting redistribution to sites of interaction.

We found the C-terminal PDZ-binding domain of Necl-4 is required for and binds preferentially to the first PDZ domain of Par-3 based on coprecipitation and colocalization assays, respectively. As the PDZ binding sequences in the CTR of the four Necl proteins, are identical (Maurel et al. 2007), they likely bind to a common set of proteins. In agreement, Necl-2 was previously shown to interact with Par-3. Thus, in the testes, a tertiary complex composed of Necl-2, Par-3 and junctional adhesion molecule C (JAM-C) at the spermatid-Sertoli cell junction is necessary for spermatid differentiation (Fujita et al. 2007). In these studies, Necl-2 was also shown to interact preferentially with the PDZ1 domain of Par-3 (Fujita et al. 2007). These findings suggest that Necl-2, which is likewise concentrated with Necl-4 along the adaxonal membrane and in the incisures, may cooperate in the recruitment of Par-3 to these sites.

Par-3 is a multimodular scaffolding protein and interacts with many other membrane receptors as well as cell polarity regulators (Ebnet et al. 2017). Given its multimeric, multi-PDZ domain structure, other mechanisms are likely to cooperate with the Necls in the targeting of Par-3 to the adaxonal membrane. Of note in this regard, p75^{NTR} associates with and is also required for targeting of Par-3 to the adaxonal membrane of Schwann cells; it does by a direct interaction with the first PDZ domain of Par-3 (Chan et al. 2006; Lewallen et al. 2011). An attractive possibility is that Necl-4 may initiate targeting of Par-3 to the adaxonal compartment, which then recruits and stabilizes p75^{NTR} at this site; alternatively, interactions of all proteins may be required to recruit and stabilize the complex. Par-3 targeting in Schwann cells also requires N-Cadherin although this does not appear to be via a direct interaction (Lewallen et al. 2011).

In addition to its three central PDZ domains, Par-3 contains an N-terminal domain (NTD) and a C-terminal region (CTR). The NTD mediates self-association (Mizuno et al. 2003), promoting formation of filament-like structures (Zhang et al. 2013). Par-3 can also be targeted to the membrane by interactions of its C-terminal region with phosphoinositide lipids (Krahn et al. 2010). Interactions with PI(4,5)P₂ are known to control the apical targeting of PAR-3/Bazooka to the plasma membrane in *Drosophila* follicular epithelia (Claret et al. 2014). As deletion of its C-terminal region did not impair targeting of Par-3 mediated by Necl-4 in CHO cells (Fig. 5C), such interactions may not be essential in Schwann cells.

The SLIs are also highly enriched in Necl-4 and Par-3 (Maurel et al. 2007; Poliak et al. 2002; Spiegel et al. 2007), a junction-rich location in which a number of polarity components have been localized (Ozcelik et al. 2010; Poliak et al. 2002). We were unable to assess whether Necl-4 is required for Par-3 localization to the clefts as knockdown of Necl-4 inhibits Schwann cell myelination in our cocultures (Maurel et al. 2007). However, as Necl-1 and Necl-2 have similar PDZ-binding motifs and are also expressed in the clefts, loss of Necl-4 by itself may not preclude accumulation of Par-3. The function of polarity components in the incisures remains to be established. Clefts are topologically continuous with the inner, adaxonal Schwann cell membrane (Salzer 2015). In agreement, clefts contain adhesion molecules and polarity components similar to those in the adaxonal membrane (Tricaud 2017). Incisures are also enriched in autotypic junctions between adjacent Schwann cell membranes (Salzer 2003). Par-3, is therefore a candidate to regulate junction assembly and cytoskeletal organization at these sites (Tricaud 2017; Yano et al. 2017).

Necl-4 recruitment of Par-3: implications for Schwann cell polarity and myelination

Schwann cells are highly polarized cells with both longitudinal and radial axes of polarity (Masaki 2012; Pereira et al. 2012; Salzer 2015). This polarity is essential for proper myelination and the fidelity of domain formation, including the organization of the underlying axon (Zollinger et al. 2015a). Earlier studies had implicated assembly of the basal lamina in driving Schwann cell polarity (Bunge and Bunge 1986). Here we show that axonal contact localizes Par-3 to the Schwann cell adaxonal membrane, in agreement with previous reports (Chan et al. 2006; Lewallen et al. 2011) and implicate Necl-4 in this recruitment.

