

# LINGO-1, a Transmembrane Signaling Protein, Inhibits Oligodendrocyte Differentiation and Myelination through Intercellular Self-interactions<sup>[5]</sup>

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**Background:** Myelination of axons by oligodendrocytes is critical for neuronal function. LINGO-1, a cell receptor, inhibits myelination.

**Results:** An extracellular fragment of LINGO-1 binds to LINGO-1 displayed on a cell surface and also inhibits oligodendrocyte maturation and myelination.

**Conclusion:** LINGO-1 in axons and oligodendrocytes can self-interact to inhibit myelination.

**Significance:** Understanding mechanisms governing myelination may lead to the treatment of demyelinating diseases like multiple sclerosis.

Overcoming remyelination failure is a major goal of new therapies for demyelinating diseases like multiple sclerosis. LINGO-1, a key negative regulator of myelination, is a transmembrane signaling protein expressed in both neurons and oligodendrocytes. In neurons, LINGO-1 is an integral component of the Nogo receptor complex, which inhibits axonal growth via RhoA. Because the only ligand-binding subunit of this complex, the Nogo receptor, is absent in oligodendrocytes, the extracellular signals that inhibit myelination through a LINGO-1-mediated mechanism are unknown. Here we show that LINGO-1 inhibits oligodendrocyte terminal differentiation through intercellular interactions and is capable of a self-association in trans. Consistent with previous reports, overexpression of full-length LINGO-1 inhibited differentiation of oligodendrocyte precursor cells (OPCs). Unexpectedly, treatment with a soluble recombinant LINGO-1 ectodomain also had an inhibitory effect on OPCs and decreased myelinated axonal segments in cocultures with neurons from dorsal root ganglia. We demonstrated LINGO-1-mediated inhibition of OPCs through intercellular signaling by using a surface-bound LINGO-1 construct expressed ectopically in astrocytes. Further investigation showed that the soluble LINGO-1 ectodomain can interact with itself in trans by binding to CHO cells expressing full-length LINGO-1. Finally, we observed that soluble LINGO-1 could activate RhoA in OPCs. We propose that LINGO-1 acts as both a ligand and a receptor and that the mechanism by which it negatively regulates OPC differentiation and myelination is mediated by a homophilic intercellular interaction. Disruption of this protein-protein interaction could lead to

a decrease of LINGO-1 inhibition and an increase in myelination.

Understanding the cellular and molecular mechanisms regulating axo-glial communication and myelination in the developing and adult CNS is essential for the generation of treatments to induce effective repair in demyelinating disorders like multiple sclerosis. To date, only a few regulators of this process have been described. Notably,  $\gamma$ -secretase (1), CXCR2 (2), and LINGO-1 (3) inhibit myelination, whereas neuregulin1 promotes myelination by oligodendrocytes (4, 5) and is an essential signal in the peripheral central nervous system (4). LINGO-1, in particular, has been identified as a key negative regulator of myelination during development (3), and preventing its function has been successful in promoting recovery in inflammatory and non-inflammatory animal models of demyelination (6, 7). LINGO-1 is a CNS-specific single transmembrane protein expressed in neurons and in oligodendrocytes but absent in astrocytes (3). In neurons, it functions as an essential coreceptor of the Nogo receptor complex that mediates the inhibition of axonal growth because of regulatory factors present in myelin (8). In this context, its coreceptor partners are the Nogo-66 receptor and p75NGF (8). The latter can be substituted in the complex by TAJ/TROY, another TNF family receptor (9, 10). In addition to its inhibitory role in myelination, LINGO-1 can also act as a negative regulator of BDNF signaling through direct interaction with trkB (11). Although LINGO-1 binds to other receptors to regulate their activity and is also an essential signaling coreceptor within the NgR complex, it has no known direct ligand. The ligands of the NgR complex, Nogo-66, myelin associated glycoprotein (MAG), and OMgp, all bind the glycosphatidylinositol-anchored NgR, using its coreceptors to transduce an intracellular signaling event (reviewed in Ref. 12).

In oligodendrocytes, LINGO-1 has been identified as a negative regulator of OPC differentiation and myelination using a

<sup>[5]</sup>This article contains supplemental Figs. 1–5 and experimental procedures.

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variety of molecular and genetic tools. These include loss of function studies using siRNA, blocking antibodies, and null-mutant mice (3, 6, 7) as well as gain of function studies using lentiviral overexpression and transgenic mice (3, 13). Nevertheless, in the oligodendrocyte lineage, coreceptor complexes containing LINGO-1 (analogous to those found in neurons) or ligands for LINGO-1 have not been identified. Consequently, the extracellular signal responsible for regulating myelination through LINGO-1 expressed in oligodendrocytes is currently unknown. We present experimental evidence that LINGO-1 can operate in trans, as a ligand, and inhibit OPC differentiation and myelination.

## EXPERIMENTAL PROCEDURES

**Reagents**—Unless indicated otherwise, all reagents were from Sigma-Aldrich (St. Louis, MO), except for cell culture basal media and reagents, which were from Invitrogen. Poly-D-lysine (PDL)<sup>2</sup>-coated tissue culture plates and flasks and Matrigel were from BD Biosciences.

**Statistical Analysis**—GraphPad Prism was used for all statistical analysis using one-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons, or Student's *t* test for pairwise comparisons, as appropriate and as indicated in the figure legends. A value of  $p < 0.05$  was considered significant.

**Lentiviral Constructs**—Sequences corresponding to full-length rat LINGO-1, M1-I614, (FL-LINGO-1, GenBank<sup>TM</sup> accession number NM\_001100722) and truncated LINGO-1, M1-G581 (ECTO-LINGO-1), containing the extracellular and transmembrane domains, were cloned into the NotI and BamHI sites of the lentiviral vector HRST-IRES-eGFP (14) under the control of the cytomegalovirus promoter. Virus was generated at the Harvard Gene Therapy Initiative facility by transient transfection into 293T cells, filtered through a 0.45- $\mu$ m membrane and concentrated by ultracentrifugation. Viral titer was determined by Southern blot analysis on infected U2Os cells.

**Soluble LINGO-1 Stable Cell Lines**—The cDNA sequence of the human LINGO-1 ectodomain (M1-E532, NCBI reference sequence NP\_116197.4) was cloned into the EcoRI and BglII sites of the pFUSE-hIgG1-Fc vector (Invivogen, San Diego, CA) so that the Fc domain of human IgG1 was fused to the C terminus of LINGO-1 ectodomain. All constructs were sequence-verified. CHO-K1 cells (ATCC) were cultured in F-12 medium (Invitrogen) supplemented with 10% FBS. The cells were then transfected with the pFUSE-Lingo-Fc plasmid using FuGENE6 reagent according to the product manual (Roche). Forty-eight hours after transfection, cells were treated with 0.25% Trypsin-EDTA in Hanks' buffered saline, diluted 100-fold in F-12 medium, and plated into multiple 10-cm<sup>2</sup> culture dishes. We isolated single cell clones in the presence of 1 mg/ml zeocin, and the highest expressors of secreted LINGO-1-Fc, determined by

Western blotting of the supernatant using anti-LINGO-1 mAb (R&D Systems, Minneapolis, MN), were established for protein production by further expansion. The identity of the expressed protein was verified by MALDI-TOF mass spectrometry. Production of soluble LINGO-1-Fc was performed in Bellocells (Cesco Engineering) seeded with  $2 \times 10^7$  cells in 500 ml of F-12 media containing 5% FBS, 100 IU penicillin, and 100  $\mu$ g/ml streptomycin. Growth of the cells was done using 30 s of top hold time and 5 min of bottom hold time with medium change every 2 days for 10 days. Protein production was done with no top hold time and 10 min of bottom hold time the first week and then increased to 18 to 20 min of bottom hold time for the remaining production run. Media containing soluble LINGO-1-Fc was harvested every 3 to 4 days with 450 ml of fresh medium added at each harvest.

**Purification of Soluble LINGO-1 Protein**—Bellocell supernatants were adjusted to pH 7.5 adding 1 M HEPES to a final concentration of 50 mM and centrifuged at 1000 rpm for 10 min to remove residual cell debris. Soluble LINGO-1-Fc was bound overnight to 400  $\mu$ l of rProteinA resin (GE Life Sciences) for every 500 ml of expression media. The resin was collected in a column, washed with 4 column volumes of PBS and then 3 column volumes of high-salt PBS (800 mM NaCl, 10 mM phosphate, 2.7 mM KCl (pH 7.4)), and protein was eluted with 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl (pH 2.8). Neutralization buffer (0.5 M NaH<sub>2</sub>PO<sub>4</sub> (pH 8.6)) was then added immediately (25  $\mu$ l/1 ml eluate). Pooled elutions were purified by size-exclusion chromatography on a Superdex 200 column (GE Life Sciences) in 140 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol and concentrated to 1 mg/ml in Vivaspin 20, 30 kDa molecular weight cut-off protein concentrators (Vivaproducts).

