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A central role for Necl4 (SynCAM4) in Schwann cell–axon interaction and myelination

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Myelination in the peripheral nervous system requires close contact between Schwann cells and the axon, but the underlying molecular basis remains largely unknown. Here we show that cell adhesion molecules (CAMs) of the nectin-like (Necl, also known as SynCAM or Cadm) family mediate Schwann cell–axon interaction during myelination. Necl4 is the main Necl expressed by myelinating Schwann cells and is located along the internodes in direct apposition to Necl1, which is localized on axons. Necl4 serves as the glial binding partner for axonal Necl1, and the interaction between these two CAMs mediates Schwann cell adhesion. The disruption of the interaction between Necl1 and Necl4 by their soluble extracellular domains, or the expression of a dominant-negative Necl4 in Schwann cells, inhibits myelination. These results suggest that Necl proteins are important for mediating axon-glia contact during myelination in peripheral nerves.

Myelination of axons by Schwann cells in the peripheral nervous system (PNS) allows fast and efficient saltatory propagation of action potentials along the nerve. The formation of functional myelinated fibers requires reciprocal communication between Schwann cells and their associated axons. During development, Schwann cells receive specific axonal signals that control their survival, migration and differentiation into ensheathing (nonmyelinating) or myelinating cells[1]. Although many of the axonal signals that regulate the initial differentiation of Schwann cells are known, the molecular events governing the onset and progression of myelination are less well understood[1]. In the PNS, myelination is regulated by neuregulin-1 that is bound to the axonal membrane[2,3], indicating that Schwann cell–axon contact may be a prerequisite for myelination. Schwann cells express a number of CAMs that could mediate their association with axons. N-cadherin has been suggested to mediate the initial growth of Schwann cell processes and their alignment with axons, as well as glia-glia interaction[4]. However, the expression of N-cadherin is downregulated as precursors differentiate into immature Schwann cells, which occurs days before myelination[5]. Myelinating Schwann cells also express neurofascin 155 (NF155)[6], TAG-1 (ref. 7) and gliomedin[8], all of which are important for Schwann cell–axon interactions at and around the nodes of Ranvier, but seem to be dispensable for myelination[8–10]. Two CAMs of the immunoglobulin superfamily (IgCAMs), L1 (ref. 11) and myelin-associated glycoprotein (MAG)[12], were originally suggested to mediate Schwann cell–axon attachments, but further evidence from gene targeting studies demonstrated that neither was required to initiate axon–Schwann cell association[13–15]. Hence, although CAMs have been implicated in various developmental stages of myelinating Schwann cells[16–18], the identity of the molecules mediating axon–Schwann cell contact during myelination still remains elusive.

To identify cell surface proteins expressed by Schwann cells at the onset of myelination, we recently screened cDNA expression libraries prepared from dibutyryl cyclic AMP-treated primary Schwann cells and 3-d-old rat sciatic nerve using a signal-sequence trap[19]. Among the large variety of structurally and functionally diverse CAMs and signaling molecules identified in our screen, we isolated members of the Necl family. Necl proteins are a small group of the IgCAM superfamily that include four different members in human and rodents (Necl1, Necl2, Necl3 and Necl4; see also **Supplementary Table 1** online for nomenclature)[20,21]. All four are type I transmembrane proteins that contain three immunoglobulin-like domains in their extracellular region and a short intracellular domain that mediates their interactions with protein 4.1 and PDZ-domain proteins[22–25]. Necls mediate Ca^{2+}-independent cell adhesion by binding homophilically, as well as heterophilically, to other Necls or their related nectins[26–28]. Based on their tissue distribution and subcellular localization, as well as their interactions with scaffolding proteins, it has been proposed that the Necls are important in the organization of the plasma membrane at specific areas of cell-cell contact[21,29]. Necl2 (SynCAM1) was shown to be present at both the presynaptic and postsynaptic membranes and to induce functional presynaptic differentiation[26,30]. Necl1 (SynCAM3) was found at various contact sites between neurons, as well as between neurons and astrocytic processes surrounding synapses in the cerebellum, and along myelinated axons in the PNS[27]. In this study, we report that Necl4 (SynCAM4) mediates the Schwann cell–axon interactions necessary for myelination.

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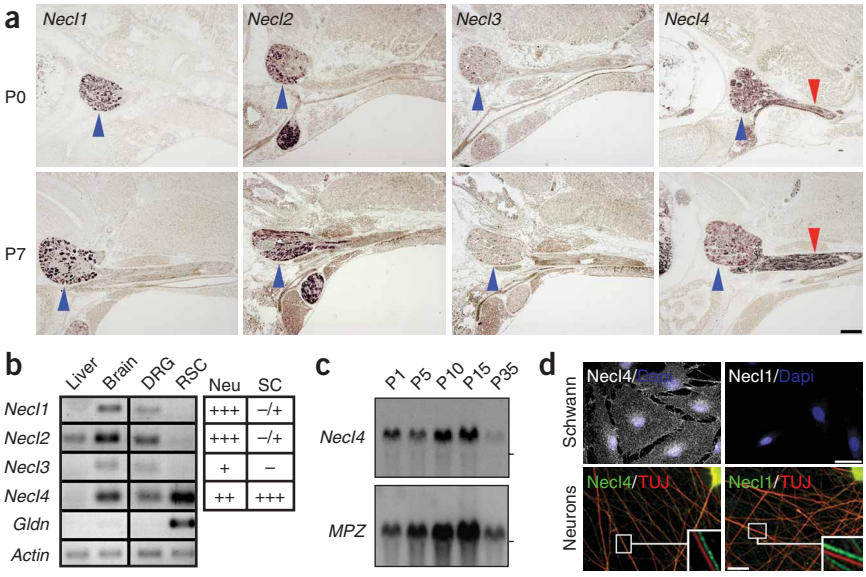

Figure 1 Differential expression of Necl proteins in the PNS. (**a**) *In situ* hybridization analysis. Cross-sections of postnatal day 0 (P0) or P7 rats were hybridized to *Necl1*, *Necl2*, *Necl3* and *Necl4* antisense probes as indicated. We saw strong expression of *Necl4* mRNA in the nerve (red arrowheads) and strong expression of *Necl1*, *Necl2* and *Necl4* mRNA in the DRG (blue arrowheads). (**b**) RT-PCR analysis. Specific primer sets for *Necl1*, *Necl2*, *Necl3*, *Necl4*, gliomedin (*Gldn*) and actin were used to amplify cDNA derived from isolated DRG neurons or isolated rat Schwann cells (RSC). Actin and gliomedin were used as controls for genes that are expressed ubiquitously or specifically in Schwann cells, respectively. The table summarizes the expression of the Necls in DRG neurons and Schwann cells (based on RT-PCR and *in situ* hybridization). (**c**) Northern blot analysis. Blots containing RNA isolated from rat sciatic nerves at the indicated postnatal days were successively hybridized with radiolabeled Necl4 and P0 (MPZ) cDNAs. Both mRNAs increased in parallel from P1 to P15. The location of the 18S ribosomal RNA is marked on the right. (**d**) Immunocytochemistry. Isolated Schwann cells or DRG neurites were immunostained with antibodies to Necl4, Necl1 and βIII tubulin (TUJ) as indicated; nuclei were labeled with Dapi (blue). The boxed regions are shown as insets, in which the two fluorescent labels are offset. Scale bars, 400 μm (**a**) and 30 μm (**d**).