Three major complexes regulate cell polarity, i.e. the Par-3 complex that also includes Par-6 and aPKC; the Crumbs complex, consisting of Crumbs, Pals1 (protein associated with Lin-7), and PatJ (Pals1 associated tight junction protein); and the Scribble complex, consisting of Scribble, discs large1 and lethal giant larva (Bilder 2003). The Par-3 and Crumbs complexes promote apical and junctional domain assembly whereas the Scribble complex drives basolateral formation (McCaffrey and Macara 2012; Pieczynski and Margolis 2011). Many components of these complexes are enriched in various sites of myelinating Schwann cells (Etienne-Manneville 2008; Pereira et al. 2012; Poliak et al. 2002). In particular, the enrichment of the Par and Crumbs complex in the adaxonal domain suggests it is an apical domain equivalent whereas components of the Scribble complex in the abaxonal Schwann cell membrane suggests it is a basolateral domain equivalent (Chan et

al. 2006; Ozcelik et al. 2010; Poliak et al. 2002). As mutual exclusion of polarity complexes is a key mechanism for establishing/reinforcing cell polarity (Rodriguez-Boulán and Macara 2014), interactions of the adaxonal membrane with the axon and the abaxonal membrane with the basal lamina are likely to mutually coordinate the establishment of Schwann cell radial polarity.

Necl-4 binding to Necl-1 on the axon is therefore likely to promote Schwann cell polarity and myelination by localizing the Par-3/Par-6/aPKC polarity complex to the adaxonal membrane. Both Par-6 and aPKC have been implicated in Schwann cell myelination. Overexpression of Par-6 (Chan et al. 2006) and of aPKC (Beirowski et al. 2011) in Schwann cells in cocultures inhibits myelination, although it is not known if these overexpression defects are downstream of Par-3. In addition, the NAD⁺ dependent deacetylase Sirt2 activates Par-3 and thereby aPKC (Beirowski et al. 2011). Sirt2 is colocalized with Par-3 in the periaxonal compartment and clefts; Schwann cells deficient in Sirt-2 show a significant delay in the onset of myelination providing support for a role of Par-3 in myelination (Beirowski et al. 2011). Necl-4 interactions with Par-3 may also recruit Pals1, a component of the Crumbs complex. Thus, Pals1 transiently colocalizes with Par-3 at the adaxonal Schwann cell membrane then in the paranodes and clefts (Ozcelik et al. 2010; Poliak et al. 2002; Zollinger et al. 2015b) where they form a biochemical complex (Zollinger et al. 2015b). Knockout/knockdown studies indicate Pals1 is required for the timely onset of Schwann cell myelination and the fidelity of radial sorting of axons (Ozcelik et al. 2010; Zollinger et al. 2015b). Future studies to determine whether Necl-4 recruits the full Par-3 polarity complex to the adaxonal membrane as well as other components of the Crumbs complex, in addition to Pals1, will be of considerable interest.

Other effectors downstream of Necl-4 recruitment of Par-3 potentially include the Rho GTPases, in particular cdc42 and Rac1, both of which are essential for Schwann cell myelination (Benninger et al. 2007; Nodari et al. 2007). cdc42 is part of the polarity complex with Par-3 and interacts with it directly to activate aPKC, which is itself essential for myelination (Beirowski et al. 2011). Par-3 also recruits and spatially regulates Tiam1, which activates Rac1, thereby coordinating cytoskeletal remodeling important for the development of cell polarity (Mertens et al. 2006). Indeed, active Rac1 signaling was reported to be localized to the axon-glial interface in Schwann cells by a Par-3-dependent polarization mechanism that involves BDNF activation of p75^{NTR} and is required for proper myelination (Tep et al. 2012). Finally, Par-3 can also interact directly with PTEN (Feng et al. 2008; von Stein et al. 2005; Wu et al. 2007) potentially supplementing other mechanisms that target PTEN to the adaxonal compartment (Cotter et al. 2010; Ozcelik et al. 2010). As PTEN has been implicated in regulating Schwann cell ensheathment and myelination (Goebbels et al. 2010; Goebbels et al. 2012), these results suggest a potential role of Necl-4/Par-3 interactions in these later events of myelination.