**OPC/Oligodendrocyte Cultures**—Rat OPC cultures were prepared according to McCarthy and DeVellis (15) with modifications (16). Briefly, cortical hemispheres from postnatal day 1 rat pups were cleared of meninges, minced with a razor blade, and incubated 15 min in 0.01% trypsin and 500,000 units of DNase I in Hanks' balanced salt solution (Invitrogen) at 37 °C. Tissue was centrifuged, supernatant was replaced by DMEM containing 20% fetal calf serum, triturated with a 10 ml serological pipette, and filtered twice through a 100- $\mu$ m cell strainer. Volume was adjusted to 1 brain/10 ml of DMEM, 20% FCS and plated onto 75-ml PDL-coated flasks at 1 brain/flask. Cultures were fed every 3 days by gently replacing the growth medium. On day 11, flasks were shaken for 1 h at 200 rpm on an orbital shaker at 37 °C to remove microglia and dead cells. The medium was discarded, replaced with fresh medium, and flasks were shaken for 18–24 h at 200 rpm and 37 °C. The medium was removed from flasks and plated on untreated Petri dishes for 30 min at 37 °C to remove microglia. Supernatant was collected, filtered twice through a 40- $\mu$ m cell strainer, and centrifuged at 1000 rpm for 30 min. The cell pellet was resuspended in oligodendrocyte basal defined medium (BDM) growth medium (16) consisting of DMEM, 4 mM L-glutamine, 1 mM pyruvate, 0.1% w/v BSA, 50  $\mu$ g/ml apo-transferrin, 5  $\mu$ g/ml insulin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone, 13 IU/ml penicillin, and 13  $\mu$ g/ml streptomycin.

<sup>2</sup> The abbreviations used are: PDL, poly-D-lysine; DRG, dorsal root ganglion; DAPT, N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester; LINGO-1, LRR and Ig domain-containing, Nogo Receptor-interacting protein; MBP, myelin basic protein; m.o.i., multiplicity of infection; ANOVA, analysis of variance; CNTF, ciliary neurotrophic factor; OPC, oligodendrocyte precursor cell.

## LINGO-1 Regulates Myelination in Trans

OPC cultures were maintained in BDM containing 10 ng/ml PDGF and 10 ng/ml FGF (Preprotech) with medium replacement every other day.

Mouse OPCs were derived from oligosphere cultures prepared as described (17). Briefly, embryonic day 14.5 mouse cortical hemispheres were mechanically triturated in NPH medium (DMEM/F-12 1:1,  $1 \times B27$  supplements, 2 mM L-Gln, 10 ng/ml EGF) and plated as suspension cultures at 2 brains/flask in 9 ml NPH into 25-cm<sup>2</sup> culture flasks. Neurospheres were passaged every 3–4 days at a 1:3 ratio for up to 6 passages. Oligospheres were induced by mechanically triturating neurospheres in OPM medium (NPH without EGF, 10 ng/ml FGF, 10 ng/ml PDGF). Cells were plated at  $1 \times 10^5$  in 9 ml OPM in 25-cm<sup>2</sup> uncoated flasks. Oligospheres were passaged up to 6 times at a ratio of 1:2 every 72 h. Mouse OPCs were obtained from oligospheres by mechanical dissociation and plated in OPM on PDL-coated tissue culture plates.

To induce differentiation of either rat or mouse OPC cultures, PDGF and FGF were removed from the medium. In some instances, differentiation was further induced by addition of 1 ng/ml CNTF, 1.5 nM T3 or 10 ng/ml CNTF, 15 nM T3, as indicated in the text. For experiments with soluble LINGO-1-Fc, 4 days after plating, the PDGF/FGF medium was removed and replaced with fresh medium containing LINGO-1-Fc with or without CNTF/T3, as indicated in the figures.

**DRG-OPC Cocultures**—DRGs were dissected from embryonic day 15 rat embryos, incubated for 30 min in 0.05% trypsin/EDTA, 0.04% DNaseI in HBSS at 37 °C, triturated with the tip of a 1000- $\mu$ l Gilson pipette and centrifuged at 800 rpm for 5 min. The cell pellet was resuspended in DMEM, 10% FCS, 50 IU penicillin, 50  $\mu$ g/ml streptomycin, 2 mM L-Gln containing, 100 ng/ml NGF (Serotec, Raleigh, NC), plus 10  $\mu$ M 5 fluoro-deoxyuridine and 10  $\mu$ M uridine to remove any contaminating fibroblasts, and cells were plated at  $1 \times 10^5$  cells/well into 24-well plates precoated with reduced growth factor Matrigel. After 1 week, the medium was switched to neurobasal medium supplemented with  $1 \times B27$ , 50 IU penicillin, 50  $\mu$ g/ml streptomycin, 2 mM L-Gln, and 100 ng/ml NGF. During week 3, OPCs purified from rats as described above were added to the DRGs at  $2 \times 10^5$  cells/well using the same growth medium without NGF and in the presence of 50  $\mu$ g/ml soluble LINGO-1-Fc or 2  $\mu$ M DAPT, a  $\gamma$ -secretase inhibitor reported to increase myelination (1). Cells were incubated an additional 2 weeks and were fed fresh media containing soluble LINGO-1-Fc, or DAPT every 3–4 days. At 14 days *in vitro*, cocultures were fixed with 4% paraformaldehyde, then stained for myelin basic protein (MBP, Covance SMI99, 1:1000) and Neurofilament H (Serotec AHP245, 1:1000) to highlight the axons, followed by Alexa Fluor-conjugated secondary antibodies (Invitrogen, Alexa Fluor 488 and 594), as described below. Because of the absence of known molecular markers specific for compact myelin, myelination in these cultures was assessed blindly by trained observers using a combination of MBP<sup>+</sup> staining together with the characteristic linear morphology displayed by myelinated axonal segments (Fig. 4). In addition, myelin segments identified by MBP staining were counted as described by Taveggia *et al.* (5) in at least 18 high-power fields in regions of the wells with comparable numbers of neurites in two independent experiments.

**Lentiviral Infection**—OPCs (rat or mouse) were plated into either 24-well or 96-well PDL-coated plates in BDM containing 10 ng/ml PDGF and 10 ng/ml FGF for 2 days. The medium was replaced with fresh PDGF/FGF medium, containing FL-LINGO-1 lentivirus, truncated-LINGO-1 lentivirus, or control lentivirus at 2.5 m.o.i. The experiments with mouse OPCs in figure 2A also show cultures infected at 0.5 m.o.i. Cells were incubated for 24 h, virus-containing medium was removed, and cells were fed with BDM medium containing CNTF and T3, as indicated in the figures, to induce differentiation. Successful infection was monitored by Western blotting (FL-LINGO-1, Abcam) and enhanced GFP expression (supplemental Fig. 4).

**Immunocytochemistry**—Cell cultures were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and rinsed 3 times for 5 min in PBS. For MBP and NG2 (Chemicon/Millipore, AB5320, 1:200) staining, cells were blocked and permeabilized in PBS containing 5% normal goat serum and 0.1% Triton X-100 for 1 h at room temperature and then incubated in primary antibody in permeabilization buffer overnight at 4 °C. Subsequently, cells were rinsed 3 times for 5 min in permeabilization buffer and incubated for 1 h at room temperature in Alexa Fluor 488 or Alexa Fluor 596 goat anti-rabbit or goat anti-mouse IgG secondary antibodies at 1:1000 in permeabilization buffer. Finally, cells were rinsed 3 times for 5 min in PBS and stored in SlowFade gold. For O4 staining, cells were incubated live in O4 primary antibody (Chemicon, 1:50) at 37 °C for 30 min, rinsed twice in PBS, and fixed in 4% paraformaldehyde. Subsequently, cells were processed as described above to double-stain with MBP primary antibodies. Cells stained with O4 received Alexa Fluor 596 goat anti-mouse IgM, 1:1000.

For quantification, plates were scanned on a Cellomics Arrayscan VTI. MBP and O4 staining intensity was measured from 9 fields/well at  $\times 5$  magnification and normalized to total cell number determined by Hoechst 33342-stained nuclei. We confirmed that changes in MBP staining intensity correlated with numbers of MBP<sup>+</sup> cells obtained by manual counting (supplemental Fig. 3B).

