Semaphorin 4D/Plexin-B1-Mediated M-Ras GAP Activity Regulates Actin-Based Dendrite Remodeling through Lamellipodin

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Semaphorins have been identified as repulsive guidance molecules in the developing nervous system. We recently reported that the semaphorin 4D (Sema4D) receptor Plexin-B1 induces repulsion in axon and dendrites by functioning as a GTPase-activating protein (GAP) for R-Ras and M-Ras, respectively. In axons, Sema4D stimulation induces growth cone collapse, and downregulation of R-Ras activity by Plexin-B1-mediated GAP activity is required for the action. Axonal R-Ras GAP activity downregulates phosphatidylinositol 3-kinase signaling pathway, and thereby induces inactivation of a microtubule assembly promoter protein, CRMP-2. However, in contrast to the well studied roles of semaphorins and plexins in axonal guidance, signaling molecules linking M-Ras GAP to dendritic cytoskeleton remain obscure. Here we identified an Ena/VASP ligand, Lamellipodin (Lpd), as a novel effector of M-Ras in dendrites. Lpd was expressed in F-actin-rich distal dendritic processes and was required for both basal and M-Ras-mediated dendrite development. Subcellular fractionation showed M-Ras-dependent membrane translocation of Lpd, which was suppressed by Sema4D. Furthermore, the Ena/VASP-binding region within Lpd was required for dendrite development, and its membrane targeting was sufficient to overcome the Sema4D-mediated reduction of dendritic outgrowth and disappearance of F-actin from distal dendrites. Furthermore, *in utero* electroporation experiments also indicated that regulation of the M-Ras-Lpd system by the GAP activity of Plexin is involved in the normal development of cortical dendrites *in vivo*. Overall, our study sheds light on how repulsive guidance molecules regulate actin cytoskeleton in dendrites, revealing a novel mechanism that the M-Ras-Lpd system regulates actin-based dendrite remodeling by Sema/Plexin in rats or mice of either sex.

Introduction

Semaphorins are a large family of secreted or transmembrane molecules that have been shown to regulate axonal pathfinding during the development of the nervous system (Kolodkin et al., 1993; Luo et al., 1993; Tamagnone et al., 1999). Semaphorins were originally identified as repulsive axonal guidance molecules. Semaphorin 4D (Sema4D), through its receptor Plexin-B1, induces axonal growth cone collapse in hippocampal and cortical neurons (Swiercz et al., 2002). The cytoplasmic regions are highly conserved among all plexin subfamilies, and the cytoplasmic region of Plexin directly encodes a GTPase activating protein (GAP) for R-Ras (Oinuma et al., 2004a; Toyofuku et al., 2005;

Uesugi et al., 2009). Axonal R-Ras GAP activity downregulates phosphatidylinositol 3-kinase (PI3-K) signaling pathway and thereby induces inactivation of a microtubule assembly promoter, collapsin response mediator protein-2 (CRMP-2), inducing growth cone collapse (Ito et al., 2006). Several modulators of cytoskeletal dynamics, including Rho family GTPases, p21-activated kinase (PAK), CasL (MICALs), and LIM kinase, have also been implicated in the downstream signaling of Sema/Plexin (Liu and Strittmatter, 2001; Whitford and Ghosh, 2001; Swiercz et al., 2002; Terman et al., 2002; Hung et al., 2010, 2011). Although signaling mechanisms inducing axonal repulsion have been extensively studied, little is known about the roles of Sema/Plexin in dendrites. We recently revealed that Plexin-B1 also displays a GAP activity for M-Ras, inhibiting dendrite development in cortical neurons (Saito et al., 2009). M-Ras regulates dendrite morphology through activation of the ERK pathway, and the inhibitory effect of Sema4D/Plexin-B1 on dendrite development is, in part, mediated by inhibition of the ERK pathway; although the ERK pathway contributes to dendrite development, signaling molecules linking M-Ras GAP to dendritic cytoskeleton remain obscure.

Lamellipodin (Lpd) has been identified as an Ena/VASP family protein-interacting molecule harboring a Ras association (RA) domain, pleckstrin-homology (PH) domain, and proline-rich region. Lpd interacts with Ena/VASP through FPPPP motifs in a proline-rich region and regulates the localization of Ena/VASP (Krause et al., 2004). Ena/VASP proteins at the tips of

Received Feb. 20, 2012; revised April 18, 2012; accepted April 30, 2012.

Author contributions: G.T. and I.O. designed research; G.T. performed research; G.T. analyzed data; G.T., M.N., and I.O. wrote the paper.

This research was supported by Japan Science and Technology Agency PRESTO Project (Development and Function of Neuronal Networks) to I. O., Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan [Scientific Research (B) 23390019 (to M. N.), Young Scientists (A) 23689005 (to I. O.), and Challenging Exploratory Research 23657127 (to I. O.)], and grants from the Uehara Memorial Foundation and Daiichi-Sankyo Foundation of Life Science (to I. O.). We are grateful to L. Tamagnone for Plexin-B1 cDNA, H. Kikutani for the soluble forms of Sema4D expression plasmids, J. Miyazaki and T. Saito for the EYFP dual-promoter expression plasmid, M. Matsuda for pCIN2 vector, and H. Takematsu for IRES-EGFP plasmids.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0799-12.2012

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lamellipodia and filopodia accelerate actin polymerization by their anti-capping and bundling activity (Krause et al., 2003), and neurons lacking these proteins fail to form neurites (Kwiatkowski et al., 2007). The *Caenorhabditis elegans* Lpd ortholog MIG-10 has important roles in neuronal migration, polarization, and axon guidance (Adler et al., 2006; Chang et al., 2006; Quinn et al., 2006). Recent studies in mammals have shown that knockdown of Lpd decreases the length of axons (Michael et al., 2010). It has also been reported that Lpd regulates migration of pyramidal neurons through the regulation of serum response factor (SRF) transcription factor activity (Pinheiro et al., 2011). However, physiological interaction between Lpd and Ras family GTPases still remains unclear, and the identity of which Ras family proteins directly bind to Lpd and function upstream of Lpd in neurons has been rarely understood.

In this work, we have identified Lpd as the effector of M-Ras in dendrite development and demonstrate a novel mechanism by which Sema/Plexin regulates actin-based dendrite remodeling through the M-Ras-Lpd system.