A key question is which functions of Necl-4 in myelination are mediated by Par-3 or may involve other effectors. As noted, Necl-4 knockout mice exhibit delayed myelination, abnormalities in the organization of the paranodal and internodal domains and focal hypermyelination/tomaculae (Golan et al. 2013). A zebrafish deficient for Par-3 in neural crest cells likewise exhibits delayed onset of myelination although has a surprisingly limited

myelin phenotype (Blasky et al. 2014). Whether mice mutant for Par-3 will similarly exhibit a limited phenotype or exhibit additional defects present in the Necl-4 mutant will be of interest for future study. Recent mass spectroscopy studies implicate the choline transporter-like 1 (CTL1) as a novel interacting partner with Necl-4 in myelinating Schwann cell. This interaction, in turn, regulates Schwann cell choline homeostasis, lipid biogenesis and myelination *in vitro* (Heffernan et al. 2017) potentially contributing to the delayed myelination in mutants.

In our array screen, we identified a number of other proteins that are candidates to interact with the cytoplasmic domain of Necl-4, including PDZ-domain proteins of all three polarity-complex proteins (see Table 1). Transcripts for all of these proteins are expressed by Schwann cells, based on RNAseq (A. Brosius Lutz, B. Barres personal communication). Not every protein identified in this screen necessarily interacts with Necl-4 *in vivo* however, notably PATJ (also called CIPP) interacts with claudins in the tight junctions of the paranodes (Poliak et al. 2002). However, many of the other proteins are likely interactors. Indeed, several proteins from our screen were previously reported to interact with members of the Necl family, including Pals2 (also called MPP6) (Kakunaga et al. 2005; Shingai et al. 2003), dlg3 (Kakunaga et al. 2005) and MPDZ (also called MUPP1) (Tanabe et al. 2013). Other proteins identified in this screen are known to be enriched in clefts, e.g. Pals2 (Terada et al. 2012), Magi-2 and MPDZ (also called MUPP1) (Poliak et al. 2002) suggesting a potential interaction. These latter proteins have been linked to acquisition of apical polarity (Padash Barmchi et al. 2016) and tight junction assembly (Adachi et al. 2009). In the future, it will be of interest to assess whether other PDZ proteins identified in this screen are likewise targeted to and complexed with the Necl-proteins in the adaxonal membrane and clefts and contribute to the organization of the myelin sheath.

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Abbreviations:

Necl-4	Nectin-like 4
Par-3	Partitioning-defective 3
Necl	nectin-like
ICD	intracellular domain
SLI	Schmidt-Lanterman incisures
PDZ	PSD-95/Dlg/ZO-1
DRG	dorsal root ganglia
CHO	Chinese Hamster Ovary