**Astrocyte Cultures and Infection with LINGO-1 Lentiviruses**—Astrocytes were obtained from P1 rat cortical hemisphere cultures used to prepare rat OPCs, with the following modification: after removing OPCs and microglia by mechanical shaking as described above, medium was added to the flasks and an additional vigorous shaking at 500 rpm was carried out for 30 min. Flasks were rinsed twice with PBS with an additional vigorous shaking by hand, and the astrocytes remaining attached to the flask were removed by incubating in 2 ml 0.05% trypsin-EDTA in HBSS at 37 °C until cells detached ( $\sim 3$ –5 min). Astrocytes were centrifuged at 1000 rpm for 10 min, the cell pellet was resuspended in BDM oligodendrocyte growth medium, and cells were plated at a density of 200,000 cells/well in poly-D-lysine-coated 96-well plates to generate quickly a confluent monolayer. Cells were allowed to attach for 1 h in a tissue culture incubator and subsequently infected with FL-LINGO-1 lentivirus, truncated-LINGO-1 lentivirus, or control lentivirus at 2 m.o.i. After 24 h, medium was replaced, and astrocytes were incubated for a total of 72 h. Infection was assessed by monitoring expression of the viral enhanced GFP reporter. At the end of the 72-h period, freshly isolated rat OPCs were plated on the

lentiviral-infected astrocyte monolayer at a density of 5000 OPCs/well in oligodendrocyte growth medium containing 1 ng/ml CNTF, 1.5 nM T3. Cultures were incubated for an additional 4 days and processed for MBP and O4 antibody staining as described above, except that secondary antibodies used were Alexa Fluor 596 goat anti-mouse IgG, 1:1000, for MBP and Alexa Fluor 350 goat anti-mouse IgM, 1:200, for O4.

**Western Blotting**—At the end of treatment, cells were lysed in radioimmune precipitation assay buffer (Millipore, 20-188). Protein samples were run on NuPage 4–12% Bis-Tris gels (Invitrogen) and then transferred to a nitrocellulose membrane and probed using standard techniques with antibodies against MBP (Covance, SMI99, 1:1000), LINGO-1 (Abcam, Ab23631, 1:1000) and  $\beta$  tubulin for loading controls (Santa Cruz Biotechnology, SC9104, 1:2000). Membranes were then scanned using the LiCor Odyssey machine. Densitometric quantitation from the grayscale images was performed using the gel analysis macro included in the ImageJ software (National Institutes of Health) (18).

**Full-length LINGO-1 Stable CHO Cells**—CHO cells were stably transfected to overexpress full-length LINGO-1 on the cell surface using plasmid pCLPCX (derived from pLPCX, Clontech, by insertion of a CMV promoter upstream of the 5' LTR) containing the full coding sequence of human LINGO-1 (M1-I620). We isolated single cell clones in the presence of puromycin, and the highest expressors, determined by Western blotting for total LINGO-1 expression, were chosen for subsequent binding assays.

**Soluble LINGO-1 Binding**—Parent and LINGO-1 CHO cells were plated into 96-well PDL-coated plates at  $2.5 \times 10^4$  cells/well and left to attach overnight. Soluble LINGO-1-Fc was preclustered with an equimolar concentration of alkaline phosphatase-labeled anti-Fc antibody (Chemicon, Temecula, CA, AP113A) for 45 min at room temperature. All reagents were diluted in binding buffer (HBSS, 20 mM HEPES, 1% FBS). Following preclustering, medium was removed from the cells, and 50  $\mu$ l of the complexes were added to the wells and incubated for 90 min at 37 °C. Plates were washed 5 times in ice-cold binding buffer, fixed for 10 min in 4% paraformaldehyde, and then washed an additional 3 times. After the final wash, the substrate p-Nitrophenylphosphate (Vector Laboratories, SK-5900), containing Levamisole (Vector Laboratories, SP-5000) as directed by the manufacturer, was added, and the plates were read at 405 nm on a plate reader (Molecular Devices, Spectramax) as the color developed. Competitive binding displacement to OPCs was performed on cells plated into 96-well plates at  $5 \times 10^3$  cells/well and grown for 2 days in BDM containing 10 ng/ml PDGF and 10 ng/ml FGF. Binding was carried out as described above for CHO cells, with the following exceptions: untagged soluble LINGO-1 generated by cleavage of the Fc fragment was added to the binding reagents just prior to cell incubation at the concentrations indicated in Fig. 6C, and binding buffer consisted of DMEM/F-12, 1% FBS.

Binding to purified rat OPCs was performed by adding the soluble LINGO-1 ectodomain at the concentrations indicated in Fig. 6B to OPCs grown in 96-well plates as described above. Cells were rinsed 3 times in cold PBS and processed for West-

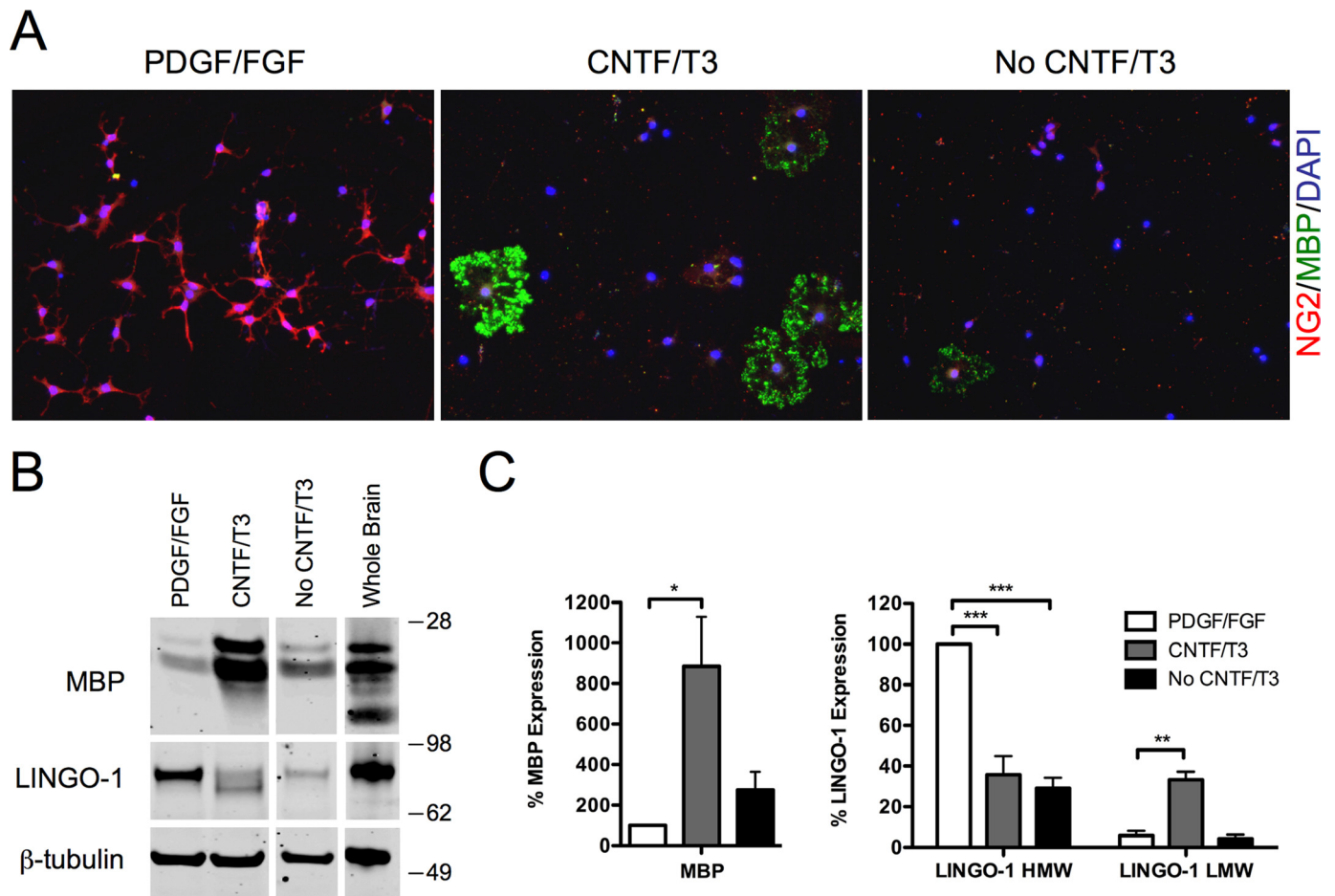
ern blotting as described. The presence of bound soluble LINGO-1 was detected with an antibody against the Fc tag.

**Analysis of RhoA Activity**—Three-day-old rat OPCs incubated in oligodendrocyte growth medium containing 10 ng/ml PDGF, 10 ng/ml FGF were treated with 100  $\mu$ g/ml soluble LINGO-1-Fc, Fc-alone, or 5 ng/ml lysophosphatidic acid for 15 and 60 min. Cells were then lysed, and GTP-bound RhoA was immunoprecipitated using a conformation-specific antibody against active RhoA (80601, NewEast Biosciences, Malvern, PA) following the instructions of the manufacturer. Active and total RhoA were analyzed by Western blotting of the immunoprecipitates and total cell lysates, respectively, using an antibody against total RhoA (NewEast Biosciences).