RESULTS

Necl proteins in the PNS

To examine the expression of Necls in the PNS, we performed *in situ* hybridization of newborn and 7-d-old rats using specific probes for Necl1–4 (**Fig. 1a**). Necl1, Necl2 and Necl4 were clearly detected in dorsal root ganglia (DRG). In addition, a strong signal for Necl4 was also detected in Schwann cells located along the nerve. The expression of Necl4 markedly increased in myelinating Schwann cells during the first postnatal week, which corresponds to the initial period of active myelination in the PNS. RT-PCR analysis of mRNA isolated from cultured DRG neurons or Schwann cells showed that, although transcripts of all four Necl proteins could be detected in DRG neurons, cultured Schwann cells expressed high levels of Necl4 and low levels of Necl2, but did not express Necl1 or Necl3 (**Fig. 1b**). Northern blot analysis of sciatic nerve from different postnatal days demonstrated that Necl4 mRNA expression increased during the first 2 weeks after birth in a manner that is reminiscent of other myelin-related genes, such as myelin P0 (MPZ; **Fig. 1c**). Altogether, these results show that members of the Necl gene family are differentially expressed by neurons and myelinating Schwann cells in peripheral nerves: sensory and motor neurons express Necl1 and Necl2 (refs. 25,31,32), whereas myelinating Schwann cells mainly express Necl4.

We generated an antiserum against Necl4 that specifically recognized Necl4, but not Necl1, Necl2 and Necl3, and an antiserum that recognized Necl1 and weakly Necl2, but not Necl3 or Necl4 (**Supplementary Fig. 1** online). Cultured rat Schwann cells immunostained with these antibodies had strong Necl4 immunoreactivity in their cell membrane and processes, but none for Necl1 (**Fig. 1d**). Staining of isolated DRG neurons revealed high levels of Necl1 and lower levels of Necl4 in the cell soma and along neurites (**Fig. 1d**). In agreement with these findings, Necl4, but not Necl1, could be immunoprecipitated from cultured rat Schwann cells (**Supplementary Fig. 1**), further indicating that it is the primary Necl expressed in these cells.

To examine the expression and localization of Necl4 during myelination, we made use of mixed sensory DRG neuron and Schwann cell cultures, which allows a refined analysis of the process[3,8]. Necl4 was weakly expressed in Schwann cells during the first week in culture, but its expression increased thereafter in Schwann cells that were aligned along axons (**Fig. 2a–d**). In these cells, Necl4 immunoreactivity was particularly prominent at axon-glial contact sites. Necl4 expression appeared to increase further after the induction of myelination and was particularly high in myelinating Schwann cells labeled for myelin basic protein (MBP) or MAG (**Fig. 2e–j**). These results demonstrate that Necl4 protein is found in myelinating Schwann cells and indicate that its expression is upregulated by axonal contact and myelination.

Complementary internodal localization of Necl4 and Necl1

We determined the localization of Necl4 and Necl1 in myelinated nerves by using antibodies to these proteins in combination with antibodies to various axonal or glial markers (**Fig. 3**). In teased sciatic-nerve fibers from adult rats, Necl4 immunoreactivity was detected along the internodes, but was absent from the nodes (**Fig. 3a–e**). This labeling was specific and was completely abolished by preincubating the antibody with a recombinant protein containing the cytoplasmic tail of Necl4, but not that of Necl1 (**Supplementary Fig. 2** online). Double labeling of teased rat sciatic-nerve fibers for Necl4 and MAG showed a marked colocalization of these proteins in Schmidt-Lanterman incisures (SLI), in paranodal loops and all along the adaxonal Schwann-cell membrane (apposing the axon) (**Fig. 3f–g**). At the paranodes, Necl4 immunoreactivity was distinct from that of Caspr, which labels the axoglial junction[17] (**Fig. 3e**). In contrast to myelinating Schwann cells, Necl4 was absent from GFAP- and L1-labeled ensheathing nonmyelinating Schwann cells[1] (**Supplementary Fig. 3** online). Immunolabeling of rat sciatic nerve for Necl1 and Na^+ channels (**Fig. 3h**), Caspr (**Fig. 3i**) or Kv1.2 (**Fig. 3j**) demonstrated that Necl1 was present along the internodes, including the juxtaparanodal region, but was absent from the nodes and paranodes. Occasionally, Necl1 labeling was also detected in the outermost ring of the SLI (**Fig. 3k**).

Double immunolabeling of cross-sections of sciatic nerves for Necl4 and for neurofilament (which labels axons) or β-dystroglycan (which labels abaxonal Schwann-cell membrane) provided further evidence that Necl4 was present at the adaxonal membrane surrounding the axon (**Fig. 3i–m**). In addition, in ~15% of the fibers in the sections, Necl4 was found in an inner, wider ring corresponding to incisures

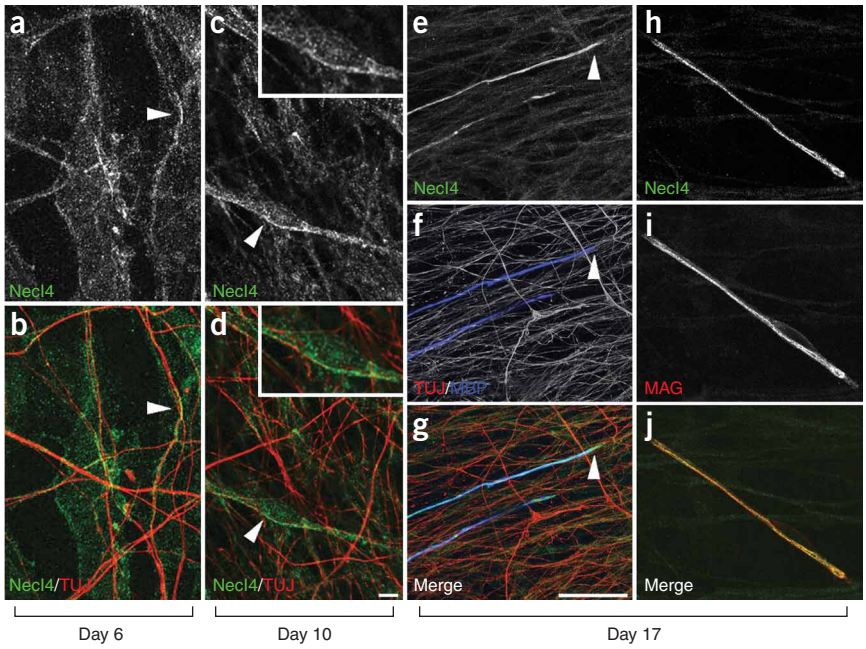

Figure 2 Axonal contact and myelination are associated with increased expression of Necl4 in Schwann cells. Cultures of dissociated DRG neurons and Schwann cells were immunostained for Necl4 and TUJ, MBP or MAG, 6, 10 or 17 d after plating as indicated; myelination was induced with ascorbic acid at day 10. (**a**–**d**) Increased labeling of Necl4 at contact sites between Schwann cells and the axons are marked by arrowheads. Insets (**c**,**d**) show higher-magnification images of the areas marked with an arrowhead. (**e**–**j**) MBP- and MAG-positive myelinating Schwann cells (arrowheads) have intense Necl4 immunoreactivity. Scale bars, 10 μm (**a**–**d**) and 30 μm (**e**–**j**).