Materials and Methods

DNA constructs and site-directed mutagenesis. Myc-tagged Plexin-B1 Δ ect (deletion of the extracellular domain (ect), amino acids 1–1306) and Myc-Plexin-B1 Δ ect-RA (R1677A/R1678A/R1984A) have been described previously (Oinuma et al., 2004b). Myc-Plexin-B1-N-Cyt-GGA (Oinuma et al., 2006) was inserted into pCXN2. Myc-tagged M-RasQL, R-RasQL, H-RasGV, GFP-tagged M-RasQL, and GST-fused M-Ras have been described previously (Katoh et al., 2000; Oinuma et al., 2004a, 2006; Saito et al., 2009). Human Rap1 was obtained by reverse transcription (RT)-PCR from HeLa cells. A constitutively active mutant of Rap1, Rap1GV (G12V), was generated by PCR-mediated mutagenesis and subcloned into pCMV-Myc (Clontech). Dominant-negative mutants of M-Ras (M-RasSN) and an effector loop mutant of M-Ras (M-RasQLDA) were generated by PCR-mediated mutagenesis. For *in vivo* electroporation experiments, M-RasQL was subcloned into pCAG-IRES-EGFP. Human Lpd-RA (amino acids 1–380) was obtained by RT-PCR from HEK293T cells, and subcloned into pGEX-6P-1 (GE Healthcare). Full-length Lpd was obtained by ligation of Lpd-RA to KIAA1681 cDNA (Kazusa DNA Research Institute, Kisarazu, Chiba, Japan), and was subcloned into pcDNA3 (Invitrogen) to produce NH₂-terminal HA-tagged version of Lpd, or subcloned into pCMV-Myc (Clontech) to produce NH₂-terminal Myc-tagged version of Lpd. Lpd- Δ RA (deletion of amino acids 270–345), Lpd- Δ C (amino acids 1–592), and Lpd-CT (amino acids 683–1250) were subcloned into pcDNA3 (Invitrogen) or pCMV-Myc (Clontech). A CAAX sequence (where C represents cysteine, A is an aliphatic amino acid, and X is a terminal amino acid) was fused to the COOH terminus in frame with full-length or truncated forms of Lpd as described previously (Katoh et al., 2002). The short hairpin RNAs (shRNAs) for Lpd were designed to target 19 nucleotides of the rat transcript and were expressed by using an shRNA expression vector, pSilencer (Ambion). The target sequences for the Lpd shRNA constructs are as follows: Lpd shRNA #1 (nucleotides 1521–1539, 5'-GGACTATCGGAA-CAAATAC-3'); Lpd shRNA #2 (nucleotides 219–237, 5'-CTTGAA TGAAGCTCTGAAT-3'). The shRNA for Lpd transcript (nucleotides 210228, 5'-CATATACAACCTGAATGAA-3'), which had no effect on Lpd expression, was used as a control shRNA. The Lpd shRNA-resistant construct rLpd was obtained by creating silent mutagenesis within the target sequence of Lpd shRNA #2 (nucleotides 219–237, 5'-CTTG AATGAAGCTCTGAAT-3' were replaced with 5'-CTTGAATGAAG CGCTTAAC-3'). The shRNA construct for M-Ras has been described previously (Saito et al., 2009).

Antibodies and reagents. We used the following antibodies: a mouse monoclonal antibody against Myc and Na⁺/K⁺-ATPase α subunit (Millipore); mouse monoclonal antibodies against α -tubulin, β -actin, and microtubule-associated protein 2 (MAP2) (2a+2b) (Sigma-Aldrich); a mouse monoclonal antibody against GFP (B2; Santa Cruz Biotechnology); a rabbit polyclonal antibody against Myc (MBL); a rat monoclonal

antibody against HA (3F10; Roche); rabbit polyclonal antibodies against Lpd and GAPDH (Santa Cruz Biotechnology); a rabbit polyclonal antibody against Lpd (Sigma-Aldrich); a goat polyclonal antibody against M-Ras (R&D Systems); secondary antibodies conjugated to HRP (Dako-Cytomation); and secondary antibodies conjugated to Alexa Fluor 488 or 555 (Invitrogen). Poly-L-lysine was purchased from Sigma-Aldrich and used at 25 μ g/ml. F-actin was visualized with rhodamine phalloidin (Invitrogen). A soluble form of mouse Sema4D fused to human IgG₁-Fc was from H. Kikutani (Osaka University, Osaka, Japan), and Sema4D stimulation was carried out by replacing the culture medium with Sema4D-containing conditioned medium, which contained $\sim$ 150 nM Sema4D-Fc.

Cell culture and transfection. HEK293T cells were cultured in DMEM containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 0.2 mg/ml streptomycin under humidified air containing 5% CO₂ at 37°C, and transient transfections were carried out using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Primary cortical neurons were prepared from the cortices of embryonic day 19 (E19) rats of either sex as described previously (Saito et al., 2009) and seeded on poly-L-lysine-coated glass coverslips (circular, 13 mm in diameter; Matsunami Glass) at a density of 3×10^4 cells. Transient transfections were carried out at 4 or 5 days *in vitro* (DIV) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. All animal experiments were conducted according to the guidelines of the Kyoto University Animal Research Committee.

In vitro binding assays. Recombinant GST-fusion proteins were purified from *E. coli* according to the method of Self and Hall (1995). For purification of GST-M-Ras protein, we added 1 mM GTP throughout the purification procedure. We loaded GST-fused M-Ras with guanine nucleotides by incubating the protein with 10 μ M GDP- β S or GTP- γ S in loading buffer (20 mM Tris-HCl, pH 8.0, 1.5 mM GTP, 1 mM DTT, 5 mM MgCl₂, 20 mM EDTA, and 10% glycerol, 0.5 mg/ml BSA) at 37°C for 10 min. The reaction was stopped by addition of MgCl₂ to a final concentration of 20 mM.

For a dot-blot assay, 5 μ g of GST or GST-fused Lpd-RA was spotted onto a nitrocellulose membrane and allowed to dry for 1 h at room temperature. The membrane was blocked in buffer A containing 5% low fat milk for 1 h at 4°C. The membrane was then incubated for 1 h at 4°C in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT) containing 5 μ g of GST-fused purified M-Ras protein preloaded with GDP- β S or GTP- γ S. The membrane was washed with buffer A and then incubated with 0.3% low fat milk in TBS containing anti-M-Ras antibody.

For a pull-down assay, HEK293T cells (10^6 cells) transfected with HA-tagged Lpd or Myc-tagged M-RasQL, R-RasQL, or Rap1GV were rinsed with PBS and lysed with the ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1% NP-40, 1 mM PMSF, 1 mM DTT, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin). Cell lysates were then centrifuged for 10 min at $16,000 \times g$ at 4°C. The supernatants were incubated for 1 h at 4°C with 5 μ g of GST, GST-fused Lpd-RA, or GST-fused M-Ras preloaded with GDP- β S or GTP- γ S and subsequently with glutathione-Sepharose beads for 1 h at 4°C. After the beads were washed with the ice-cold cell lysis buffer, the bound proteins were eluted in Laemmli sample buffer, analyzed by SDS-PAGE, and immunoblotting.