## RESULTS

**LINGO-1 Expression Is Down-regulated during OPC Maturation**—As a first step in understanding the role of LINGO-1 in OPC differentiation and maturation, we analyzed its levels of protein expression in purified OPC cultures as a function of differentiation *in vitro*. We assessed maturation and differentiation in the cultures by immunocytochemistry, staining for the OPC marker NG2 and mature oligodendrocyte marker MBP. In the presence of PDGF/FGF, cultures contained abundant numbers of NG2<sup>+</sup> cells and no MBP expression was detected under those conditions (Fig. 1A, left panel). Removal of PDGF/FGF was accompanied by a decrease in NG2 expression and a subsequent appearance of a few MBP<sup>+</sup> cells (Fig. 1A, right panel). The addition of CNTF and T3 greatly increased the proportion of mature oligodendrocytes that extended sheets of MBP<sup>+</sup> membrane (Fig. 1A, center panel). Western blot analysis indicated that LINGO-1 was abundantly expressed by OPCs as an ~95-kDa protein in the presence of PDGF/FGF. This protein band decreased substantially by approximately two thirds after PDGF/FGF removal with or without CNTF/T3 stimulation (Fig. 1, B and C, and supplemental Fig. 1). Occasionally, the single, ~95-kDa band corresponding to LINGO-1 appeared as a doublet containing a lower molecular weight species after treatment with CNTF/T3 (Fig. 1B). The expression levels of the smaller band represented approximately one-third the amount of LINGO-1 present in PDGF/FGF (Fig. 1C). MBP expression showed the opposite pattern, with two characteristically abundant 21.5- and 18.5-kDa isoforms in cultured oligodendrocytes (19) increasing as the cells differentiated (Fig. 1B), consistent with our results from immunostainings of OPC cultures (Fig. 1A). These results indicate that LINGO-1 expression is inversely correlated with OPC differentiation and maturation.

**Extracellular Domain of LINGO-1 Is Sufficient to Inhibit OPC Differentiation**—To test the effect of sustained LINGO-1 expression on OPC differentiation, mouse and rat OPC cultures were infected with a lentiviral construct expressing full-length LINGO-1 or a control virus in the presence of PDGF/FGF. After 48 h, PDGF and FGF were removed from the medium, and cells were allowed to differentiate. In some cultures, differentiation was further stimulated by adding CNTF and T3. We assessed OPC differentiation by levels of MBP protein expression. Overexpression of full-length LINGO-1 in OPC cultures (supplemental Fig. 4A) led to reduced expression of MBP, as detected by Western blotting (Fig. 2, A and B, and

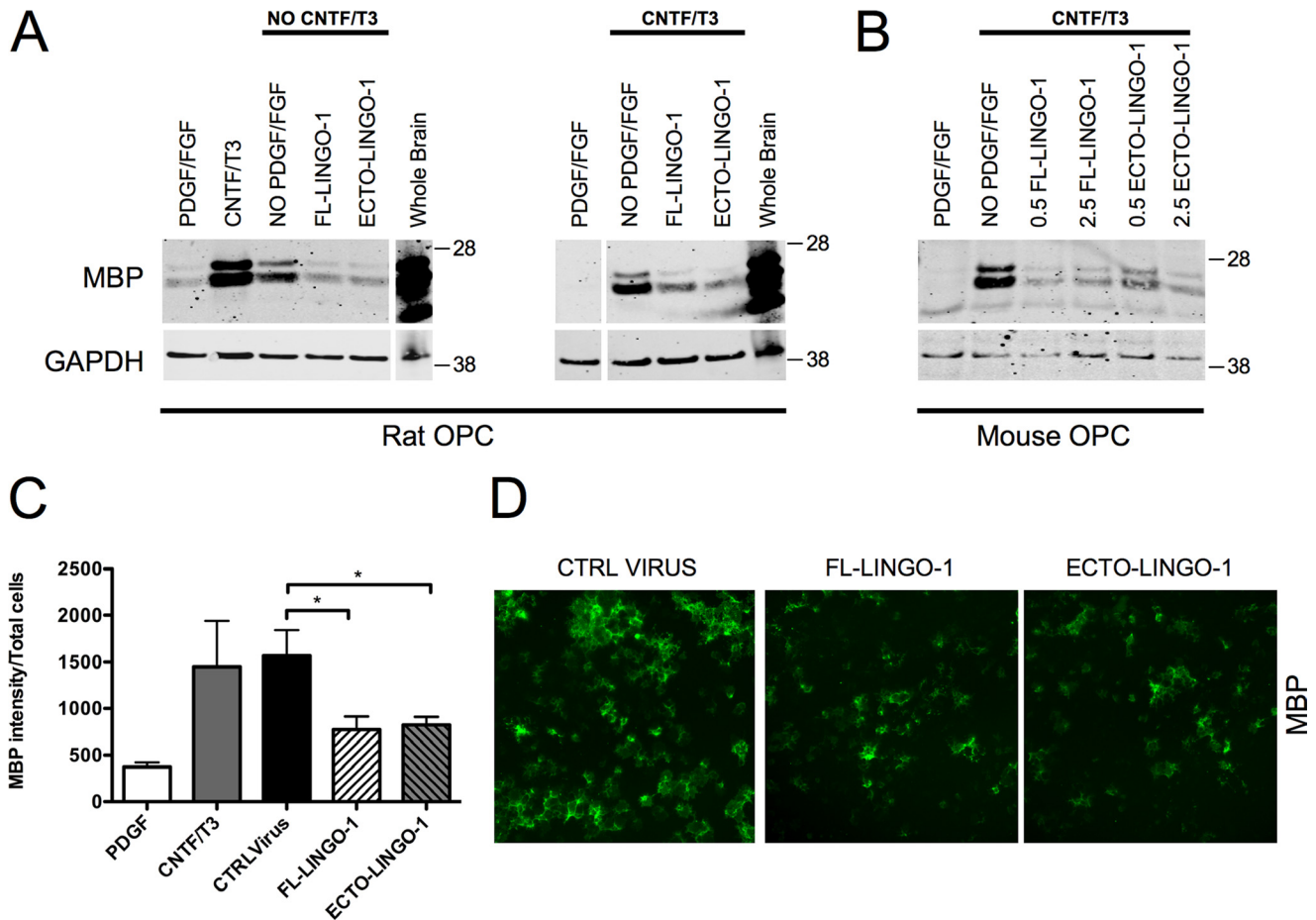


**FIGURE 1. LINGO-1 expression decreases during OPC maturation *in vitro*.** Newborn rat OPCs were grown for 3 days in PDGF/FGF as described under "Experimental Procedures." PDGF/FGF was removed and replaced for 10 ng/ml CNTF and 15 nM T3 for an additional 4 days to promote oligodendrocyte differentiation. **A**, OPC differentiation in culture was assessed by immunocytochemistry and fluorescence microscopy for NG2 and MBP. **B**, representative Western blot analyses of cultured rat OPCs show an increase in MBP protein expression that correlated with a decrease in LINGO-1 expression as cells differentiated for 4 days in the absence or presence of CNTF/T3. **C**, densitometric quantitation of four independent experiments for changes in MBP and LINGO-1 expression, normalized to GAPDH. Expression is represented as percent of levels in PDGF/FGF. *HMW*, LINGO-1 high molecular weight band; *LMW*, LINGO-1 low molecular weight band; *MBP*, \* $p < 0.05$  one-way ANOVA with Bonferroni post hoc test; LINGO-1, \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  two-way ANOVA with Bonferroni post hoc test.

supplemental Fig. 2) and quantitative immunofluorescence microscopy (Fig. 2, C and D). Unexpectedly, OPC cultures infected with a truncated lentiviral construct containing a membrane-bound ectodomain (the extracellular and transmembrane domains) but lacking the cytoplasmic domain of LINGO-1 also prevented an increase in MBP expression (Fig. 2, A–D, and supplemental Fig. 2, A and B). We observed a similar response using OPCs from either rat (Fig. 2A) or mouse (Fig. 2, B–D). These observations indicated that the extracellular domain of LINGO-1 was sufficient to inhibit OPC differentiation.

**Soluble LINGO-1 Ectodomain Prevents OPC Differentiation and Myelination**—To gain a better understanding of the role of the LINGO-1 ectodomain in OPC differentiation, we prepared a soluble recombinant LINGO-1-Fc ectodomain. Consistent with our observations using the membrane-bound ectodomain, the soluble LINGO-1 extracellular domain also prevented OPC maturation in a dose-dependent manner, as assessed by MBP expression using Western blot analyses (Fig. 3A) and immunofluorescence (Fig. 3, B–D). This observation was evaluated blindly and reproduced by different investigators in both rat and mouse cells (supplemental Fig. 3A).