(**Fig. 3m**). In cross-sections, Necl1 was localized to the circumference of the axons (marked by neurofilament staining), indicating that it was localized at the axon–Schwann cell interface (**Fig. 3n–o**). These results revealed a complementary localization of Necl4 and Necl1 at the axoglial interface along the internodes (**Fig. 3p**).

Differential binding of Necls to neurons and Schwann cells

To characterize possible axon-glia interactions mediated by members of the Necl family, we tested whether soluble Fc fusion proteins containing their extracellular domain could bind to sensory neurons or Schwann cells (**Fig. 4a**,**b**). Soluble extracellular domains of Necl1 and Necl3 bound to Schwann cells, whereas no binding was detected with the extracellular domain of Necl2 or Necl4. In contrast, the extracellular domains of all four Necl proteins bound to the neurites of cultured DRG neurons (**Fig. 4b**): Necl4-Fc robustly labeled the axons, Necl2-Fc showed moderate binding, and Necl1-Fc and Necl3-Fc showed very weak binding. These findings are consistent with the idea that Schwann cells express Necl4, which mediates Schwann cell–axon interactions by binding to axonal Necl1.

We next examined whether the binding of Necl1–Fc to Schwann cells and Necl4–Fc to DRG neurites could be mediated by their interactions with members of the Necl family. We tested the ability of Fc fusion proteins containing the extracellular domains of the Necl proteins to bind COS7 cells expressing Necl1 or Necl4 (**Supplementary Fig. 4**

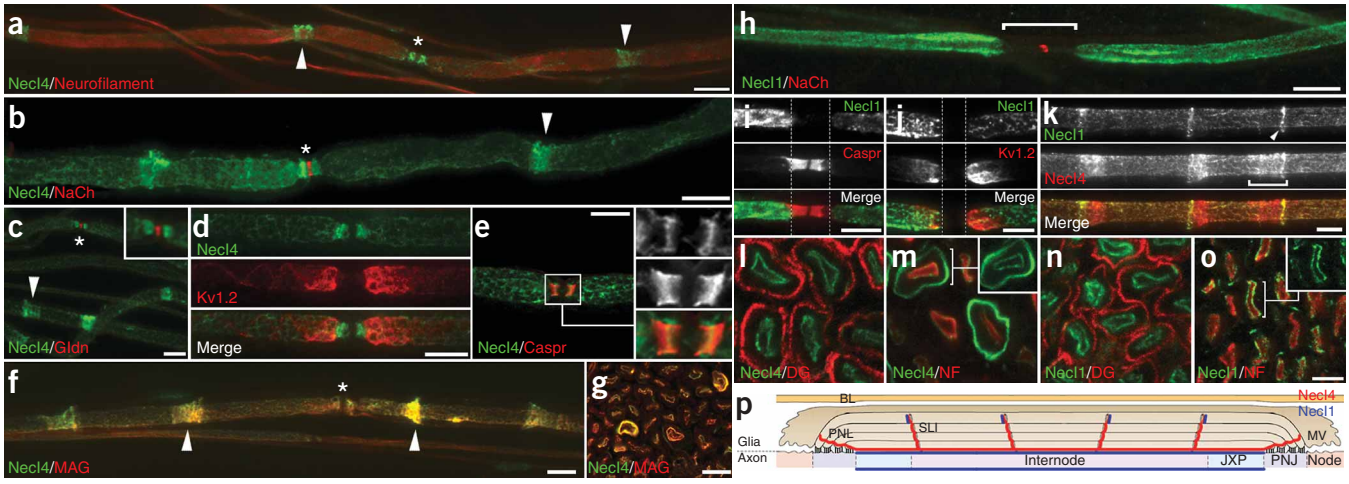

Figure 3 Necl4 and Necl1 are localized along the internodes. (**a**–**f**) Immunofluorescence staining of teased adult rat sciatic nerves for Necl4 and neurofilament (NF, **a**), Na^+ channels (NaCh; to label nodes, **b**), gliomedin (Gldn; to label nodes, **c**), Kv1.2 (to label juxtaparanodes, **d**), Caspr (to label paranodes, **e**) or MAG (**f**), as indicated. Inset in **c** shows a higher magnification of the nodal region. Insets in **e** show a higher magnification of the paranodes labeled for Necl4 (upper), Caspr (middle) or the merged image (lower). (**g**) Cross-sections of adult rat sciatic nerves labeled for Necl4 and MAG. Arrowheads and asterisks mark the incisures and the nodes of Ranvier, respectively. (**h**–**k**) Teased fibers immunolabeled for Necl1 and Na^+ channels (**h**), Caspr (**i**), Kv1.2 (**j**) or Necl4 (**k**) as indicated. The locations of the nodes and paranodes are marked with a horizontal line in **h** and with dashed vertical lines in **i**,**j**. Note that Necl1 was present along the axonal internodes, but was absent from the paranodes or the nodes of Ranvier. The SLIs, or their outermost ring (arrowhead), are labeled in **k**. (**l**–**p**) Cross-sections of adult rat sciatic nerves labeled for Necl4 (**l**,**m**) or Necl1 (**n**,**o**), together with NF or dystroglycan (DG), as indicated. (**p**) A schematic view of a longitudinal section of a myelinated axon, summarizing the localization of Necl4 and Necl1. BL, basal lamina; JXP, juxtaparanodal region; MV, microvilli; PNL, paranodal loops; PNJ, paranodal junction. Scale bars, 10 μm (**a**–**k**) and 5 μm (**i**–**o**).

online). Necl1-Fc and Necl4-Fc strongly bound to cells expressing either Necl1 or Necl4. Necl2-Fc and Necl3-Fc bound to cells expressing Necl1, and weakly to COS7 cells expressing Necl4. Neither Necl1-Fc nor Necl4-Fc bound to COS7 cells expressing other IgCAMs that mediate axon-glial contact, such as contactin, neurofascin, L1, MAG, TAG-1 and NrCAM (data not shown). These results support the notion that Necl1 and Necl4 comprise a binding pair in peripheral nerves.