For immunoprecipitation, HEK293T cells cotransfected with the indicated plasmids were lysed for 10 min with ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1% NP-40, 1 mM PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin). After centrifugation for 10 min at $16,000 \times g$, the supernatants were incubated with anti-Myc antibody for 2 h, followed by incubation with protein G-Sepharose (GE Healthcare) for 1 h. Then the beads were washed with the cell lysis buffer. A Seize X Protein G Immunoprecipitation Kit (Pierce) was used to see endogenous interactions in cultured cortical neurons. Primary cultured cortical neurons were lysed for 10 min with ice-cold cell lysis buffer. After centrifugation for 10 min at $16,000 \times g$, the supernatants were incubated with anti-Lpd antibody conjugated with immobilized protein G Plus for 2 h. Then the beads were washed with binding/wash buffer, and bound proteins were eluted with ImmunoPure IgG Elution buffer and analyzed by SDS-PAGE and immunoblotting.

Immunoblotting. Proteins were separated by SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 3% low-fat milk in TBS and then incu-

Figure 1. Sema4D induces the disappearance of F-actin from distal dendrites through downregulation of M-Ras activity. **A**, Cultured cortical neurons cotransfected with GFP and Myc-tagged M-RasQL at 5 DIV were fixed and stained with rhodamine-conjugated

bated with primary antibodies. The primary antibodies were detected with HRP-conjugated secondary antibodies and an enhanced chemiluminescence detection kit (GE Healthcare). Images were captured using a LAS3000 analyzer (Fuji) equipped with Science Lab software (Fuji).

Separation of membrane and cytosolic fractions. Transiently transfected HEK293T cells or 7 DIV cortical neurons were suspended in buffer B (20 mM Tris-HCl, pH7.5, 100 mM NaCl, 1 mM EGTA, 5 mM MgCl₂, 250 mM sucrose, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell suspensions were then lysed by sonication (5 times at 5 s each), and the lysates were centrifuged at 1000 × g for 10 min to remove unbroken cells and nuclear fractions. The supernatants were further fractionated at 100,000 × g for 1 h into particulate and cytosolic fractions. After the particulate pellet was washed with buffer B, the pellet was resuspended with Laemmli sample buffer and defined as the membrane fractions. The fractions were analyzed by SDS-PAGE and immunoblotting.

Nucleofection. Cortical neurons from E19 rats (2.5×10^6 cells) of either sex were suspended in 100 μl of Nucleofector solution (Amaxa), mixed with total 4 μg of plasmid DNA for knockdown of Lpd (GFP:shRNA, 1:4), and nucleofected (program O-003) before plating using Nucleofector device (Amaxa).

Immunofluorescence microscopy. Primary cultured cortical neurons on coverslips were fixed with 4% PFA in PBS. After residual PFA had been quenched with 50 mM NH₄Cl in PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS and incubated with 10% FBS in PBS. Cells on coverslips were mounted on 90% glycerol containing 0.1% *p*-phenylenediamine dihydrochloride in PBS. The cells were photographed with a Leica DC350F digital camera system (Leica) equipped with a Nikon Eclipse E800 microscope (Nikon).

For visualizing endogenous Lpd and MAP2 or F-actin in cultured cortical neurons, neurons were permeabilized with the buffer (0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, and 3 mM MgCl₂) and incubated with 10% FBS in PBS. After incubation with 10% FBS in PBS, neurons were incubated for 1 h with anti-Lpd and anti-MAP2 antibodies diluted with Can Get Signal® Immuno-stain Immunoreaction Enhancer Solution A (TOYOBO), followed by incubation with Alexa Fluor 555-conjugated anti-rabbit IgG (1:

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phalloidin at 7 DIV after stimulation with Sema4D for 1.5 h. Transfected cells are shown by GFP fluorescence. Boxed regions in the top are enlarged and shown in the bottom. Scale bars, 20 μm (top sets of panels) and 5 μm (bottom sets of panels). **B**, The sum fluorescence pixel intensity values for F-actin within 5 μm of distal or proximal parts of dendrites (as represented in the bottom sets of panels in **A**) were measured, and the relative F-actin intensity ratio of distal over proximal was determined as described in Materials and Methods. Results are the means ± SEM of three independent experiments ($n = 15$; * $p < 0.05$).

2000 dilution) and Alexa Fluor 488-conjugated anti-mouse IgG (1:2000 dilution) for 1 h. For visualizing F-actin, neurons were incubated for 1 h with rhodamine phalloidin (Invitrogen) with Can Get Signal® Immunostain Immunoreaction Enhancer Solution A (TOYOBO). To obtain a z-plane image, optical sections of images were captured through the cell in 0.20 μ m steps using a C1 laser-scanning confocal imaging system (EZ-C1 version 3.20 software; Nikon) and a microscope (Eclipse TE2000-U; Nikon) with a 60 $\times$ numerical aperture, 1.40 oil objective (Nikon), and a digital camera (DXM1200C; Nikon). The images were arranged and labeled using Photoshop 7.0 (Adobe).

Quantification of F-actin intensity in dendrites. Images taken by confocal laser scanning microscopy were imported to Photoshop. We adjusted brightness and contrast uniformly across entire fields maintaining signal linearity, confirming that fluorescence intensity was not saturated. Then, the sum fluorescence pixel intensity values for GFP (G) and F-actin (A) within $\sim$ 5 μ m distal (dist) or proximal (prox) parts of dendrites were measured with Photoshop software. For compensation of the volume of the regions of interest, values of (A-dist)/(G-dist) and (A-prox)/(G-prox) were calculated first, and then relative F-actin intensity ratio of distal over proximal was determined. At least 15 neurons per construct were collected from three independent experiments, and more than five dendrites per each neuron were measured. Statistical significance was determined by using the ANOVA test (Dunnett) or *t* test.

Quantification of dendrite morphology. Total dendritic length and total dendritic branch tip number (TDBTN) were measured as described previously (Saito et al., 2009) using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). At least 60 neurons per construct were collected from three independent experiments, and statistical significance was determined by using the ANOVA test (Dunnett) or *t* test. Dendritic tips were scored when they were longer than 3 μ m as described previously (Saito et al., 2009).