aPKC atypical protein kinase C

REFERENCES

- Adachi M, Hamazaki Y, Kobayashi Y, Itoh M, Tsukita S, Furuse M, Tsukita S. 2009 Similar and distinct properties of MUPP1 and Patj, two homologous PDZ domain-containing tight-junction proteins. *Mol Cell Biol* 29:2372–89. [PubMed: 19255144]
- Beirowski B, Gustin J, Armour SM, Yamamoto H, Viader A, North BJ, Michan S, Baloh RH, Golden JP, Schmidt RE and others. 2011 Sir-two-homolog 2 (Sirt2) modulates peripheral myelination through polarity protein Par-3/atypical protein kinase C (aPKC) signaling. *Proc Natl Acad Sci U S A* 108:E952–61. [PubMed: 21949390]
- Benninger Y, Thurnherr T, Pereira J, Krause S, Wu X, Chrostek-Grashoff A, Herzog D, Nave K, Franklin R, Meijer D and others. 2007 Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development. *J Cell Biol* 177:1051–61. [PubMed: 17576798]
- Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET, Sudhof TC. 2002 SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525–31. [PubMed: 12202822]
- Bilder D 2003 PDZ domain polarity complexes. *Curr Biol* 13:R661–2.
- Blasky AJ, Pan L, Moens CB, Appel B. 2014 Pard3 regulates contact between neural crest cells and the timing of Schwann cell differentiation but is not essential for neural crest migration or myelination. *Dev Dyn* 243:1511–23. [PubMed: 25130183]
- Bunge MB, Bunge RP. 1986 Linkage between Schwann cell extracellular matrix production and ensheathment function. *Ann N Y Acad Sci* 486:241–7. [PubMed: 3471156]
- Chan JR, Jolicoeur C, Yamauchi J, Elliott J, Fawcett JP, Ng BK, Cayouette M. 2006 The polarity protein Par-3 directly interacts with p75NTR to regulate myelination. *Science* 314:832–6. [PubMed: 17082460]
- Claret S, Jouette J, Benoit B, Legent K, Guichet A. 2014 PI(4,5)P2 produced by the PI4P5K SKTL controls apical size by tethering PAR-3 in *Drosophila* epithelial cells. *Curr Biol* 24:1071–9. [PubMed: 24768049]
- Cotter L, Ozcelik M, Jacob C, Pereira JA, Locher V, Baumann R, Relvas JB, Suter U, Tricaud N. 2010 Dlg1-PTEN interaction regulates myelin thickness to prevent damaging peripheral nerve overmyelination. *Science* 328:1415–8. [PubMed: 20448149]
- Ebnet K, Kummer D, Steinbacher T, Singh A, Nakayama M, Matis M. 2017 Regulation of cell polarity by cell adhesion receptors. *Semin Cell Dev Biol*.
- Einheber S, Meng X, Rubin M, Lam I, Mohandas N, An X, Shrager P, Kissil J, Maurel P, Salzer JL. 2013 The 4.1B cytoskeletal protein regulates the domain organization and sheath thickness of myelinated axons. *Glia* 61:240–53. [PubMed: 23109359]
- Einheber S, Zanazzi G, Ching W, Scherer S, Milner T, Peles E, Salzer J. 1997 The axonal membrane protein Caspr, a homologue of neuroligin IV, is a component of the septate-like paranodal junctions that assemble during myelination. *J Cell Biol* 139:1495–506. [PubMed: 9396755]
- Etienne-Manneville S 2008 Polarity proteins in glial cell functions. *Current Opinion in Neurobiology* 18:488–94. [PubMed: 18840525]
- Feng W, Wu H, Chan L-, Zhang M. 2008 Par-3-mediated junctional localization of the lipid phosphatase PTEN is required for cell polarity establishment. *Journal of Biological Chemistry* 283:23440–23449. [PubMed: 18550519]
- Fogel AI, Akins MR, Krupp AJ, Stagi M, Stein V, Biederer T. 2007 SynCAMs organize synapses through heterophilic adhesion. *J Neurosci* 27:12516–30. [PubMed: 18003830]
- Fujita E, Tanabe Y, Hirose T, Aurrand-Lions M, Kasahara T, Imhof BA, Ohno S, Momoi T. 2007 Loss of partitioning-defective-3/isotype-specific interacting protein (par-3/ASIP) in the elongating spermatid of RA175 (IGSF4A/SynCAM)-deficient mice. *Am J Pathol* 171:1800–10. [PubMed: 18055550]
- Goebbels S, Oltrogge JH, Kemper R, Heilmann I, Bormuth I, Wolfer S, Wichert SP, Mobius W, Liu X, Lappe-Siefke C and others. 2010 Elevated phosphatidylinositol 3,4,5-trisphosphate in glia triggers

cell-autonomous membrane wrapping and myelination. *J Neurosci* 30:8953–64. [PubMed: 20592216]