Necl4 is the glial partner for axonal Necl1

To determine directly whether Necl4 is the Schwann cell binding partner for axonal Necls, we examined the ability of Necl1-Fc to bind to Schwann cells that were transfected with a short interfering RNA (siRNA) designed to knockdown the expression of Necl4. In contrast to cells transfected with a control siRNA, Schwann cells transfected with Necl4-specific siRNA showed reduced binding of Necl1-Fc in proportion to the reduction of Necl4 expression (**Fig. 4c**). Aggregating Necl1-Fc on the surface of Schwann cells using a secondary antibody to human Fc (hFc) specifically induced the coclustering of Necl4, but not of other membrane proteins such as gliomedin, which was clustered by NF155-Fc (**Fig. 4d**). In a reciprocal set of experiments, reduction of Necl1 expression in DRG neurons by transfection of siRNA abolished the binding of Necl4-Fc (**Fig. 4e**). Furthermore, aggregating Necl4-Fc on the surface of DRG neurites specifically induced coclustering of Necl1, but not of Necl2, Necl4 or other IgCAMs such as the axonal isoform of neurofascin, NF186 (**Fig. 4f** and data not shown). Altogether, these results demonstrate that Necl4 mediates the binding of axonal Necl1 to Schwann cells and that Necl1 is required for the interaction of Necl4 with the axon.

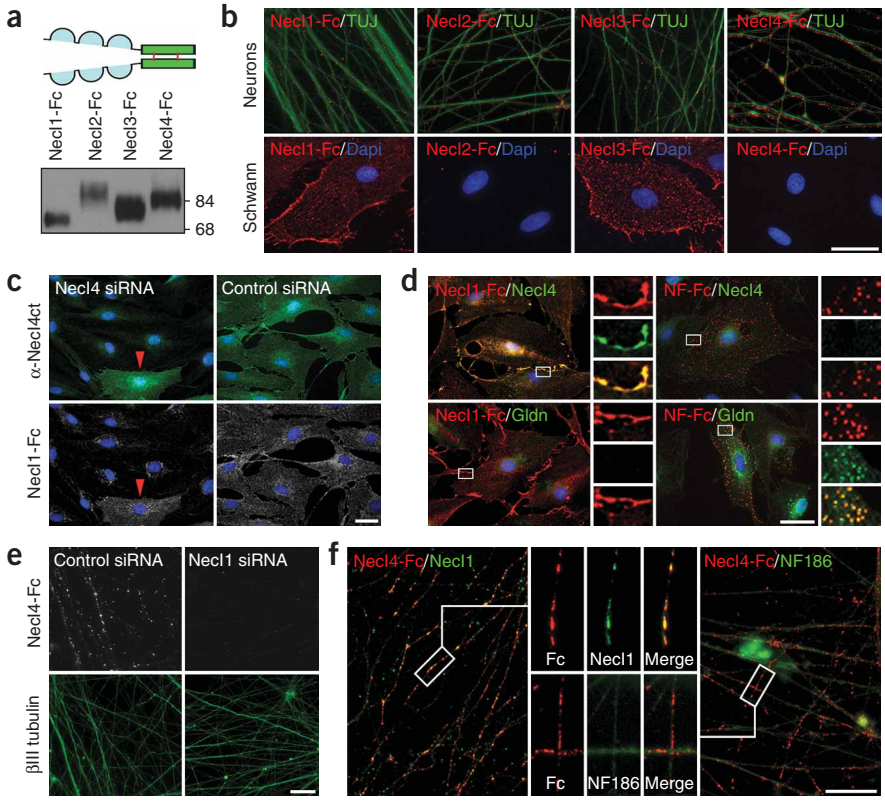

Figure 4 Necl4 is the glial binding partner for axonal Necl1. (**a**) Immunoblot of secreted Fc fusion proteins containing the extracellular domains of the four Necl proteins. (**b**) Differential binding of the four Necl proteins to DRG neurons and Schwann cells. Binding of Fc fusion proteins was detected using a secondary antibody to hFc, neurites were labeled with an antibody to βIII tubulin (TUJ), and DAPI was used to label Schwann cell nuclei. The images in the upper panels were shifted to permit better visualization. (**c**) Downregulation of Necl4 in Schwann cells abolished Necl1 binding. Necl1-Fc binding to Schwann cells transfected with a Necl4 or a control siRNA. Binding of Necl1-Fc and the expression of Necl4 (α-Necl4ct) are indicated. (**d**) Clustering of Necl4 in Schwann cells by Necl1-Fc. Soluble Necl1-Fc or NF155-Fc (NF-Fc) were aggregated on Schwann cells followed by immunolabeling for Necl4 or gliomedin (green) as indicated. Bound Necl1-Fc colocalized with Necl4. Insets show the separate channels and the merged image of the boxed area. (**e**) Downregulation of neuronal Necl1 abolished Necl4 binding. Binding of Necl4-Fc to DRG neurons transfected with a Necl1 or control siRNA. Neurites were labeled with an antiserum to βIII tubulin in the lower panels. (**f**) Necl4-Fc induces the clustering of axonal Necl1. Necl4-Fc was aggregated on DRG neurons followed by immunolabeling for Necl1 or the neuronal isoforms of neurofascin (NF186) as indicated. A higher magnification of the boxed region is shown as a single channel or the merged image as indicated. Scale bar, 40 μm.

To determine whether the interaction between Necl1 and Necl4 is sufficient to mediate axon-glial adhesion, we tested the ability of purified Schwann cells to adhere to an otherwise nonadhesive plastic surface coated with Necl1-Fc or Necl4-Fc, using hFc and laminin as negative and positive controls, respectively (**Fig. 5**). Schwann cells adhered to and spread on Necl1-Fc and laminin, but not on Necl4-Fc or hFc. In contrast to the adherence on laminin, efficient adherence of Schwann cells on Necl1-Fc did not require the presence of Ca^{2+} ions. Pretreating Necl1-Fc substrates with Necl4-Fc, but not with Necl1–Fc, before the addition of the Schwann cells completely abolished their ability to adhere to Necl1-Fc substrate.

Disruption of Necl1-Necl4 interaction inhibits myelination

The above results suggest that the *trans*-interaction between Necl4 and Necl1 mediates Schwann cell–axon adhesion. To determine whether disrupting this interaction affects myelination, we added Necl1-Fc, Necl4-Fc or Zig1-Fc (Zig1 is a related IgCAM expressed in Schwann cells[19]) to cocultures of DRG neurons and Schwann cells. Fc fusions were added before the induction of myelination, after Schwann cells had already aligned with axons. The cultures were supplemented with fresh Fc fusion proteins for an additional 10 d and then fixed and immunolabeled for MBP (**Fig. 6a–d**). The addition of Necl-Fc fusion proteins resulted in a marked reduction (~90% by Necl1-Fc and ~80% by Necl4-Fc, $P < 0.005$) in the number of myelin segments as compared with control-treated (Zig1-Fc) or untreated cultures (**Fig. 6e**). In contrast, we observed no effect on myelination using soluble extracellular domain of MAG (**Supplementary Fig. 5** online), indicating that although Necl4 and MAG colocalized in the nerve, they have different roles in PNS myelination. No effect on myelination was reported for the extracellular domains of other IgCAMs, including neurofascin[8,33] and NrCAM[34], further supporting the specificity of the effect of the Necl proteins. Notably, the addition of Necl-Fc fusion proteins to the culture had no effect on Schwann cell proliferation, as determined by the number of DAPI-labeled nuclei