In vivo electroporation. Pregnant ICR mice were purchased from Japan SLC and treated in accordance with the guidelines for the Animal Care and Use Committee of the Graduate School of Biostudies of Kyoto University (Kyoto, Japan). *In vivo* electroporation was performed as described previously (Saito and Nakatsuji, 2001). In brief, timed pregnant ICR mice bearing E15 embryos were deeply anesthetized and the uterine horns carrying either sex of embryos were exposed through a midline abdominal incision. Two microliters of plasmid solutions (0.5–1.5 μ g/ μ l diluted with saline) were injected into the lateral ventricle of the embryos using a micropipette made from a glass capillary. Electric pulses (50 ms at 950 ms intervals) were delivered five times with forceps-type electrodes (CUI 650P5; Nepagene) and an electroporator (CUI21EDIT; Nepa Gene) at 50 V. The uterine horns were then placed back into the abdominal cavity and the abdominal wall was sutured.

Immunohistochemistry. After the *in utero* electroporation of the indicated expression vectors at E15, mice at postnatal day postnatal day 7 (P7) were anesthetized and transcardially perfused. Isolated brains were fixed with 4% PFA, then saturated with 30% sucrose in PBS overnight at 4°C, and frozen with dry ice. Brains were sliced in 50- μ m-thick coronal sections using a cryostat (CM3050S; Leica). Sections were washed with PBS, permeabilized with 0.3% Triton X-100 in PBS for 15 min and then blocked with PBS containing 1% blocking reagent (Roche), 1% goat

Figure 2. GTP-bound active form of M-Ras specifically binds to the RA domain of Lpd. **A**, Lpd constructs used in this study. Numbers indicate amino acid positions within the sequence. **B**, HEK293T cell lysates expressing constitutively active mutants of various Ras family proteins were used in a pull-down assay with GST-Lpd-RA. **C**, **D**, Cell lysates from HEK293T cells expressing the indicated plasmids were immunoprecipitated with anti-Myc antibody. **E**, HEK293T cell lysates expressing HA-tagged Lpd were used in pull-down assay with GST-M-Ras preloaded with GTP- γ S or GDP- β S. **F**, Purified GST or GST-Lpd-RA proteins spotted onto nitrocellulose membranes were probed with GST-M-Ras loaded with GDP- β S or GTP- γ S. Bound GST-M-Ras was detected with anti-M-Ras antibody.

serum, and 0.15% Triton X-100 for 1 h at room temperature. The sections were incubated with Alexa Fluor 488-conjugated anti-GFP antibody (Invitrogen) for 24 h at 4°C. Then they were washed five times with PBS containing 0.1% Tween 20 and mounted using 90% glycerol containing 0.1% *p*-phenylenediamine dihydrochloride in PBS. Confocal images were acquired using a laser-scanning confocal imaging system (FLUOVIEW FV1000-D; Olympus) and a microscope equipped with spectral system (IX81-S; Olympus) and a 10 $\times$ NA 0.4 dry objective (Olympus). The images were arranged and labeled using Photoshop (Adobe).

Results

Sema4D regulates actin-based dendrite remodeling through downregulation of M-Ras activity

We recently reported that the Sema4D receptor Plexin-B1 induces reduction in dendritic outgrowth by functioning as M-Ras GAP (Saito et al., 2009). To examine whether this action is accompanied by actin cytoskeletal rearrangement, we observed distribution of phalloidin-stained F-actin in primary cultured cortical neurons with or without Sema4D stimulation. Sema4D stimulation for 1.5 h induced the disappearance of F-actin from the distal part of the dendritic processes (Fig. 1A), which was quantified by measuring the ratio of F-actin fluorescence intensity between distal and proximal regions in dendrites (Fig. 1B). The Sema4D-mediated reduction of F-actin in distal dendrites

Figure 3. Lpd is required for M-Ras-mediated dendritic maturation. **A**, Developmental expression of Lpd in cultured cortical neurons was analyzed by immunoblotting. **B**, **C**, Cultured cortical neurons at 7 DIV were double-stained with antibodies against Lpd and MAP2 (**B**) or rhodamine-conjugated phalloidin (**C**). Scale bar, 20 μm. **D**, Lysates from cortical neurons (*Figure legend continues*.)

was blocked by ectopic expression of a constitutively active form of M-Ras, M-RasQL. These results indicate that Sema4D regulates actin-based dendrite remodeling of cortical neurons through downregulation of M-Ras activity.

M-Ras specifically and directly binds to the RA domain of an Ena/VASP ligand Lpd

To search for signaling molecules linking M-Ras to actin-based dendrite remodeling, we performed a BLAST search for RA domain, which is found in various Ras effectors such as Raf-1 and PI3-K. As a result, we identified an Ena/VASP ligand Lpd as a candidate effector for M-Ras. To define the RA domain-binding specificity among the Ras family proteins, we examined the interaction of the RA domain of Lpd with various Ras family members, including M-Ras, R-Ras, Rap1, and H-Ras, by pull-down assays. Myc-tagged constitutively active M-Ras, R-Ras, Rap1, and H-Ras were expressed in HEK293T cells and used in pull-down assays with purified GST-fused Lpd-RA (RA domain of Lpd; amino acids 1–380) (Fig. 2*A*). GST-Lpd-RA interacted with active mutants of M-Ras and R-Ras. However, active mutants of H-Ras or Rap1 did not interact with GST-Lpd-RA, showing specificity for M-Ras and R-Ras among Ras family members (Fig. 2*B*). To further confirm the specificity of the interaction, we examined the interaction between Lpd and various mutants of M-Ras by immunoprecipitation assays. HA-tagged full-length Lpd and Myc-tagged various mutants of M-Ras were coexpressed in HEK293T cells, and the cell lysates were incubated with an anti-Myc antibody. As shown in Figure 2*C*, full-length Lpd interacted with M-RasQL, but not with M-RasSN, a dominant-negative mutant, or M-RasQLDA, an effector loop mutant. These data indicate that active M-Ras specifically binds Lpd in cells. To define the M-Ras-interacting region within Lpd, we generated Lpd-ΔRA (Fig. 2*A*), which lacks RA domain, and used it in immunoprecipitation studies. We observed the interaction of M-RasQL with wild-type (WT) Lpd, but not Lpd-ΔRA (Fig. 2*D*). These data suggest that the RA domain is necessary and sufficient for M-Ras binding. Next, to examine the GTP-dependent interaction of M-Ras with Lpd, HA-tagged Lpd expressed in HEK293T cells was used in a pull-down assay with GST-fused M-Ras preloaded with GDP-βS or GTP-γS. Lpd interacted with GTP-bound active M-Ras much more strongly than GDP-bound inactive M-Ras (Fig. 2*E*). A dot blot assay using *E. coli*-derived recombinant Lpd and GDP-βS- or GTP-γS-loaded M-Ras protein purified from *E. coli* as a probe revealed that the GTP-bound active M-Ras directly interacted with the RA domain of Lpd (Fig. 2*F*). Collectively, these results suggest that active M-Ras specifically and directly binds to the RA domain of Lpd.