- Goebbels S, Oltrogge JH, Wolfer S, Wieser GL, Nientiedt T, Pieper A, Ruhwedel T, Groszer M, Sereda MW, Nave KA. 2012 Genetic disruption of Pten in a novel mouse model of tomaculous neuropathy. *EMBO Mol Med*.
- Golan N, Kartvelishvili E, Spiegel I, Salomon D, Sabanay H, Rechav K, Vainshtein A, Frechter S, Maik-Rachline G, Eshed-Eisenbach Y and others. 2013 Genetic deletion of *Cadm4* results in myelin abnormalities resembling Charcot-Marie-Tooth neuropathy. *J Neurosci* 33:10950–61. [PubMed: 23825401]
- Heffernan C, Jain MR, Liu T, Kim H, Barretto K, Li H, Maurel P. 2017 Nectin-like 4 complexes with Choline Transporter-Like protein-1, and regulates Schwann cell choline homeostasis and lipid biogenesis in vitro. *J Biol Chem*.
- Ivanovic A, Horresh I, Golan N, Spiegel I, Sabanay H, Frechter S, Ohno S, Terada N, Mobius W, Rosenbluth J and others. 2012 The cytoskeletal adapter protein 4.1G organizes the internodes in peripheral myelinated nerves. *J Cell Biol* 196:337–44. [PubMed: 22291039]
- Kakunaga S, Ikeda W, Itoh S, Deguchi-Tawarada M, Ohtsuka T, Mizoguchi A, Takai Y. 2005 Nectin-like molecule-1/TSLL1/SynCAM3: a neural tissue-specific immunoglobulin-like cell-cell adhesion molecule localizing at non-junctional contact sites of presynaptic nerve terminals, axons and glia cell processes. *J Cell Sci* 118:1267–77. [PubMed: 15741237]
- Krahn MP, Klopfenstein DR, Fischer N, Wodarz A. 2010 Membrane targeting of Bazooka/Par-3 is mediated by direct binding to phosphoinositide lipids. *Curr Biol* 20:636–42. [PubMed: 20303268]
- Lewallen KA, Shen YA, De la Torre AR, Ng BK, Meijer D, Chan JR. 2011 Assessing the role of the cadherin/catenin complex at the Schwann cell-axon interface and in the initiation of myelination. *J Neurosci* 31:3032–43. [PubMed: 21414924]
- Masaki T. 2012 Polarization and myelination in myelinating glia. *ISRN Neurol* 2012:769412. [PubMed: 23326681]
- Maurel P, Einheber S, Galinska J, Thaker P, Lam I, Rubin MB, Scherer SS, Murakami Y, Gutmann DH, Salzer JL. 2007 Nectin-like proteins mediate axon Schwann cell interactions along the internode and are essential for myelination. *J Cell Biol* 178:861–74. [PubMed: 17724124]
- McCaffrey LM, Macara IG. 2012 Signaling pathways in cell polarity. *Cold Spring Harb Perspect Biol* 4.
- Mertens AE, Pegtel DM, Collard JG. 2006 Tiam1 takes PARt in cell polarity. *Trends Cell Biol* 16:308–16. [PubMed: 16650994]
- Miyoshi J, Takai Y. 2007 Nectin and nectin-like molecules: biology and pathology. *Am J Nephrol* 27:590–604. [PubMed: 17823505]
- Mizuno K, Suzuki A, Hirose T, Kitamura K, Kutsuzawa K, Futaki M, Amano Y, Ohno S. 2003 Self-association of PAR-3-mediated by the conserved N-terminal domain contributes to the development of epithelial tight junctions. *J Biol Chem* 278:31240–50. [PubMed: 12756256]
- Monk KR, Feltri ML, Taveggia C. 2015 New insights on Schwann cell development. *Glia* 63:1376–93. [PubMed: 25921593]
- Nodari A, Zambroni D, Quattrini A, Court FA, D'Urso A, Recchia A, Tybulewicz VL, Wrabetz L, Feltri ML. 2007 Beta1 integrin activates Rac1 in Schwann cells to generate radial lamellae during axonal sorting and myelination. *J Cell Biol* 177:1063–75. [PubMed: 17576799]
- Ogita H, Takai Y. 2006 Nectins and nectin-like molecules: roles in cell adhesion, polarization, movement, and proliferation. *IUBMB Life* 58:334–43. [PubMed: 16754328]
- Ozcelik M, Cotter L, Jacob C, Pereira JA, Relvas JB, Suter U, Tricaud N. 2010 Pals1 is a major regulator of the epithelial-like polarization and the extension of the myelin sheath in peripheral nerves. *J Neurosci* 30:4120–31. [PubMed: 20237282]
- Padash Barmchi M, Samarasekera G, Gilbert M, Auld VJ, Zhang B. 2016 Magi Is Associated with the Par Complex and Functions Antagonistically with Bazooka to Regulate the Apical Polarity Complex. *PLoS One* 11:e0153259. [PubMed: 27074039]
- Pereira JA, Lebrun-Julien F, Suter U. 2012 Molecular mechanisms regulating myelination in the peripheral nervous system. *Trends in Neurosciences* 35:123–134. [PubMed: 22192173]