To better characterize the stage within the oligodendrocyte lineage at which LINGO-1 exerts its effect, we double-immunostained OPC cultures treated with soluble LINGO-1 for expression of MBP and the sulfatide O4. The O4 surface marker appears at an intermediate stage of oligodendrocyte differentiation, after down-regulation of NG2 but before expression of MBP, and is present in immature MBP<sup>−</sup> as well as in mature MBP<sup>+</sup> oligodendrocytes. We found that treatment with LINGO-1 after PDGF/FGF removal did not alter the proportion of O4<sup>+</sup> cells present in the cultures, but we observed a reduced number of O4<sup>+</sup>/MBP<sup>+</sup> double-labeled cells, indicating that exposure to soluble LINGO-1 prevented an increase in MBP expression (Fig. 3, C and D). Similarly, lentiviral overexpression of full-length LINGO-1 had no effect on the number of O4<sup>+</sup> cells (supplemental Fig. 2B). Therefore, exposure to soluble LINGO-1 prevented the later stages of differentiation into MBP<sup>+</sup> mature oligodendrocytes without affecting the transition from NG2<sup>+</sup> OPCs to the intermediate O4<sup>+</sup> stage. This also suggests that the decrease in LINGO-1 protein observed during differentiation of cultured OPCs (Fig. 1) may be necessary for terminal differentiation of oligodendrocytes into the MBP<sup>+</sup> stage.



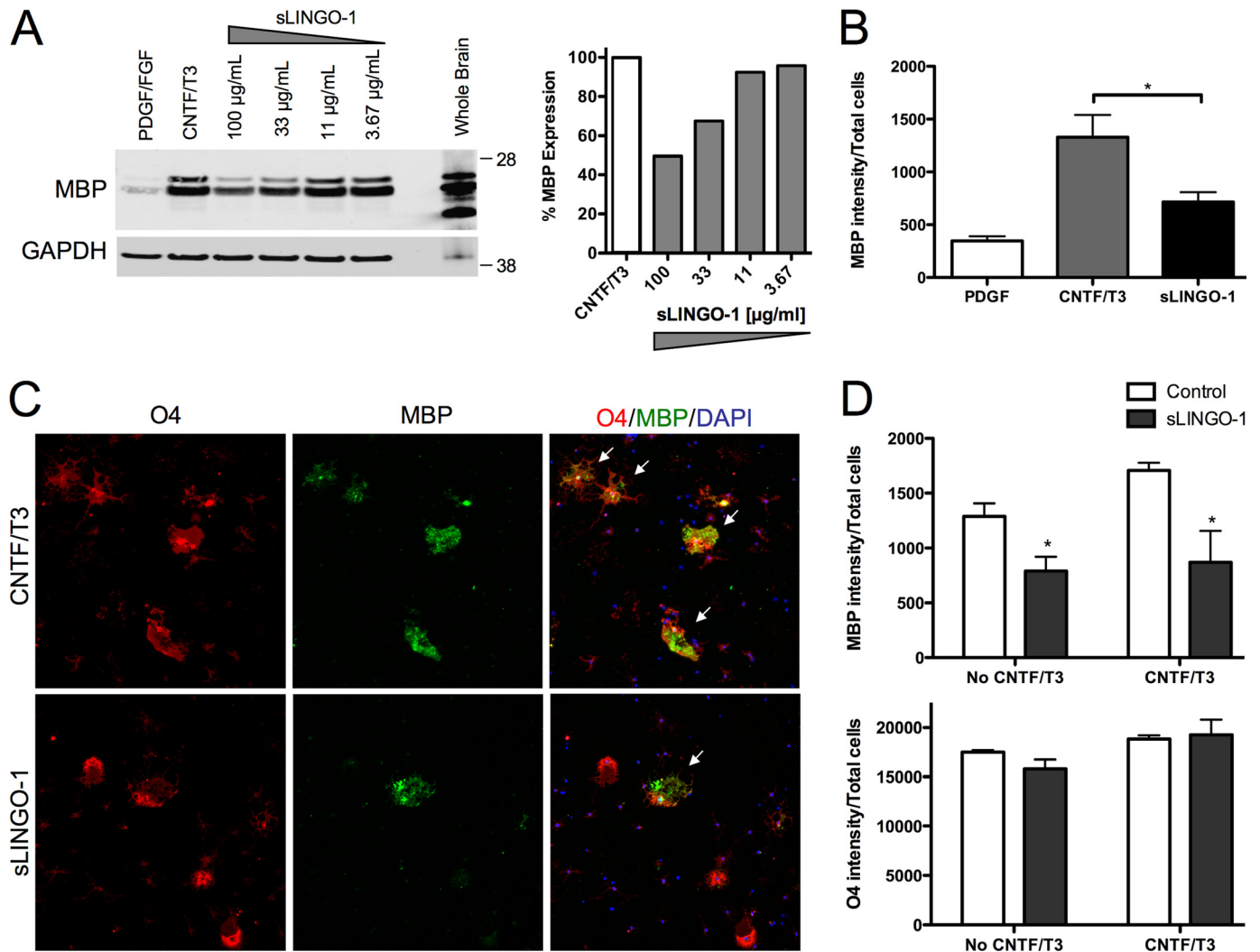
**FIGURE 2. Overexpression of LINGO-1 prevents OPC maturation.** Rat and mouse OPC cultures were infected with lentiviral constructs expressing full-length LINGO-1 (*FL-LINGO*), a truncated form containing the extracellular and transmembrane domains of LINGO-1 (*ECTO-LINGO*), or a control virus (*CTRL VIRUS*), as described under "Experimental Procedures." After PDGF/FGF removal, cells were treated for an additional 4 days for rat and 6 days for mouse in the absence or presence of 1 ng/ml CNTF and 1.5 nM T3. *A*, representative Western blot analysis of eight independent experiments in rat OPCs infected at 2.5 m.o.i. shows that overexpression of *FL-LINGO-1* or *ECTO-LINGO-1* prevented expression of MBP. Each panel contains samples run on the same gel. *B*, representative Western blot analysis of mouse OPCs infected at 0.5 and 2.5 m.o.i., as indicated, also shows decreased MBP expression after overexpression of these two LINGO-1 constructs. GAPDH was used as a loading control for Western blot analyses. *C*, decreased differentiation of mouse OPCs by *FL-LINGO* and *ECTO-LINGO* confirmed by quantitative immunofluorescence microscopy for MBP expression. Data are representative of three experiments. Values are mean  $\pm$  S.E. of four replicate cultures/condition. \*,  $p < 0.05$ , one-way ANOVA with Bonferroni post hoc test. *D*, representative images of oligodendrocyte differentiation in infected mouse cultures stained for MBP show reduced numbers of mature cells.

Because LINGO-1 has been implicated as a regulator of myelination (3, 7), we tested whether the soluble LINGO-1 ectodomain could also affect this process using an *in vitro* coculture system. Because DRG axons are myelinated by oligodendrocytes on their central branches, we tested cocultures of DRG neurons with OPCs, a system that has been used extensively for the study of myelination *in vitro* (3, 5, 8, 13). Myelination was identified as linear segments of MBP (green) colocalizing with axons (red, neurofilament-H) in double-immunostained rat DRG-OPC cocultures, visualized by fluorescence microscopy (Fig. 4A). We detected an expected increase in myelinated MBP<sup>+</sup> segments in response to DAPT, a  $\gamma$ -secretase inhibitor reported to increase myelination (1). In accord with our observations that soluble LINGO-1 inhibited maturation of OPCs in pure cultures (Fig. 3), we demonstrated that this protein decreased myelination in rat DRG-OPC co-cultures (Fig. 4, A and B).

**LINGO-1 Inhibits OPC Differentiation in Trans**—Our observations that a soluble LINGO-1 ectodomain inhibited myelination and OPC differentiation, combined with reports that developmental overexpression of LINGO-1 in neurons can

decrease myelination *in vivo* (13), suggested that in axo-glia interactions the extracellular domain of this molecule may be capable of acting directly on oligodendrocytes as an inhibitor of myelination. Our initial attempts to test if LINGO-1 can exert an inhibitory effect on OPC differentiation through intercellular interactions used OPCs plated on confluent monolayers of CHO cells stably expressing full-length or *ECTO-LINGO-1*. These experiments were unsuccessful because of incompatibilities in the growth medium requirements for CHO cells and OPCs (data not shown). We developed an alternative strategy to test this hypothesis by infecting astrocytes with lentiviruses encoding full-length LINGO-1 or its membrane-bound ectodomain (supplemental Fig. 4B). After a three-day period allowing for expression of the protein constructs, we seeded purified OPCs on these astrocytes and monitored the extent of differentiation after 4 days. Because cultured astrocytes do not express LINGO-1 (3), this ensured that effects on OPC differentiation would be produced by the ectopically expressed protein present on the astrocytic cell surface. The ectodomain displayed in this manner is expected to be identical for both