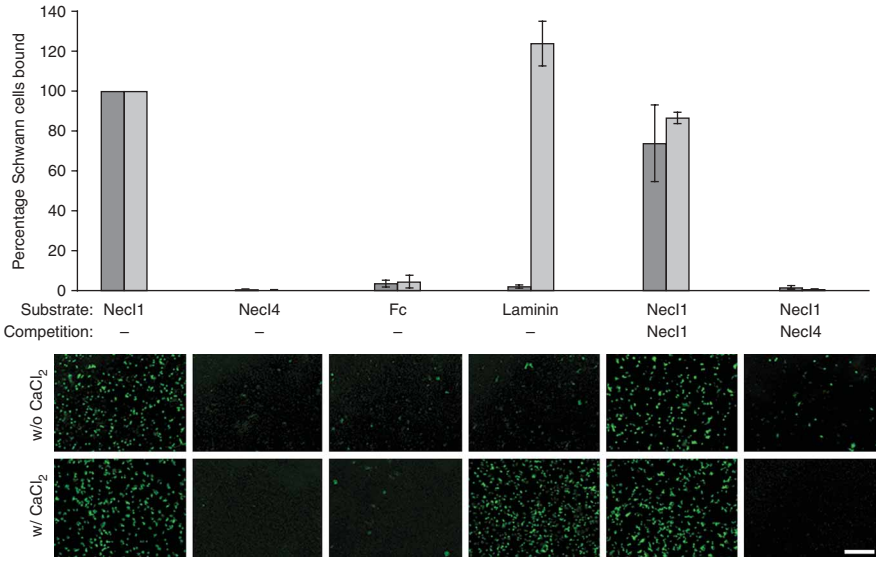

Figure 5 Interaction between Necl4 and Necl1 mediates Ca^{2+}-independent adhesion of Schwann cells. Schwann cells were added to a plastic dish precoated with Necl1-Fc or Necl4-Fc, the Fc region of human IgG (Fc), or laminin. Adherent cells were counted and were compared with the percentage of the cells adhering to Necl1-Fc in the presence (light gray bars) or absence (dark gray bars) of Ca^{2+} as indicated. Schwann cells adhered to Necl1-Fc and to laminin, but not to Necl4-Fc. Pretreating Necl1-Fc substrates with Necl4-Fc, but not with Necl1, before addition of Schwann cells completely abolished their adhesion. Low-magnification pictures taken from a representative experiment are shown below the graph. Scale bar, 1 mm.

per field of view (Necl1-Fc, 478 ± 39; Necl4-Fc, 438 ± 86; Zig1-Fc, 474 ± 40; no Fc, 461 ± 75; $n = 7$), or the percentage of BrdU-incorporated nuclei (Necl1-Fc, 8.3 ± 2.2; Necl4-Fc, 11.2 ± 2.1, Zig1-Fc, 7.4 ± 2.3; no Fc, 10.2 ± 1.9; $n = 7$). These results indicate that disruption of the axon-glia interactions mediated by Necl proteins inhibits myelination.

Electron microscopy analysis of the cultures 9 d after the induction of myelination revealed that many axons were ensheathed by Schwann cells in both the hFc control and the Necl4-Fc–treated cultures (**Fig. 6f–h**). However, while in the control cultures 68% ($n = 87$) of the Schwann cell processes wrapped around the axon at least 1.5 turns, in the Necl4-Fc–treated cultures membrane wrapping by Schwann cells was only detected in 12% ($n = 118$) of the cases (**Fig. 6h,i**). Instead, most (88%) of the Schwann cell processes that contacted an axon in the Necl4-Fc–treated cultures surrounded it only once or less. In these cultures we frequently detected Schwann cells sending long membrane protrusions (occasionally sufficient to make 1.5 turns around the axon) that failed to wrap around the axon, resulting in a horseshoe configuration (upper scheme in **Fig. 6h**). This analysis suggests that the Necl proteins are not required for the initial axon-glia contact, but rather for the complete ensheathment of the

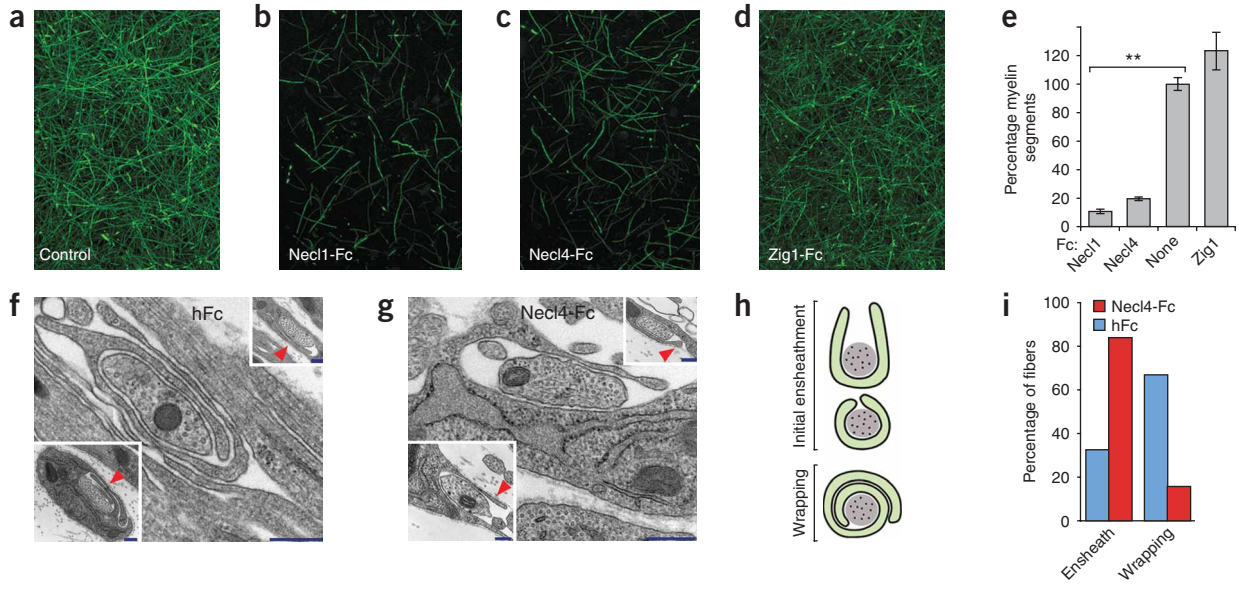

Figure 6 Necl-mediated axon-glia interaction is required for myelination. (**a–e**) Myelinated DRG cultures were left untreated (**a**) or grown in the presence of 50 μg ml^{-1} Necl1-Fc (**b**), Necl4-Fc (**c**) or Zig1-Fc (**d**) for 10 d, and then immunostained for MBP. The number of MBP-positive segments present in each condition is shown as a percentage of that in the untreated cultures (**e**). Cultures grown in the presence of the Necl-Fc proteins contained significantly (**$P < 0.005$) fewer myelin segments than did untreated or control-treated (Zig1-Fc) cultures. (**f–i**) Electron microscopy analysis of myelinating cultures. Representative images of 9-d-old DRG and Schwann cells cultures, grown in the presence of hFc (**f**) or Necl4-Fc (**g**). Two additional examples are shown in the insets. Although Schwann cell processes in the control Fc-treated cultures already circled 1.5 times around the axon, they did not do so when the cultures were grown in the presence of Necl4-Fc (red arrowheads). Schematic representation of axons ensheathed (U shape and O shape) and wrapped (1.5 turns and more) by myelinating Schwann cells (**h**). The numbers of ensheathed and wrapped axons in each culture are shown as a percentage of the total sites counted (hFc, $n = 87$; Necl4-Fc $n = 187$; **i**). Scale bars, 100 μm (**a–d**) and 20 nm (**g**,**h**).