- Pieczynski J, Margolis B. 2011 Protein complexes that control renal epithelial polarity. *Am J Physiol Renal Physiol* 300:F589–601. [PubMed: 21228104]
- Poliak S, Matlis S, Ullmer C, Scherer SS, Peles E. 2002 Distinct claudins and associated PDZ proteins form different autotypic tight junctions in myelinating Schwann cells. *J Cell Biol* 159:361–72. [PubMed: 12403818]
- Rodriguez-Boulán E, Macara IG. 2014 Organization and execution of the epithelial polarity programme. *Nat Rev Mol Cell Biol* 15:225–42. [PubMed: 24651541]
- Sakisaka T, Takai Y. 2004 Biology and pathology of nectins and nectin-like molecules. *Curr Opin Cell Biol* 16:513–21. [PubMed: 15363801]
- Salzer J. 2003 Polarized domains of myelinated axons. *Neuron* 40:297–318. [PubMed: 14556710]
- Salzer JL. 2015 Schwann cell myelination. *Cold Spring Harb Perspect Biol* 7:a020529. [PubMed: 26054742]
- Salzer JL, Brophy PJ, Peles E. 2008 Molecular domains of myelinated axons in the peripheral nervous system. *Glia* 56:1–9. [PubMed: 17910053]
- Shi S, Jan L, Jan Y. 2003 Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* 112:63–75. [PubMed: 12526794]
- Shingai T, Ikeda W, Kakunaga S, Morimoto K, Takekuni K, Itoh S, Satoh K, Takeuchi M, Imai T, Monden M and others. 2003 Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. *J Biol Chem* 278:35421–7. [PubMed: 12826663]
- Spiegel I, Adamsky K, Eshed Y, Milo R, Sabanay H, Sarig-Nadir O, Horresh I, Scherer SS, Rasband MN, Peles E. 2007 A central role for Nect4 (SynCAM4) in Schwann cell-axon interaction and myelination. *Nat Neurosci* 10:861–9. [PubMed: 17558405]
- Stiffler MA, Chen JR, Grantcharova VP, Lei Y, Fuchs D, Allen JE, Zaslavskaya LA, MacBeath G. 2007 PDZ domain binding selectivity is optimized across the mouse proteome. *Science* 317:364–9. [PubMed: 17641200]
- Susuki K, Raphael AR, Ogawa Y, Stankewich MC, Peles E, Talbot WS, Rasband MN. 2011 Schwann cell spectrins modulate peripheral nerve myelination. *Proceedings of the National Academy of Sciences of the United States of America* 108:1–6.
- Suzuki A, Ohno S. 2006 The PAR-aPKC system: lessons in polarity. *J Cell Sci* 119:979–87. [PubMed: 16525119]
- Tanabe Y, Fujita E, Hayashi YK, Zhu X, Lubbert H, Mezaki Y, Senoo H, Momoi T. 2013 Synaptic adhesion molecules in Cadm family at the neuromuscular junction. *Cell Biol Int* 37:731–6. [PubMed: 23505055]
- Tep C, Kim ML, Opincariu LI, Limpert AS, Chan JR, Appel B, Carter BD, Yoon SO. 2012 Brain-derived neurotrophic factor (BDNF) induces polarized signaling of small GTPase (Rac1) protein at the onset of Schwann cell myelination through partitioning-defective 3 (Par3) protein. *J Biol Chem* 287:1600–8. [PubMed: 22128191]
- Terada N, Saitoh Y, Ohno N, Komada M, Saitoh S, Peles E, Ohno S. 2012 Essential function of protein 4.1G in targeting of membrane protein palmitoylated 6 into Schmidt-Lanterman incisures in myelinated nerves. *Molecular and cellular biology* 32:1–8.
- Tricaud N. 2017 Myelinating Schwann Cell Polarity and Mechanically-Driven Myelin Sheath Elongation. *Front Cell Neurosci* 11:414. [PubMed: 29354031]
- von Stein W, Ramrath A, Grimm A, Muller-Borg M, Wodarz A. 2005 Direct association of Bazooka/ PAR-3 with the lipid phosphatase PTEN reveals a link between the PAR/aPKC complex and phosphoinositide signaling. *Development* 132:1675–86. [PubMed: 15743877]
- Wu H, Feng W, Chen J, Chan LN, Huang S, Zhang M. 2007 PDZ domains of Par-3 as potential phosphoinositide signaling integrators. *Mol Cell* 28:886–98. [PubMed: 18082612]
- Yano T, Kanoh H, Tamura A, Tsukita S. 2017 Apical cytoskeletons and junctional complexes as a combined system in epithelial cell sheets. *Ann N Y Acad Sci* 1405:32–43. [PubMed: 28763830]
- Zhang C, Rasband MN. 2016 Cytoskeletal control of axon domain assembly and function. *Curr Opin Neurobiol* 39:116–21. [PubMed: 27203619]