M-Ras interacts with Lpd in cortical neurons during dendritic development

M-Ras is predominantly expressed in the CNS, especially in the cortex (Kimmelman et al., 2002). We have reported that M-Ras, but not R-Ras, is required for normal dendrite development, and the expression of M-Ras protein is upregulated during the stage of dendritic development (Saito et al., 2009). Lpd has been reported to be localized at the tips of axonal growth cones and involved in axonal morphogenesis (Krause et al., 2004; Michael et al., 2010), but its expression in dendrites has been obscure. Our Western blot analysis with primary cultured rat cortical neurons revealed that Lpd was expressed in cortical neurons throughout the periods of axonal and dendritic development (Fig. 3*A*), and immunofluorescent confocal microscopy with anti-Lpd and anti-MAP2 antibodies showed that Lpd localized at the tips and shafts of MAP2-positive dendrites in primary cultured rat cortical neurons at 7 DIV (Fig. 3*B*). Moreover, double-staining for Lpd and F-actin revealed that Lpd was enriched in F-actin-rich distal dendritic processes (Fig. 3*C*). We next examined the endogenous interaction of Lpd with M-Ras and R-Ras in cultured cortical neurons at 7 DIV. As shown in Figure 3*D*, endogenous M-Ras was coimmunoprecipitated with Lpd by an anti-Lpd antibody, but not by an anti-HA antibody used as a negative control. Conversely, we did not detect an interaction between Lpd and R-Ras. These results indicate that M-Ras specifically interacts with Lpd during dendrite development.

Knockdown of Lpd reduces dendritic outgrowth and branching

Next, to assess the role of Lpd in dendrite morphogenesis, we generated two shRNA expression vectors designed to target two different regions of the Lpd transcript. These shRNAs effectively reduced the amount of endogenous Lpd in cortical neurons (Fig. 3*E*, *F*). At 4 DIV, cultured cortical neurons were transfected with yellow fluorescent protein (YFP) and shRNAs, and their dendrite morphology was observed at 7 DIV. Expression of the control shRNA did not affect the dendrite development of cortical neurons, but expression of the two Lpd-specific shRNAs impaired dendritic outgrowth and branching (Fig. 3*G–I*). These results indicate that Lpd is required for normal dendrite development in cortical neurons.

Lpd is required for M-Ras-mediated dendritic maturation

We have previously shown that ectopic expression of M-RasQL promotes dendritic maturation in cultured cortical neurons (Saito et al., 2009). To address the biological role of interaction between Lpd and M-Ras during dendrite development, we examined the effect of knockdown of Lpd on dendrite morphology in M-RasQL-expressing neurons. Ectopic expression of M-RasQL with control shRNA enhanced dendritic outgrowth and branching, whereas Lpd shRNAs suppressed dendritic outgrowth and branching promoted by overexpression of M-RasQL, indicating that Lpd acts downstream of M-Ras to induce dendrite maturation (Fig. 3*L–N*). To rule out potential off-target effects of the Lpd shRNA, we generated a shRNA-resistant construct of Lpd for a rescue analysis (rLpd; Fig. 3*J*), which effectively restored Lpd expression (Fig. 3*K*). Expression of rLpd with Lpd shRNA in neurons rescued the M-RasQL-dependent dendritic outgrowth and branching, confirming that the suppression induced by the Lpd shRNA is not the result of off-target effects (Fig. 3*L–N*).

M-Ras induces membrane translocation of Lpd

Next, we sought to identify the physiological role of the interaction of M-Ras with Lpd. It has been well known that Ras family proteins are

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(Figure legend continued.) at 7 DIV were immunoprecipitated with an antibody against Lpd. **E**, **F**, Effects of Lpd-specific shRNAs on endogenous Lpd protein in 7 DIV neurons were verified by immunoblot of whole-cell lysates (**E**) or immunofluorescence cell staining (**F**). Scale bar, 20 μm. **G**, **L**, Cultured cortical neurons were cotransfected with YFP and the indicated plasmids at 4 DIV and fixed at 7 DIV. Transfected neurons were shown by the fluorescence of YFP. Arrows and arrowheads indicate axons and dendrites, respectively. Scale bar, 20 μm. **H**, **I**, **M**, **N**, Total length of dendrites and TDBTN of the transfected neurons were measured. Results are the means ± SEM of three independent experiments ($n = 60$; * $p < 0.05$; *** $p < 0.005$). **J**, Construction of shRNA-resistant Lpd (rLpd) by silent mutagenesis. Sequence alignment of Lpd and rLpd in the Lpd shRNA #2 region is shown. Numbers indicate amino acid position within the sequence, and asterisks indicate identical nucleotides. **K**, Effects of the Lpd shRNA #2 on exogenously expressed Lpd. Whole-cell lysates from HEK293T cells cotransfected with Myc-tagged Lpd and the shRNA constructs were immunoblotted with an antibody against Myc or α-tubulin.

Figure 4. Lpd-mediated dendritic development requires M-Ras binding and membrane targeting. **A**, **B**, Cellular homogenates from HEK293T cells (**A**) or cultured cortical neurons at 7 DIV (**B**) transfected with the indicated expression plasmids were separated into membrane (M) and cytosolic (C) fractions. The fractionated samples were analyzed by immunoblotting with anti-HA or anti-GFP antibody (**A**) and anti-Lpd or anti-M-Ras antibody (**B**). These fractions were also immunoblotted with anti-Na⁺/K⁺-ATPase antibody for the control membrane protein and with anti-GAPDH antibody for the control cytosolic protein. The results shown are representative of three independent experiments that yielded similar results. **C**, Lpd-CAAX and -ΔRA-CAAX constructs used in this study. **D**, Subcellular localization of Myc-Lpd-CAAX and Myc-Lpd-ΔRA-CAAX were analyzed as described in the legend to **A**. **E**, Cultured cortical neurons were cotransfected with YFP and the indicated plasmids at 5 DIV and fixed at 7 DIV. Transfected neurons were shown by the fluorescence of YFP. Arrows indicate axons. Scale bar, 20 μm. **F**, **G**, Total length of dendrites (**F**) and TDBTN (**G**) of the transfected neurons were measured. Results are the means ± SEM of three independent experiments ($n = 60$; * $p < 0.05$; *** $p < 0.005$).

targeted to the membranes by a series of modifications of the COOH-terminal CAAX motif, and this membrane anchoring of Ras family proteins is crucial for their signaling and cellular functions (Ehrhardt et al., 2002). We examined whether subcellular localiza-

tion of Lpd is regulated by M-Ras. We prepared crude membrane and cytosolic fractions from cellular homogenates of HEK293T cells expressing HA-tagged Lpd-WT and either GFP-fused M-RasQL or M-RasSN and analyzed the distribution of Lpd by immunoblotting.