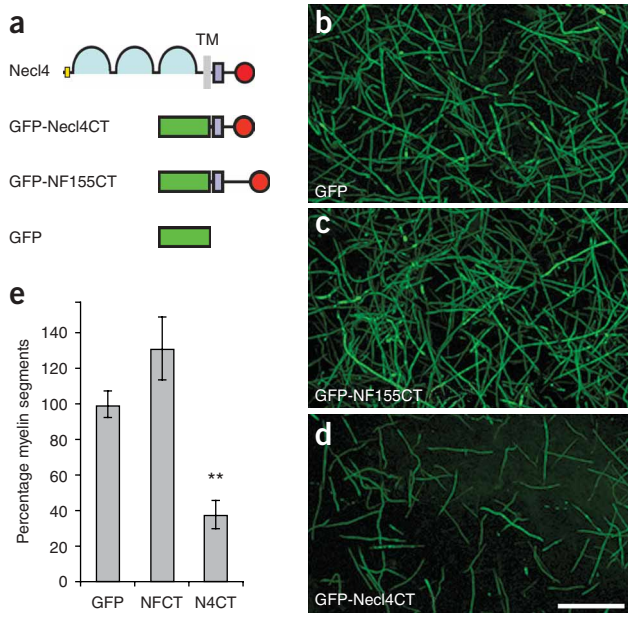

Figure 7 Expression of a dominant-negative Necl4 inhibits myelination. (**a**) Schematic of Necl4 and the different constructs used; the cytoplasmic domains of Necl4 and neurofascin 155 contain a 4.1 (purple square) and a PDZ (red circle) binding sequence. (**b**–**e**) Schwann cells were infected with retroviruses that direct the expression of GFP (**b**), GFP-NF155CT (**c**) or GFP-Necl4CT (**d**). Infected cells were allowed to myelinate sensory DRG axons, and were then fixed and immunolabeled for MBP. The extent of myelination was determined by counting the number of MBP-positive segments present in each condition (**e**). Note that the expression of GFP-Necl4ct in Schwann cells inhibited myelination by 65% (** $P < 0.001$), whereas that of the C terminus of neurofascin had no significant effect, as compared with cultures infected by GFP. Scale bar, 100 μm.

axon by Schwann cells and the transition from the ensheathing stage to myelin wrapping.

Inhibition of myelination by a dominant-negative Necl4

To further examine the role of Necl4 in myelination, we generated a dominant-negative Necl4 by fusing its cytoplasmic domain to GFP (GFP-Necl4CT; **Fig. 7a**). A similar mutant has been shown to interfere with the function of Necl2 in synapse formation in cultured hippocampal neurons[26], and it would be expected to disrupt the intracellular interactions of Necl4 with cytoskeletal and signaling components in Schwann cells. As a control for GFP-Necl4CT, we used a GFP fusion protein containing the cytoplasmic domain of neurofascin (GFP-NF155CT) that, like Necl4, contains consensus sequences for the binding of PDZ domains[35] and FERM (4.1-ezrin-radixin-moesin) proteins[36]. Both GFP-Necl4CT and GFP-NF155CT were incorporated into a retrovirus, and 3T3 fibroblasts infected with these retroviruses showed intracellular staining of the fusion protein (**Supplementary Fig. 6** online). The same viral stocks were then used to infect proliferating Schwann cells in dissociated DRG cultures[8] and myelination was analyzed 18 d later by counting MBP-positive myelin segments (**Fig. 7b–d**). Cultures infected with viruses encoding GFP-Necl4CT contained significantly fewer myelinated segments (reduced by ~60–65%; $P < 0.001$) than did GFP-infected cultures, whereas expression of GFP-NF155CT had no significant effect (**Fig. 7e**). There were no differences in the number or proliferation of Schwann cells between cultures infected with the different viruses. We thus concluded that expression of the cytoplasmic domain of Necl4 in Schwann cells specifically inhibits myelination.

Soluble Necl4 inhibits remyelination of sciatic nerve

To evaluate the significance of Necl1-Necl4 interactions during myelination *in vivo*, we made use of a PNS remyelination procedure[37]. In this experimental setting, demyelination is induced by lysolecithin and is followed by a period of remyelination that is fundamentally similar to developmental myelination. An advantage of this model is that it allows for an examination of the effect on myelination of various substances introduced directly into the nerve. Furthermore, it avoids the technical difficulties that arise when working with premyelinated axons of newborn animals because of the smaller size of their nerves and the fact that the perineurial barrier has not yet fully formed[38]. We induced a focal demyelinating lesion of sciatic nerves by intraneural injection of lysolecithin, and then injected either Necl4-Fc or hFc fusion proteins directly into the demyelinated site 5 d later and again at 8 d, at a time when Schwann cells actively remyelinate the lesion[39]. If Necl4 is indeed required for myelination, then addition of the Necl4-Fc fusion protein should function to inhibit remyelination in this model by competing with endogenous Schwann cell Necl4 for the binding of axonal Necl1.