- Zhang Y, Wang W, Chen J, Zhang K, Gao F, Gao B, Zhang S, Dong M, Besenbacher F, Gong W and others. 2013 Structural insights into the intrinsic self-assembly of Par-3 N-terminal domain. *Structure* 21:997–1006. [PubMed: 23643951]
- Zhu Y, Li H, Li K, Zhao X, An T, Hu X, Park J, Huang H, Bin Y, Qiang B and others. 2013 Necl-4/SynCAM-4 is expressed in myelinating oligodendrocytes but not required for axonal myelination. *PLoS One* 8:e64264. [PubMed: 23700466]
- Zollinger DR, Baalman KL, Rasband MN. 2015a The ins and outs of polarized axonal domains. *Annu Rev Cell Dev Biol* 31:647–67. [PubMed: 26566119]
- Zollinger DR, Chang KJ, Baalman K, Kim S, Rasband MN. 2015b The Polarity Protein Pals1 Regulates Radial Sorting of Axons. *J Neurosci* 35:10474–84. [PubMed: 26203142]

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Figure 1. Necl-4 and Par-3 are co-expressed in the internodal membrane and clefts
Expression of Par-3 (red) and Necl-4 (green) in teased sciatic nerves is shown; the merged image (orange) shows extensive co-localization along the internode (arrow) and in the Schmidt-Lanterman incisures (clefts), one of which is indicated by the arrowhead. Bar 5 μ m.

Figure 2. Necl-4/Necl-1 interactions recruit Par-3 to the membrane.

(A) Equal numbers of Necl-1 and Necl-4 expressing CHO cells were mixed at the time of plating and stained for Necl-4 (red) and Par-3 (green). Endogenous Par-3 (green) is recruited to the membrane and cleared from the cytoplasm of only the Necl-4 positive cells (asterisk). (B) Equal numbers of HA/Necl-1 (green) and V5/Necl-4 (blue) expressing CHO cells were mixed at the time of plating. Endogenous Par-3 (red) specifically accumulates at sites of contact of Necl-4 and Necl-1 expressing cells (pink in merge). (C) Schwann cells (Sc) were infected with either a scrambled or a Necl-4 shRNA lentiviral construct (infected cells

shown in green), seeded onto DRG neurons and allowed to myelinate for 7 days. Cells were stained for Par-3 (red) and GFP (green). Sc in the control and scrambled conditions still display asymmetrical Par-3 localization (asterisk). In the Necl-4 knockdown Sc, the periaxonal localization of Par-3 is largely absent (#) or severely disrupted (arrow). (D) Quantitation of percentage of Sc with asymmetric Par-3 localization in WT, scrambled and shNecl-4 Schwann cells. * Student's T-Test: $p < 0.0001$

Figure 3. Necl-4 interacts with Par-3 through the PDZ-binding domain of Necl-4.

(A) Schematic diagram of wild type Necl-4 and Necl-4- PDZ deletion constructs. (B) Western blot of CHO cells expressing HA-tagged Necl-1, V5-tagged Necl-4 or Necl-4- PDZ constructs. (C) Equal numbers of HA/Necl-1 (green) and V5/Necl-4 or V5/Necl-4- PDZ(blue) expressing CHO cells were mixed at the time of plating. Endogenous Par-3 (red) specifically accumulates at sites of contact of Necl-4 and Necl-1 expressing cells (arrows). Deletion of the Necl-4 PDZ binding domain abolishes Par-3 recruitment to sites of contact (open arrows).

Figure 4. The PDZ-binding segment of Nec1-4 is required for effective co-immunoprecipitation of Par-3.

(A) Schwann cells expressing V5-tagged recombinant Nec1-4, either wild type (lanes 1 and 2) or Nec1-4 lacking the PDZ binding-domain (lanes 3 and 4), were lysed and subjected to immunoprecipitation with an anti-V5 antibody. Both initial lysates (input) or immunoprecipitates (IP) were subjected to SDS PAGE and Western blotting for Nec1-4 (V5) or for Par-3. (B) Quantification of immunoprecipitation showing the results from four separate experiments; values represent the percent of Par3 immunoprecipitated from input

Par3/the percent of Necl-4 immunoprecipitated from the input of Necl-4 $\pm$ SEM ($p < 0.01$, T-test).