Figure 5. Decreased membrane localization of Lpd by Plexin-B1-mediated M-Ras GAP activity is responsible for Sema4D-mediated dendrite remodeling. **A**, Cellular homogenates from cultured cortical neurons at 7 DIV stimulated with Sema4D for 1.5 h were separated into membrane (M) and cytosolic (C) fractions. The results shown are representative of three independent experiments that yielded similar results. **B, E**, Cortical neurons were cotransfected with YFP and indicated plasmids at 5 DIV and fixed at 7 DIV. Neurons were stimulated with Sema4D for 1.5 h before fixation (**B**). Transfected neurons are shown by YFP fluorescence. Arrows indicate axons. Scale bar, 20 μ m. **C, D, F, G**, Total length of dendrites and TDBTN of the transfected neurons were measured. Results are the means $\pm$ SEM of three independent experiments ($n = 60$; *** $p < 0.005$).

Figure 6. Proline-rich region of Lpd is responsible for dendrite development. *A*, Lpd-ΔC and -CT-CAAX constructs used in this study. CT, COOH-terminal region. *B*, Cell lysates from HEK293T cells expressing the indicated plasmids were immunoprecipitated with anti-Myc antibody. *C*, Cellular homogenates from HEK293T cells transfected with the indicated expression plasmids were separated into membrane (M) and cytosolic (C) fractions. The fractionated samples were analyzed by immunoblotting with anti-Myc antibody. These fractions were also immunoblotted with anti- Na^+/K^+ -ATPase antibody and anti-GAPDH antibody. *D*, Cultured cortical neurons were cotransfected with YFP and the indicated plasmids at 5 DIV and fixed at 7 DIV. Transfected neurons were shown by the fluorescence of YFP. Arrows indicate axons. Scale bar, 20 μm . *E*, *F*, Total length of dendrites and TDBTN of the transfected neurons were measured. Results are the means $\pm$ SEM of three independent experiments ($n = 60$; * $p < 0.05$; *** $p < 0.005$).

As shown in Figure 4*A*, Lpd by itself was predominant in the cytosol and hardly detected in the membrane fraction. In contrast, when coexpressed with M-RasQL, Lpd was partly translocated to the membrane fraction, whereas such translocation was not observed with M-RasSN. Moreover, depletion of endogenous M-Ras by M-Ras-specific shRNA (Saito et al., 2009) suppressed membrane localization of endogenous Lpd in cortical neurons (Fig. 4*B*). These results suggest that M-Ras binding to the RA domain induces membrane localization of Lpd.

Lpd-mediated dendritic development requires M-Ras binding and membrane targeting

Given that active M-Ras recruits Lpd to the membrane, we examined whether M-Ras binding and membrane translocation of Lpd

are required for Lpd to induce dendrite development in cortical neurons. In control shRNA-expressing neurons, overexpression of Lpd-WT, but not Lpd-ΔRA, promoted dendritic maturation. Furthermore, Lpd-WT-mediated promotion of dendritic maturation was suppressed by knockdown of M-Ras by M-Ras shRNA, suggesting that M-Ras binding to the RA domain is required for Lpd-mediated dendritic maturation (Fig. 4*E–G*). To further examine whether artificial targeting of Lpd to the membrane would affect dendrite morphology in M-Ras knockdown neurons, we fused the CAAX sequence of K-Ras to the COOH terminus of Myc-tagged Lpd full-length and Lpd-ΔRA to create membrane targeting forms (Myc-Lpd-FL-CAAX and Myc-Lpd-ΔRA-CAAX), and their membrane localization was verified by subcellular fractionation analysis (Fig. 4*C, D*). Knockdown of endogenous M-Ras suppressed dendritic outgrowth and branching, and expression of Lpd-FL-CAAX or Lpd-ΔRA-CAAX was sufficient to overcome the reduction of dendritic outgrowth and branching induced by knockdown of M-Ras (Fig. 4*E–G*). These results suggest that M-Ras binding and membrane targeting of Lpd are required for Lpd to induce dendrite development.

Decreased membrane localization of Lpd by Plexin-B1-mediated M-Ras GAP activity is responsible for Sema4D-mediated dendrite remodeling

We examined whether the M-Ras-Lpd system is involved in Sema4D/Plexin-B1-mediated dendrite remodeling. Subcellular fractionation revealed that Sema4D stimulation suppressed membrane localization of Lpd in cortical neurons (Fig. 5*A*), so we examined whether decreased membrane localization of Lpd is correlated with Sema4D action. Expression of Lpd-CAAX, but not Lpd-WT, was sufficient to overcome the reduction of dendritic outgrowth and branching induced by Sema4D (Fig. 5*B, C*). We recently reported that the extracellular domain-deleted Plexin-B1, Plexin-B1Δect, shows constitutive ligand-independent activity of M-Ras GAP; also, expression of Plexin-B1Δect-WT but not Plexin-B1Δect-RA, which has mutations at arginine motifs of GAP domains essential for GAP activity, reduces dendritic outgrowth and branching in cultured cortical neurons (Saito et al., 2009). Ectopic expression of Lpd-CAAX, but not Lpd-WT, suppressed the M-Ras GAP activity-dependent reduction of dendritic outgrowth and branching (Fig. 5*E–G*). These results suggest that decreased membrane localization of Lpd by Plexin-B1-mediated M-Ras GAP activity is responsible for Sema4D-mediated dendrite remodeling.