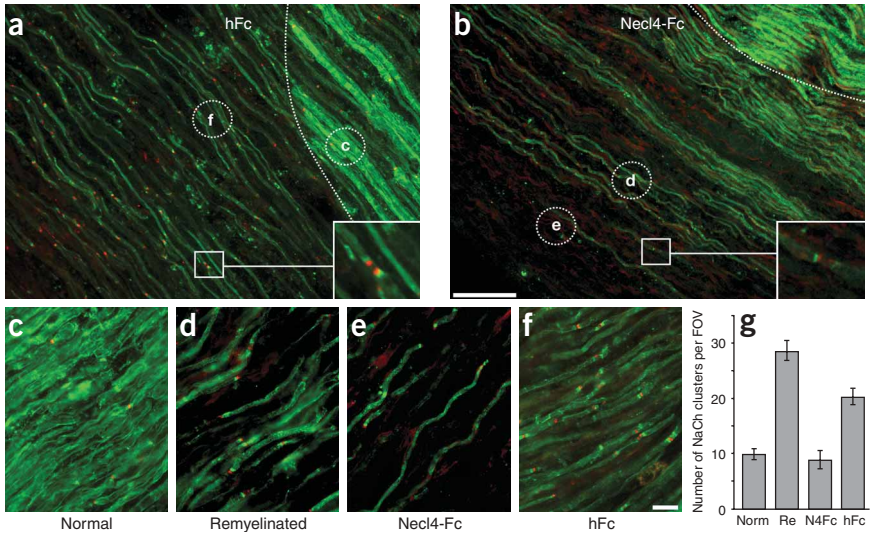

Figure 8 Necl4-Fc inhibits remyelination *in vivo*. (**a**–**f**) Demyelination of adult rat sciatic nerves was induced by lysolecithin and followed by intraneural injections of hFc (**a**) or Necl4-Fc (**b**). Nerves were collected 11 d after injection and longitudinal sections of the injected region were immunostained for MBP (green) and Na^+ channels (red). The border between the demyelinated and unaffected regions is marked by a dotted line. Insets depict higher-magnification images of selected nodes from remyelinated regions, many of which have binary clusters of Na^+ channels. Circles mark the locations of representative regions shown in **c**–**f**: unaffected nerve regions (**c**), remyelinated (**d**) or inhibited (**e**) areas in nerves injected with Necl4-Fc, as well as in remyelinated regions of nerves injected with hFc (**f**). Regions of inhibited remyelination were detected only in the Necl4-Fc nerves and not in the hFc control-injected nerves. (**g**) The number of Na^+ channel clusters found in the various regions defined in **c**–**f** was counted and is shown as an average number (mean ± s.e.m.) per field of view, FOV (a minimum of ten FOVs were counted for each region). Norm, normal; Re, remyelinated. Scale bars, 100 μm (**a**,**b**) and 20 μm (**c**–**f**).

We examined the injection sites at 11 d after injection by immunofluorescence labeling for MBP, as a marker for compact myelin, and for Na^+ channels, as a marker for nodes of Ranvier (**Fig. 8**). Na^+ channel clustering was used because new myelin segments are shorter after remyelination, resulting in an increased number of nodes of Ranvier[39]. In both the Necl4-Fc and hFc-injected nerves, we found unaffected regions that had intact myelin sheaths with relatively few nodes of Ranvier (**Fig. 8a**, region c), as well as remyelinated nerve fibers (**Fig. 8a**, region f, and **b**, region d). Remyelinated regions were clearly identified by reduced MBP immunoreactivity and the frequent occurrence of binary clusters of Na^+ channels (**Fig. 8a**,**b**, inset). However, in contrast to the control hFc-injected nerves, in the Necl4-Fc injected nerves we also observed large areas that had relatively few remyelinated axons (**Fig. 8b**, region e, and **Fig. 8e**). Compared with the remyelinated regions (**Fig. 8d**,**f**), these zones had 2–3-fold fewer Na^+ channel clusters (**Fig. 8e**,**g**), further indicating that the presence of a soluble extracellular domain of Necl4 in the demyelinated nerve inhibits remyelination.

DISCUSSION

In the present study we provide evidence that Necl4 (SynCAM4) and Necl1 (SynCAM3) mediate critical interactions between Schwann cells and axons during myelination. (i) These two CAMs are expressed early at axon-glial contact sites and are found on apposed cell membranes along the internode of myelinated axons (Schwann cells express Necl4 and axons express Necl1). (ii) Axonal contact increases the expression of Necl4 in Schwann cells, especially at the onset of myelination. (iii) Necl4 is the glial binding partner of axonal Necl1, and is both necessary and sufficient for Necl1 binding. (iv) Necl4 and Necl1 can recruit and cluster each other specifically to sites of higher ligand concentration on axons and Schwann cells, respectively. (v) The interaction between Necl1 and Necl4 mediates Schwann cell adhesion. (vi) Interfering with the Necl4-Necl1 interaction in myelinated cultures by using a soluble ectodomain of either molecule inhibits myelination. (vii) Expression of a dominant-negative mutant form of Necl4 in Schwann cells markedly reduces myelination. (viii) Intraneural injection of a soluble Necl4 protein inhibits remyelination of demyelinated sciatic nerves. On the basis of these results, we propose that Necl4 and Necl1 mediate the intricate Schwann cell–axon interaction that is required for myelination in the PNS.

Myelinating Schwann cells express several members of the immunoglobulin superfamily, including the major peripheral myelin protein P0 (ref. 40), which is essential for the generation of compact myelin, MAG, which is required for the maintenance of myelinated axons, and NF155 (ref. 6) and TAG-1 (ref. 7), both of which are necessary for the local differentiation of the axonal membrane around the nodes of Ranvier[17]. The Necl molecules described here represent a previously unknown axoglial cell-adhesion system in myelinated nerves. In the PNS, this adhesion system consists predominantly of two components, glial Necl4 and axonal Necl1. Binding experiments using transfected COS cells showed that the four Necls could bind to each other (heterophilically) and to themselves (homophilically). This result adds to previous studies showing that Necl1 mediates cell adhesion by binding to itself and to Necl2, as well as to some of the nectins[27]. *In situ* hybridization and immunohistochemistry revealed that several members of the Necl family are differentially distributed in the PNS: Necl4 is mainly found in myelinating Schwann cells, whereas sensory neurons express Necl1, Necl2 and Necl4. Nevertheless, in spite of the presence of these three Necls in DRG neurons, binding of Necl4 to axons requires Necl1, and aggregation of Necl4-Fc on DRG neurons specifically induced the clustering of Necl1, but not of the other Necls. In the reciprocal experiment, Necl1-Fc, but not Necl2-Fc or Necl4-Fc, bound to Necl4 in Schwann cells. These results indicate that *trans*-binding of Necl1 and Necl4 mediates the interaction between sensory axons and myelinating Schwann cells. The same interaction probably occurs between Schwann cells and the axons of motor neurons, as the latter express Nelcl1 (refs. 25,31), and Necl1 was detected in all myelinated axons in the sciatic nerve. Necls are well suited to transverse the 14–20-nm periaxonal space along the internodes, as transcellular recognition of Necl1 is mediated by its immunoglobulin-like V domain, which generates an antiparallel dimeric structure of the appropriate size[41].

Schwann cells regulate the molecular composition of the axonal membrane they ensheathe, thereby allowing rapid saltatory movement of action potentials along the nerve. The axolemma contains a unique set of cell recognition molecules and cytoskeletal linker proteins at the node of Ranvier, the paranodal junction, the juxtaparanodal region[17] and, as we show here, the internodal region. Immunolabeling of rat sciatic nerve revealed an axonal localization of Necl1 along the internodal membrane. Necl1 was also present at the juxtaparanodal region that is found at the end of each internodal segment, but was completely absent from the paranodal junction and the nodes of Ranvier. In cross-sections, Necl1 immunoreactivity was detected at the axonal perimeter, suggesting that it was located at the axolemma. This conclusion is further supported by recent immuno–electron microscopy data[27], which shows that Necl1 is concentrated along the axonal membrane.