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Figure 5. The PDZ1 domain of Par-3 is sufficient for recruitment by Necl-4.

(A) Schematic diagram of FLAG-tagged Par-3 PDZ domains. (B) V5-Necl-4 (blue) was clustered with anti-V5 antibodies and compared to co-recruitment of endogenous Par-3 (red) or FLAG-tagged PDZ domain constructs (green) expressed in CHO cells. Endogenous Par-3 consistently co-clustered with V5-Necl-4, with examples highlighted by the arrows. PDZ1 (top row) and PDZ3 (bottom row) constructs also co-localize with the V5-Necl-4 and endogenous Par-3 clusters. While PDZ1 consistently co-localizes with the V5-Necl-4 clusters, PDZ3 only partially co-localizes as seen in the inset. PDZ2 (middle row) does not co-localize with the V5-Necl-4/Par-3 clusters.

Figure 6. Necl-4 interacts with the Par-3 PDZ1 domain.

(A) Schematic diagram of GFP-tagged full length Par-3, Par-3 constructs with PDZ domain deletions and a Par-3 construct lacking the C-terminal tail. (B) Western blot of CHO cells expressing each construct. (C) V5/Necl-4 was used to cluster the different EGFP tagged Par-3 deletion constructs in CHO cells. Clustering of the full-length Par-3 (FL) and PDZ2 deletion (ΔPDZ2) constructs were clustered normally. Clustering was impaired in both the PDZ1 deletion (ΔPDZ1) and PDZ3 deletion (ΔPDZ3) constructs, with ΔPDZ1 being most significantly reduced. Deletion of the C terminal of Par-3, including part of the aPKC binding domain, had no effect on clustering. (D) Quantification of clustering experiments,

comparing the full length (FL) construct to each of the deletion constructs. * Student's T-Test: $p < 0.001$.

Figure 7. Schematic of proposed interactions between Necl-4 and Par3.

Illustration shows Necl-1 on the axon surface interacts in *trans* with Necl-4 on the adaxonal (inner) Schwann cell membrane. These interactions trigger the binding of the cytoplasmic tail of Necl-4 to the 1st PDZ domain of Par3, recruiting the latter to the Schwann cell membrane; other components of the Par3 complex are shown as not interacting directly. A similar complex of Necl-1 and Necl-4 occurs in the Schmidt-Lanterman incisures, which are

topologically continuous with the Schwann cell adaxonal membrane. This interaction similarly recruits Par3 in the incisures, which is not shown to simplify the illustration.

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TABLE 1

Legend.

Protein microarray and fluorescence polarization results using the Necl-4 PDZ-binding domain. The fluorescently labeled PDZ-binding C-terminus of Necl-4 was used as a probe to interrogate a genome-wide PDZ protein microarray. Positive array hits were then retested and quantified by fluorescence polarization (FP) in solution; in the latter case, peptides that exhibited saturation binding with a $kD < 100 \mu M$ were scored as FP positives. Proteins and their specific PDZ domains from both positive array hits and FP are listed on the left list. The numerator in parentheses refer to the PDZ domain(s) of the protein that Necl-4 bound to; the denominator is the number of PDZ domains in that protein. In some cases, a series of PDZ domains were synthesized in tandem and are designated by commas (e.g. 1,2,3). PDZ domains from proteins that were not of sufficient quality for the FP assay are listed as probable array hits only on the right list. Par-3 is highlighted in red.

Array hits confirmed by fluorescence polarization		Array hits but not of quantity for fluorescence polarization	
Protein	PDZ domain	Protein	PDZ domain
Scrb1	(3/4)	OMP25	(1/1)
Pals2	(1/1)	Pdzk3	(2/5)
Mpp7	(1/1)	MPDZ	(7/13)
Dlg3	(1/1)	beta1-syntrophin	(1/1)
PATJ	(3,5/10)	Magi-2	(2,3,4/6)
Par-3	(3/3)	Par-3	(1,2,3/3)
Dvl3	(1/1)	ZO-3	(1,2,3/3)
MPDZ	(5/13)		
Pdzk3	(5/5)		
Magi-1	(2/6)		
Magi-2	(2/6)		
Magi-3	(2/6)		
Par3B	(1,2,3/3)		