The COOH-terminal Ena/VASP-binding region within Lpd is required for dendrite development

Lpd has been well known as an Ena/VASP-binding protein and localizes at the tips of F-actin in axonal growth cones (Krause et al., 2004). Lpd contains four Ena/VASP-binding motifs in the COOH-terminal region, and the region is supposed to be responsible for reconstructing actin network (Krause et al., 2004). To determine whether the COOH-terminal region is required for Lpd-mediated dendrite development, we assessed the ability of truncated forms of Lpd to affect dendrite development in cortical neurons. We generated Lpd- Δ C (amino acids 1–592), which lacks all of the Ena/VASP-binding motifs (Fig. 6A), and verified its ability to interact with M-RasQL by immunoprecipitation study (Fig. 6B). We transfected cortical neurons with Lpd- Δ C at 5 DIV, observed them at 7 DIV, and found that Lpd-WT-induced promotion of dendritic outgrowth and branching was impaired by deletion of the COOH-terminal region (Fig. 6D–F). To further characterize the biological significance of the COOH-terminal region of Lpd in dendrite development, we generated Lpd-CT (amino acids 683–1250), which lacks RA and PH domains but harbors Ena/VASP-binding motifs, and the CAAX-fused forms of Lpd- Δ C and Lpd-CT (Fig. 6A), which are efficiently targeted to the membrane (Fig. 6C). Quantification analysis of the cortical neurons expressing the CAAX-fused constructs revealed that ectopic expression of Lpd-CT-CAAX promoted dendritic outgrowth and branching, whereas expression of Lpd- Δ C-CAAX did not (Fig. 6D–F). These results suggest that the COOH-terminal Ena/VASP-binding region within Lpd is required for dendrite development and its membrane targeting is sufficient.

Lpd-CT-CAAX is sufficient to block the Sema4D-mediated reduction of dendritic outgrowth and disappearance of F-actin from distal dendritic processes

Next, we examined whether the COOH-terminal Ena/VASP-binding region within Lpd mediates Sema4D-induced actin-based dendrite remodeling. Sema4D stimulation suppressed dendritic outgrowth and branching, and expression of Lpd-CT-CAAX was sufficient to overcome the reduction of dendritic outgrowth and branching induced by Sema4D treatment (Fig. 7A–C). We further analyzed the distribution of phalloidin-stained F-actin of primary cultured cortical neurons expressing Lpd-CT-CAAX with or without Sema4D stimulation. Quantification analysis of the ratio of F-actin fluorescence intensity between distal and proximal regions in dendrites showed that Sema4D-mediated reduction of F-actin in distal dendrites was blocked by ectopic expression of Lpd-CT-CAAX (Fig. 7D). These results indicate that the COOH-terminal Ena/VASP-binding region within Lpd is responsible for Sema4D-mediated actin-based dendrite remodeling of cortical neurons.

Figure 7. Lpd-CT-CAAX is sufficient to block the Sema4D-mediated reduction of dendritic outgrowth and the disappearance of F-actin from distal dendrites. **A**, Cultured cortical neurons cotransfected with YFP and the indicated plasmids at 5 DIV were fixed at 7 DIV after stimulation with Sema4D for 1.5 h. Transfected cells are shown by YFP fluorescence. Arrows indicate axons. Scale bar, 20 μm. **B**, **C**, Total length of dendrites and TDBTN were measured. Results are the means $\pm$ SEM of three independent experiments ($n = 60$; *** $p < 0.005$). **D**, Relative F-actin intensity ratio of distal over proximal was determined as described in the legend to Figure 1. Results are the means $\pm$ SEM of three independent experiments ($n = 15$; * $p < 0.05$).

Regulation of the M-Ras-Lpd system by the GAP activity of Plexin is involved in the normal development of cortical dendrites *in vivo*

To further characterize the physiological relevance of the Plexin-mediated regulation of M-Ras-Lpd system in dendrite development, we carried out *in vivo* electroporation. Embryos were electroporated *in utero* at E15 with the plasmids encoding Myc-Plexin-B1-N-Cyt-GGA, which blocks the GAP activity of Plexin-B1 in a dominant-negative manner (Oinuma et al., 2006), M-RasQL, and Myc-Lpd-CT-CAAX, and cerebral cortical sections were prepared from P7 brains. As shown in Figure 8, ectopic expression of Plexin-B1-N-Cyt-GGA (Fig. 8B), M-RasQL (Fig. 8C), and Lpd-CT-CAAX (Fig. 8D) induced aberrant morphology of dendrites and entanglement of the apical projection compared with control cells (Fig. 8A). These results indicate that regulation of the M-Ras-Lpd system by the GAP activity of Plexin is involved in the normal development of cortical dendrites *in vivo*.

Figure 8. *In vivo* expression of Plexin-B1-N-Cyt-GGA, M-RasQL, and Lpd-CT-CAAX in cerebral cortex causes aberrant projection and morphology of apical dendrites. **A–D**, Embryos were electroporated *in utero* at E15 with the plasmids encoding GFP (**A**), GFP plus Myc-Plexin-B1-N-Cyt-GGA (**B**), M-RasQL/IRES-GFP (**C**), and GFP plus Myc-Lpd-CT-CAAX (**D**), and cerebral cortical sections were prepared from P7 brains. Confocal images of the cortical sections stained with anti-GFP antibody are shown. Dashed lines indicate the boundary of the apical surface. Scale bar, 50 μ m.

Discussion

We recently reported that Sema4D receptor, Plexin-B1, is a dual functional GAP for R-Ras and M-Ras, remodeling axon and dendrite morphology, respectively (Oinuma et al., 2004a; Saito et al., 2009). Axonal R-Ras GAP inhibits PI3-K-mediated signaling, leading to inactivation of a microtubule assembly promoter protein, CRMP-2. However, signaling molecules linking M-Ras GAP to the dendritic cytoskeleton remain obscure. In this study, we have identified an Ena/VASP ligand, Lpd, as the effector of M-Ras in dendrites and revealed a novel mechanism for actin-based dendrite remodeling by Sema/Plexin-mediated M-Ras GAP activity (Fig. 9).

M-Ras stimulates the ERK pathway by activating B-Raf, thereby promoting neuronal differentiation and neurite outgrowth in PC12 cells (Kimmelman et al., 2002; Sun et al., 2006). M-Ras regulates dendrite morphology through activation of the ERK pathway, and the inhibitory effect of Sema4D/Plexin-B1 on dendrite development is, in part, mediated by inhibition of the ERK pathway (Saito et al., 2009). However, Sema4D-induced dendrite remodeling is a rapid process and observed within 1.5 h, indicating that cytoskeletal regulators are also involved in Sema4D/Plexin-B1-mediated action. Consistent with this, we have identified an Ena/VASP ligand, Lpd, one of the actin regulator proteins, as an effector of M-Ras, and the M-Ras-Lpd system regulates actin-based dendrite remodeling by Sema4D/PlexinB1-mediated M-Ras GAP activity. The COOH-terminal Ena/VASP-binding region within Lpd is required for dendrite development, and its membrane targeting is sufficient. These results suggest that Sema4D regulates dendrite remodeling through orchestral regulation of Ena/VASP-mediated, actin-based cytoskeletal remodeling and ERK-mediated MAPK signaling pathway.