## LINGO-1 Regulates Myelination in Trans

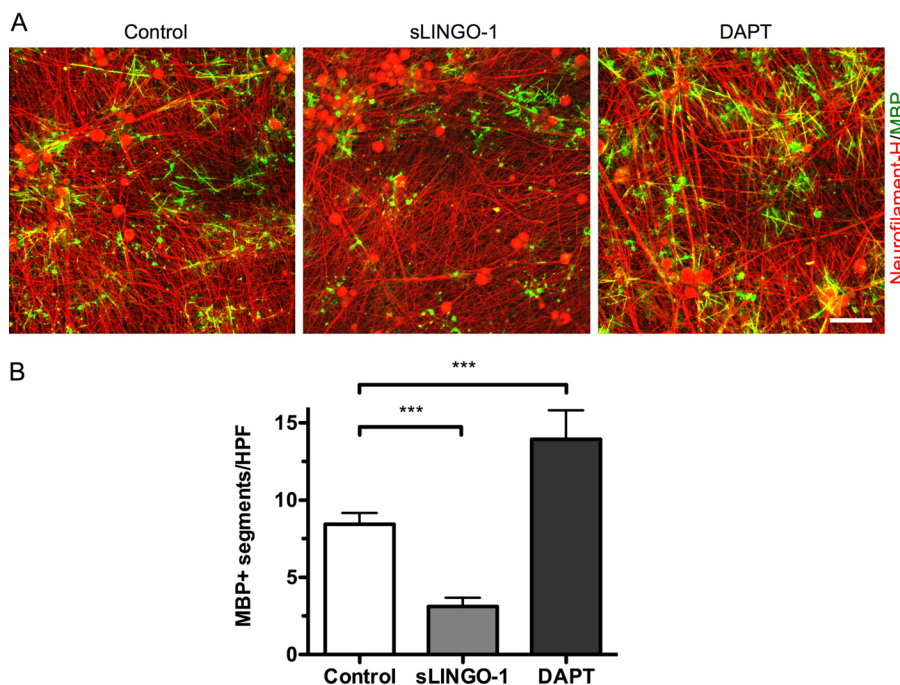


**FIGURE 3. The soluble LINGO-1 ectodomain prevents oligodendrocyte maturation.** Newborn rat OPC cultures were grown for 4 days in medium containing PDGF/FGF and subsequently treated for 4 days with 50 µg/ml recombinant soluble LINGO-1 ectodomain (B–D) or at the doses indicated (A) after removal of PDGF/FGF and addition of 1 ng/ml CNTF and 1.5 nM T3. **A**, representative Western blot analysis of five independent experiments showing a dose-responsive decrease in the amount of MBP protein expression after exposure to soluble LINGO-1. The graph in the right panel represents the densitometric quantitation of the MBP bands normalized to GAPDH. Values are expressed as percent of levels in CNTF/T3. **B**, quantitative immunofluorescence microscopy analysis for MBP showing that treatment with soluble LINGO-1 extracellular domain prevented OPC differentiation. Data are representative of three experiments. \*,  $p < 0.05$  one-way ANOVA with Bonferroni post-hoc test. **C** and **D**, rat OPC cultures were double-immunostained for O4 and MBP. Visual (**C**) and quantitative (**D**) immunofluorescence show that the effect of soluble LINGO-1 was restricted to reducing MBP expression (green cells) and did not affect the expression of O4 (red cells). The arrows in **C** indicate O4<sup>+</sup>/MBP<sup>+</sup> double-labeled cells. Data are representative of two experiments. Quantitative immunofluorescence microscopy values were obtained as in Fig. 2. \*,  $p < 0.05$ , Student's *t* test.

constructs because they share the same complete extracellular and transmembrane domains of LINGO-1. Compared with control lentivirus, only half of the OPCs plated over these astrocytes infected with LINGO-1 differentiated into MBP<sup>+</sup> cells (Fig. 5). There was no significant difference in the magnitude of the inhibition exerted by full-length or ectodomain-only LINGO-1 constructs (Fig. 5). Similar to our observations using the recombinant soluble LINGO-1 ectodomain, OPC differentiation into the O4 stage was not affected in these cocultures (Fig. 5B). These observations demonstrate that the extracellular domain of LINGO-1 can inhibit OPC differentiation through intercellular interactions in trans.

**LINGO-1 Binds to Itself in Trans**—The dual ability of LINGO-1 to inhibit oligodendrocyte differentiation by endogenous overexpression in OPCs or by the action of the ectodomain on OPCs through intercellular interactions in trans sug-

gested that LINGO-1 ectodomains expressed in adjacent cells might be capable of self-interaction to generate an inhibitory signal. To test this hypothesis, we constructed cell lines stably expressing full-length LINGO-1 and examined the ability of the soluble LINGO-1 ectodomain to bind to LINGO-1 present on the cell surface. Using this system, we demonstrated significantly increased, saturable, and dose-dependent binding of soluble LINGO-1 to CHO cells engineered to express full-length-LINGO-1 (Fig. 6A) compared with the control parent cells that lack LINGO-1 expression (supplemental Fig. 5A). To confirm that this interaction was restricted to the LINGO ectodomain portion of our soluble recombinant protein and did not involve its Fc tag, we performed two control experiments. First, we determined that an IgG did not exhibit preferential binding to LINGO-1 CHO cells compared with control CHO cells in the absence of soluble LINGO-1 (supplemental Fig. 5B). Second,



**FIGURE 4. Soluble LINGO-1 ectodomain prevents myelination.** Two-week-old DRG cultures were cocultured with newborn rat OPCs for an additional 2 weeks in the presence of DAPT or soluble LINGO-1 ectodomain, as indicated. *A*, representative pictures of myelination assessed by fluorescence microscopy show bundles of MBP+ linear segments aligning with axons identified with Neurofilament-H in control cultures (*left panel*). Addition of 2  $\mu$ M DAPT increased myelination (*right panel*), whereas incubation with 50  $\mu$ g/ml soluble LINGO-1 ectodomain prevented the appearance of myelinated axons in these cultures (*center panel*). Scale bar = 40  $\mu$ m. *B*, myelination was assessed blindly by two trained independent observers using fluorescence microscopy, and MBP+ myelin segments were counted in high-power fields (field area 65  $\mu$ m  $\times$  65  $\mu$ m). Values represent the mean  $\pm$  S.E. of at least 18 fields from duplicate wells in two independent experiments. \*\*\*,  $p < 0.001$  one-way ANOVA with Bonferroni post hoc test.

we produced a separate soluble LINGO-1 ectodomain construct that replaced the Fc tag in the C terminus for an alkaline phosphatase tag fused to its N-terminal domain (AP-LINGO-1) and assessed binding to CHO cells expressing full-length LINGO-1. We determined that, similar to soluble LINGO-1-Fc, soluble AP-LINGO-1 present in conditioned medium also showed increased, dose-dependent binding to CHO cells expressing full-length LINGO-1 compared with control CHO cells (supplemental Fig. 5C). These experiments indicated that LINGO-1 is capable of a self-association in trans. Moreover, we detected that the soluble LINGO-1 ectodomain also bound to pure OPC cultures (Fig. 6B). To characterize the specificity of this interaction, we performed a competitive binding displacement experiment using increasing concentrations of untagged soluble LINGO-1 as a competitor for binding of the Fc-tagged protein used at a constant concentration. We observed that untagged soluble LINGO-1 competed for binding of Fc-tagged soluble LINGO-1 to OPCs with micromolar affinity (half maximal binding inhibition  $\sim 1 \mu$ M, Fig. 6C).

**RhoA Is Activated by LINGO-1 Trans Interactions**—Previous reports have indicated that the activation of the small GTPase RhoA is an important second messenger downstream of LINGO-1 (8) that negatively regulates OPC differentiation (3, 20). To determine whether LINGO-1 interactions in trans can activate RhoA, we incubated OPCs with soluble LINGO-1 and analyzed the extent of RhoA activation by immunoprecipitation using a conformation-specific antibody for the active GTP-bound form. Lysophosphatidic acid, a well characterized activator of RhoA (21, 22), was used as a positive control and induced a transient response in cells exposed for 15 min that

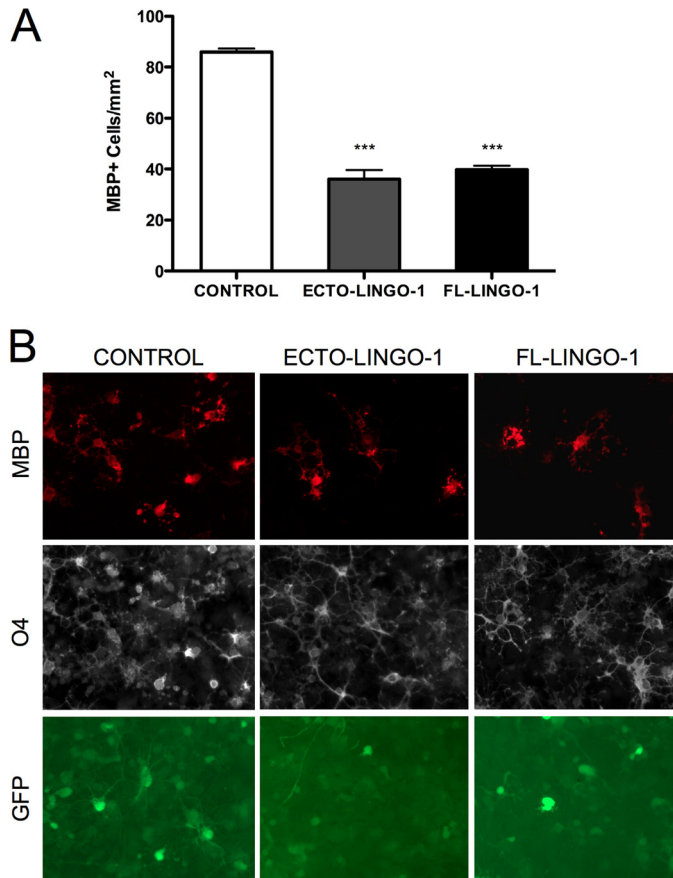
had already returned to basal levels after a 1-h exposure. Incubation with soluble LINGO-1 produced a more sustained activation of RhoA that was detected at 15 min and was still stimulated after 60 min (Fig. 7). These observations are consistent with our findings that LINGO-1 interactions in trans inhibit oligodendrocyte differentiation and suggest that this activity may be mediated by RhoA activation.