In myelinating Schwann cells, Necl4 colocalized with MAG at the periaxonal membrane, as well as in SLIs, the paranodal loops and the inner mesaxon. Analysis of *Mag*-deficient mice shows that MAG is required to maintain the normal separation of the axonal and adaxonal Schwann cell membrane, but is not necessary for myelination[16]. Consistent with this, we found that Necl4-Fc, but not MAG-Fc, inhibited myelination of DRG neurons by Schwann cells in culture (**Supplementary Fig. 5**). Nevertheless, the remarkable colocalization of Necl4 with MAG, along with the ability of Necls to bind cytoskeletal adaptor molecules[25,27], raises the possibility that these two IgCAMs may have some overlapping functions in Schwann cells. In addition to its role in mediating Schwann cell–axon contact, Necl4 may also be important in the generation of autotypic junctions formed between the myelin lamellae at the Schmidt-Lanterman incisures and paranodal loops[18]. Necl proteins interact with several adaptor molecules[25,27] that are present at the incisures and paranodal loops[42,43], thereby providing a link between membrane adhesion and the enriched actin and spectrin cytoskeleton found at these sites[44].

Schwann cells appear to interact with axons at all developmental stages, from the early migration of precursor cells and the alignment of pro-myelinating Schwann cells along individual axons, to the ensheathment and spiral wrapping of the myelin membrane, as well as the formation of the nodes of Ranvier[16–18]. Our results suggest that Necl proteins mediate axon-glia interaction at the onset of myelination. First, the expression of Necl4 was markedly induced in Schwann cells that have aligned with axons. Second, Necls were already located at the axon-glia interface before the initial MAG^+ ensheathing stage and continued to be present throughout the process of myelination. The concentration of Necl4 at the axoglial interface during early Schwann cells–axon contact (**Fig. 2**) is reminiscent of the asymmetric localization of the polarity proteins Par-3 and Par-6, which have recently been suggested to serve as a scaffold for organizing CAMs and receptors during the initiation of myelination[45]. Third, interfering with the interaction between Necl4 and Necl1 resulted in inhibition of myelination, but had no effect either on the

proliferation or migration of Schwann cells, or on their longitudinal extension and alignment with the axon. Collectively, our results suggest an important role for Necl proteins during the late stage of axonal ensheathment and the initiation of myelination. Initial experiments using cultures of mixed cortical cells showed that, similar to our findings in the PNS, Necl4-Fc binds to neurons, whereas Necl1-Fc and Necl3-Fc bind to oligodendrocytes (data not shown), suggesting that these molecules may have a similar role in the CNS. The identification of Necls described here thus represents an important step toward understanding the molecular mechanisms that operate during myelination.

METHODS

RNA expression analysis. Semi-quantitative RT-PCR was carried out using the primers listed in the **Supplementary Methods** online. Total RNA was extracted with TriReagent (Sigma) and cDNA was prepared with the ImProm II Reverse Transcription System (Promega) using PolyT-primers; the amounts of the resulting cDNA were normalized using actin-specific primers. Northern blot analysis of Necl4 was performed as previously described[43] using the same probe that was used for *in situ* hybridizations. *In situ* hybridization was carried out using cRNA probes for Necl1–4 as described in the **Supplementary Methods**; hybridization was carried out at very stringent hybridization conditions (71.5 °C), as previously described[46].

Fc fusion binding, clustering and perturbation experiments. Binding experiments were carried out by incubating the cells with medium containing different Fc fusions proteins preincubated with anti–human Fc–Cy3, as described previously[8]. For clustering experiments, purified DRG neurons or isolated Schwann cells were incubated with medium containing the respective Fc fusion protein as described above, washed once, and grown for an additional 24 h (DRG) at 37 °C or 4 h (Schwann) before fixing. Fc perturbation experiments were carried out by adding 50 μg ml^{-1} purified proteins to the medium of dissociated DRG cultures 2 d before the induction of myelination. Medium containing the Fc fusion proteins was replaced every second day with fresh medium and the cultures were fixed and stained after 11–12 d of myelination. For illustration of the extent of myelination, pictures of all the MBP-positive segments of a representative slide were overlaid. Myelination was assayed by counting the MBP-positive segments on the coverslip, which were manually screened in a fluorescence microscope at low magnification using equal settings of the CCD camera (Hamamatsu) for all coverslips in a given experiment. All MBP-positive segments were photographed and counted with an in house–developed application for MatLab7.0 (MathWorks; the application will be provided on request). These experiments were repeated two or three times with three or four samples per treatment, and the differences between the means of various treatments were calculated with Welch's test for a two-sample comparison of means with unequal variances between the averages with and without treatment[47].

Electron microscopy of myelinating cultures. Cultures grown on coverslips were washed three times in Kosnovsky fixative (2% glutaraldehyde, 3% PFA and 3% sucrose in 0.1 M cacodylate buffer), fixed in the same fixative for 2.5 h at 22 °C and an additional 48 h at 4 °C, washed four times in 0.1 M cacodylate buffer and kept at 4 °C until further use. Osmification was made in 1% OsO_4, 0.5% $K_2Cr_2O_7$, 0.5% $K_4[Fe(CN)_6]\cdot 3H_2O$, 3% sucrose (in 0.1 M cacodylate buffer) for 2 h at 22 °C before being washed twice in 3% sucrose (in 0.1 M cacodylatebuffer) and three times in double-distilled water. For enhancement, cultures were impregnated with 2% uranyl acetate for 1 h. Dehydrated samples were incubated with increasing concentrations of Epon ('hard'). Glass coverslips were removed by 2-h treatment with 30% fluoric acid, washed in double-distilled water and dried overnight in an oven. The Epon blocks of the cultures were then roughly cut into small pieces and re-embedded. Finally, 70–100-nm ultra-thin slices were cut from each block, mounted onto grids and analyzed in a CM-12 Philips electron microscope equipped with a BioCam CCD camera.

Demyelination and intraneural injection of fusion proteins. All experiments involving animals were performed in accordance with the US National Institutes of Health Guidelines for the humane treatment of animals. Adult Sprague-Dawley rats were anesthetized and the sciatic nerve was exposed. Nerves were injected with 2–3 μl of 1% lysolecithin in sterile Locke's solution (pH 7.4) by using a glass micropipette. To mark the injection sites and observe the filling of each nerve, 0.05% Fast Green (Sigma) was included in the lysolecithin solution. Each incision was then closed and rats were returned to cages for recovery. Rats were anesthetized 5 d after the original lysolecithin injection, and then again at 8 d, and each demyelinated site was injected with 3 μl of 3 mg ml^{-1} hFc or Necl4-Fc (three rats for each fusion protein) diluted in PBS containing 0.05% Fast Green. Each incision was then closed and rats were returned to cages for recovery. Finally, 11 d after the initial injection of lysolecithin, the rats were killed and the injected nerves were collected and fixed for 30 min using 4% paraformaldehyde. Nerves were cryoprotected overnight in 20% sucrose, cut into 16-μm-thick sections, and immunostained as described previously[48].

All other methods are described in the **Supplementary Methods** section.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

I.S. cloned and constructed all expression constructs and probes, generated and purified antibodies, and designed and performed most of the experiments. He was assisted by R.M. in analyzing the myelination data. K.A. performed the cell adhesion assay. O.S.N. and S.S.S contributed to gene expression analysis, Y.E. to the coculture experiments and I.H. to the immunohistochemical analysis. H.S. performed the electron microscopy and M.N.R. conducted the remyelination experiments. E.P. headed the project and prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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