## DISCUSSION

LINGO-1 has been documented as a negative regulator of myelination. However, the molecular and cellular interactions that control its function are poorly understood. In support of this role, we observed that LINGO-1 expression was inversely correlated with oligodendrocyte differentiation *in vitro*. Removal of the mitogens PDGF/FGF, with or without addition of CNTF/T3, led to an increase in MBP expression and a substantial decrease in endogenous LINGO-1 expression. In agreement with previous reports on LINGO-1 gain of function *in vitro* and *in vivo* (3, 13), we observed that overexpression of full-length LINGO-1 inhibited OPC differentiation in pure cultures. Unexpectedly, we found that a truncated construct containing only the extracellular domain of LINGO-1 was also capable of inhibiting OPC differentiation into mature MBP<sup>+</sup> oligodendrocytes. This response occurred irrespective of a presentation as a membrane-bound isoform (also reported to act as a dominant negative construct (3, 8)) or when added exogenously in soluble form. Further examination revealed that exposure to LINGO-1 did not affect the initial differentiation of OPCs expressing the marker NG2 into an immature O4<sup>+</sup>/MBP<sup>-</sup> oligodendrocyte. The inhibitory effect was restricted to

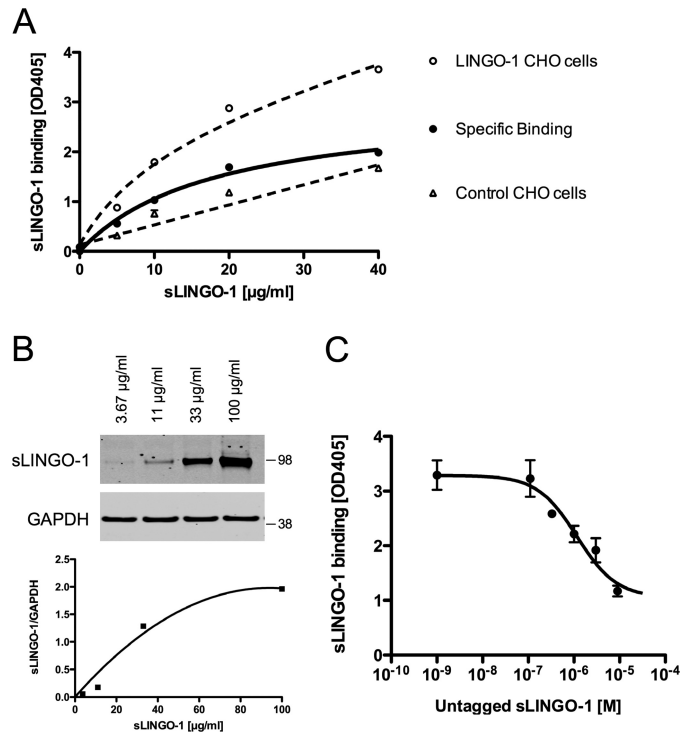


## LINGO-1 Regulates Myelination in Trans



**FIGURE 5. LINGO-1 inhibits OPC differentiation through intercellular interactions.** Confluent astrocyte cultures were infected with control, FL-LINGO-1, and ECTO-LINGO-1 lentiviruses at 2 m.o.i. After 3 days *in vitro*, freshly isolated OPCs were seeded on the infected astrocytes and examined 4 days later to assess oligodendrocyte differentiation by immunocytochemistry for MBP and O4. **A**, MBP<sup>+</sup> cell counts show that exposure to astrocytes expressing LINGO-1 decreased OPC differentiation. The effects of FL-LINGO-1 or ECTO-LINGO-1 were similar. Values represent the mean  $\pm$  S.E. of three replicate cultures/condition. \*\*\*,  $p < 0.001$  one-way ANOVA with Bonferroni post hoc test. **B**, representative images of OPCs cocultured with infected astrocytes and double-stained for MBP and O4 show that exposure to astrocytes infected with LINGO-1 inhibited expression of MBP (top panels, red cells) without a noticeable change in the expression of O4 (center panels, grayscale). Successful lentiviral infection was confirmed by expression of the eGFP fluorescent reporter downstream of the IRES (see "Experimental Procedures" and supplemental Fig. 4).

the subsequent transition from immature O4<sup>+</sup>/MBP<sup>-</sup> oligodendrocytes to a mature phenotype expressing MBP. Moreover, the soluble extracellular domain of LINGO-1 also inhibited myelination of axonal segments in OPC-DRG cocultures, a response expected from an inhibitor of oligodendrocyte terminal differentiation. Together, these experiments suggested that the extracellular domain of LINGO-1 can operate in trans as an inhibitory signal for oligodendrocyte differentiation and myelination. Our findings differ from a previous report in which a recombinant soluble LINGO-1 ectodomain induced OPCs to differentiate (3) but agree in the inhibitory effect of a gain of function by overexpression (3, 13). It is possible that discrepancies observed in the use of recombinant proteins may arise as a result of differences in experimental systems. In particular, methods used for production and purification of secreted recombinant proteins could affect their biological activity, including their ability to interact with other proteins because of



**FIGURE 6. The soluble LINGO-1 ectodomain binds to itself in trans.** **A**, CHO cells stably expressing FL-LINGO-1 and control cells were incubated with increasing doses of soluble LINGO-1 extracellular domain, and bound protein was measured by alkaline phosphatase activity as described under "Experimental Procedures." Specific binding (solid line) was calculated by subtracting the signal obtained with control CHO cells from the signal obtained with CHO cells stably transfected with FL-LINGO-1 (dashed lines). The soluble LINGO-1 ectodomain displayed specific, saturable binding to CHO cells stably transfected with FL-LINGO-1 compared with control CHO cells. Each point represents the mean  $\pm$  S.E. of four replicate wells. Binding experiments were performed independently at least eight times with three separate batches of purified recombinant sLINGO-1 protein. **B**, newborn rat OPCs were exposed to increasing doses of soluble LINGO-1 ectodomain, rinsed, and processed for Western blotting. A representative Western blot analysis shows the bound exogenous LINGO-1 ectodomain detected with an antibody against the Fc tag. The graph below represents a densitometric quantitation of the soluble LINGO-1 bands normalized to GAPDH. The curve was fit by non-linear regression using GraphPad Prism. **C**, competitive binding displacement of sLINGO-1-Fc with untagged sLINGO-1. OPCs were incubated as described above for CHO cells, with 50  $\mu$ g/ml tagged soluble LINGO-1-Fc and variable concentrations of untagged soluble LINGO-1. The value for half maximal binding inhibition was  $\sim 1 \mu$ M, determined by non-linear regression with the competitive binding, one site-fit logIC50 model, using GraphPad Prism. Each point represents mean  $\pm$  S.E. of three replicate wells.

differences in posttranslational modifications introduced by artificial cell expression systems (23). In fact, LINGO-1 contains several *N*-glycosylation sites that could negatively and unpredictably affect its capacity for self-interaction either in cis or in trans (24, 25), for interaction with its other partners, or with gangliosides that modulate those interactions (23, 26, 27). Instead, the use of gene expression vectors or transgenic animals to drive expression in primary cells would be expected to be less prone to this kind of modification in biological activity. We used lentiviral expression of ectodomain and full-length LINGO-1 in astrocytes to demonstrate the existence of an intercellular activity in trans that could inhibit oligodendrocyte differentiation and was consistent with the effect obtained with numerous independent preparations of our protein. This confirmed the ability of this domain to inhibit OPC differentiation

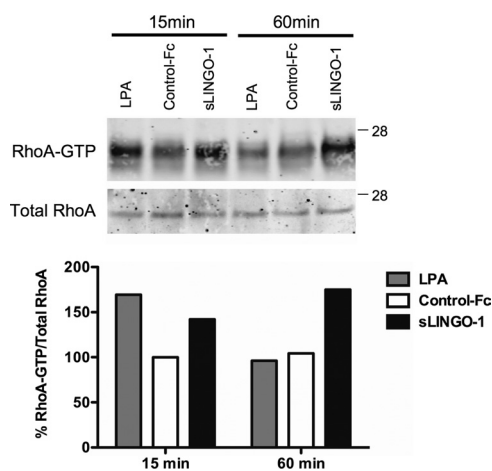


FIGURE 7. **LINGO-1 activates RhoA in trans.** OPCs grown for 3 days in PDGF/FGF were exposed to 5 ng/ml LPA, 100  $\mu$ g/ml soluble LINGO-1 ectodomain, or Fc control for the times indicated in the figure (see "Experimental Procedures"). Soluble LINGO-1 and LPA activated RhoA, as assessed by immunoprecipitation of GTP-bound RhoA, followed by Western blotting of the immunoprecipitates and whole cell lysates with a total RhoA antibody. The graph below shows the densitometric quantitation of the RhoA bands. Values represent the ratio of RhoA-GTP/Total RhoA expressed as percent levels with respect to Control-Fc at 15 min.

and validated the biological activity in trans of our recombinant protein.

Our data compelled us to change the way we understand the mechanism of action of LINGO-1 and to design experiments to test a model of self-interaction in trans. As explained below, this new model integrates various reports of the ability of this molecule to modulate oligodendrocyte differentiation by gain or loss of function in oligodendrocytes or neurons.

Because LINGO-1 can inhibit differentiation through two modes of action, gain of function in OPCs by full-length overexpression and signaling through its extracellular domain in a trans interaction, this raises two fundamental questions. First, when full-length LINGO-1 functions as a receptor in OPCs, what is its ligand? Second, if the extracellular domain of LINGO-1 can also act as a signaling molecule, what is its receptor? Within a neuron, LINGO-1 can bind multiple coreceptor partners (8–11) and may also be capable of a self-association in cis to form a tetrameric structure whose biological significance is presently unknown (24). In a multicellular environment, studies have shown that inhibition of myelination by LINGO-1 can occur when its expression is independently increased in either oligodendrocytes or in neurons (13). The mechanism explaining this redundancy in cellular response is unclear and cannot simply be accounted for by a receptor function of LINGO-1 in OPCs. When combined with our findings that the ectopic membrane-bound and soluble extracellular domain of LINGO-1 can also inhibit OPC differentiation and myelination, we hypothesized that LINGO-1 expressed on adjacent neuronal and oligodendrocytic membranes could interact in trans as a ligand-receptor unit. We demonstrated the ability of this ectodomain to self-interact in trans with LINGO-1 displayed in stably transfected CHO cells and observed specific, dose-dependent, saturable binding. This interaction was confirmed using two different LINGO-1 ectodomain constructs containing different tags either on the N terminus or the C terminus of the

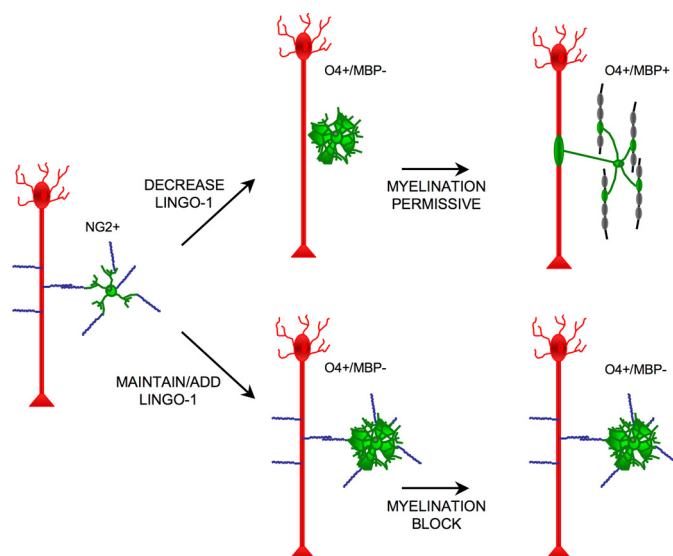


FIGURE 8. **Model for LINGO-1 inhibition of myelination.** LINGO-1, expressed by axons and NG2<sup>+</sup> OPCs, binds to itself in trans to prevent oligodendrocyte differentiation and myelination during axo-glial interactions (*left*). A developmental decrease in LINGO-1 protein in O4+/MBP<sup>-</sup> premyelinating oligodendrocytes (*center*), and possibly in the axon as well, leads to a condition permissive for myelination (*upper arrow path*). Sustained LINGO-1 expression that maintains the self-interaction in trans leads to a reduced capacity of O4+/MBP<sup>-</sup> oligodendrocytes to mature to the MBP<sup>+</sup> stage and to myelinate (*lower arrow path*). Neurons, red; OPC/Oligodendrocytes, green; LINGO-1, blue lines. Stage-specific oligodendrocyte markers used in this study are indicated. Gray objects represent additional axons myelinated by the same oligodendrocyte and their myelinated internodes originating from other oligodendrocytes (not pictured).

protein. This mechanism is consistent with the delay in oligodendrocyte differentiation produced by LINGO-1 treatment because we exposed our primary cultures at the OPC stage, when endogenous LINGO-1 expression was highest. Moreover, although LINGO-1 expression in OPCs decreased considerably with differentiation, more mature cells still maintained readily detectable levels of this protein. Finally, we demonstrated that exposure of OPCs to soluble LINGO-1 ectodomain increased activation of the small GTPase RhoA, a second messenger downstream of LINGO-1 (3, 8). This finding suggests that this mechanism may be involved in mediating the effects of the ectodomain in trans and provides further support to our model that intercellular self-interactions lead to an inhibitory signaling event in OPCs (Fig. 8).

Our data are consistent with a direct association between LINGO-1 extracellular domains, present on neighboring OPC and axonal membranes, as responsible for its inhibitory effect during axo-glial interactions prior to myelination (Fig. 8). Alternatively, our findings could be explained if LINGO-1 in trans was directly suppressing another inductive signal. For instance, BDNF can induce oligodendrocyte differentiation via TrkB (28), and there is evidence that, at least in retinal ganglion cells, LINGO-1 can interact with TrkB to inhibit phosphorylation induced by BDNF (11). We find this mechanism less likely to explain our present observations for two reasons. The first is that the inductive effect of TrkB on cultured OPCs requires exogenous addition of BDNF (28), a factor that was absent in the chemically defined media used in our experiments. The second is that BDNF induced differentiation of rat basal fore-

brain oligodendrocytes but not of cortical oligodendrocytes (28, 29), which were the cells used in our OPC cultures. Interestingly, the same investigators showed that basal forebrain oligodendrocytes expressed full-length TrkB, whereas cultured cortical oligodendrocytes expressed low levels of a truncated form of this receptor that does not respond to BDNF (29). Nevertheless, in addition to the self-interacting mechanism proposed here, it is still possible that *in vivo*, LINGO-1 may also negatively regulate endogenous BDNF signaling in areas where the full TrkB receptor is present, such as the basal forebrain or after BDNF induction in demyelination (30).

OPCs integrate multiple signals from their environment to differentiate and subsequently myelinate. Our findings suggest that at the OPC stage, a close association between neighboring OPCs could cause a LINGO-1-mediated inhibition or delay in their differentiation. *In vivo*, NG2<sup>+</sup> OPCs migrate and disperse through the parenchyma, extending a dense network of processes that contact each other (31). Whether a LINGO-1 self-interaction is taking place at those points of contact to modulate the timing of NG2 cell differentiation is an important question for future investigation.

The role of LINGO-1 in the context of axo-glial communication *in vivo* is likely to be complex. Considering the inhibitory function of this protein, other mechanisms will likely be responsible for initiating myelination. Nevertheless, studies using LINGO-1 null mice (3) and transgenic animals overexpressing LINGO-1 in neurons (13) indicate that this molecule plays an important role in determining the age of onset and the rate of myelination. Disruption of an axo-glial LINGO-1 interaction in trans may be a key step in this process, which could be induced through down-regulating LINGO-1 protein levels in premyelinating oligodendrocytes and/or in neurons (Fig. 8). In support of this hypothesis, our present observations indicate that levels of LINGO-1 protein decrease during OPC differentiation. Additionally, developmental studies in mice have shown that LINGO-1 mRNA decreases at the onset of myelination in optic nerves, which are devoid of neuronal cell bodies and contain only oligodendrocytes as the main source of mRNA. Increased mRNA expression of several mature oligodendrocyte markers, such as CNP, MBP, MAG, and Nogo-A, follows the down-regulation of LINGO-1 mRNA. In contrast, in the retina, which contains neuronal cell bodies and no oligodendrocytes, LINGO-1 expression is maintained until after myelination is complete (32). It is possible that the onset of myelination may be initiated through reduced expression of LINGO-1 in oligodendrocytes, at least in the optic nerve, although axonal regulation of this molecule may also play an important role in this process.

We have provided evidence that LINGO-1 can self-interact in trans to inhibit the later stages of oligodendrocyte differentiation and myelination. Development of effective biological reagents and chemical small molecules to disrupt this interaction would be expected to promote myelination. Therefore, a detailed molecular understanding of this binding, and the domains within LINGO-1 that participate in it, could have important consequences for developing therapies for demyelinating disorders.

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