Lpd is localized at the tips of filopodia in axonal growth cones during the stage of axonal development; knockdown of Lpd decreases axonal length and branching (Krause et al., 2004; Michael et al., 2010), but its expression in dendrites has been obscure. We revealed that Lpd was expressed in cortical neurons throughout the periods of axonal and dendritic development and localized at the F-actin-rich distal dendrites. We have previously shown that the expression of M-Ras protein is poor during the stage of axonal development, and a drastic increase in M-Ras protein is observed after 5 DIV, when dendrites begin to grow (Saito et al., 2009). By contrast, R-Ras is spatially concentrated in axon and its activity increased after plating and peaked between stages 2 and 3, when axon formation occurs, and downregulation of R-Ras activity is required for Sema4D-induced axonal growth cone collapse (Oinuma et al., 2007, 2010). Plexin-B1 is a dual functional GAP for R-Ras and M-Ras GAP, but it does not show GAP activity toward classical Ras proteins such as H-Ras (Oinuma et al., 2004a). In the present study, although both active M-Ras and R-Ras interacted with Lpd in a GST pull-down assay, we could not detect endogenous interaction of Lpd and R-Ras in cortical neurons at 7 DIV, a stage of dendritic development. Further study will be required whether the Lpd-mediated actin-based cytoskeletal regulation system is also involved in Sema4D-mediated axonal growth cone collapse. A recent report has shown that Mig-10, a *C. elegans* Lpd ortholog, interacts with active Rac, a Rho family protein, and Mig-10 regulates axon guidance downstream of Rac in response to netrin and slit (Quinn et al., 2008). The Plexin-B subfamily has been shown to bind to Rac and inhibit its signaling (Hu et al., 2001; Vikis et al., 2002). These findings suggest that not only Ras family but also Rho family proteins may interact with Lpd, and the interaction may contribute to axonal development.

Meanwhile, a very recent study reported that plexins have GAP activity for Rap1, and the activity is required for plexin-

mediated neuronal growth cone collapse (Wang et al., 2012). However, as has been reported previously by Krause and colleagues (Krause et al., 2004), we did not observe the interaction between Lpd and Rap1 in the present study, indicating that Rap GAP activity of Plexin-B1 is not involved in regulation of the Lpd-mediated dendrite development. Our *in vivo* experiments also showed that ectopic expression of a dominant-negative form of Plexin-B1 (Plexin-B1-N-Cyt-GGA), a constitutively active form of M-Ras (M-RasQL), and a membrane-targeting form of the Ena/VASP binding region of Lpd (Lpd-CT-CAAX) caused aberrant morphology of dendrites and entanglement of the apical projection, indicating that M-Ras GAP activity of Plexin-B1 enables proper dendrite development through the regulation of Lpd in cerebral cortex. Actually, in the developing cerebral cortex, Sema4D has been reported to be expressed in an apicobasal gradient with higher expression at the cortical plate at E15 (Worzfeld et al., 2004), suggesting that Sema4D regulates dendrite morphology and projection at the cortical plates. Intriguingly, in our experiments the morphological effect of the expression of M-RasQL appeared to be severer in distal dendrites than that of Plexin-B1-N-Cyt-GGA, and aberrant invasion of dendritic branches into the cortical plates was observed. Since other subtypes of semaphorins and many other guidance molecules are expressed in cerebral cortex, and cortical dendrites are exposed to multiple factors during development, further investigations are required to examine whether and how the M-Ras-Lpd system is spatiotemporally regulated by multiple guidance molecules.

Lpd has been identified as a regulator for Ena/VASP family proteins, and Lpd is localized at the areas of dynamic actin reorganization such as the leading edges of lamellipodia and the tips of filopodia in fibroblasts (Krause et al., 2004). It has also been shown that Lpd interacts with Mena, mammalian Ena, in F-actin-rich growth cones of cultured hippocampal neurons (Krause et al., 2004). Knockdown of Lpd impairs F-actin accumulation or decreases F-actin content through the inhibition of Ena/VASP-mediated signaling, and regulation of F-actin is a fundamental role for Lpd in many cell types (Krause et al., 2004; Lyulcheva et al., 2008; Bae et al., 2010; Smith et al., 2010). The M-Ras antibody used in our study is not suitable for immunostaining, so we could not observe endogenous localization of M-Ras, but overexpressed Myc-tagged M-Ras was localized predominantly in dendrites, and knockdown of endogenous M-Ras decreases F-actin content in dendrites (data not shown), suggesting a coordinate role of Lpd and M-Ras in F-actin regulation in dendrites. In the present study, we have shown that the COOH-terminal Ena/VASP-binding region within Lpd is required and its membrane targeting is sufficient for inducing dendrite development. Furthermore, both Lpd-CT-CAAX and M-RasQL overexpression are sufficient to overcome the reduction of F-actin in distal dendrites induced by Sema4D stimulation. These findings suggest that Lpd, translocated to membrane by M-Ras, may regulate actin polymerization through

Figure 9. Schematic model showing the proposed mechanism underlying Sema4D/Plexin-B1-mediated actin-based dendrite remodeling. In the absence of Sema4D stimulation, Plexin-B1 does not display M-Ras GAP activity, and GTP-bound active M-Ras in dendrites can bind to Lpd. This binding recruits Lpd to the membrane, and membrane targeting of Lpd promotes dendrite development through Ena/VASP-mediated actin cytoskeletal rearrangement (left, boxed in red). In the presence of Sema4D, Plexin-B1 with Rnd1 functions as an M-Ras GAP, suppressing M-Ras activity in dendrites. GDP-bound inactive M-Ras is still localized to the membrane but cannot bind to Lpd. This causes delocalization of Lpd from the membrane and the resultant disappearance of F-actin from distal part of the dendritic processes, leading to reduction in dendrite outgrowth (right, boxed blue).

Ena/VASP proteins at the tips of dendrites during dendrite development. Interestingly, Lpd has been shown to exist in protein complex with N-WASP, Scar/WAVE, and Ena to regulate actin cytoskeleton (Salazar et al., 2003). Further study will be required to elucidate whether these protein complex is involved in M-Ras-Lpd-mediated F-actin regulation in dendrites.

It has recently been reported that Lpd is a substrate for c-Abl, and phosphorylation of Lpd by c-Abl positively regulates Lpd activity by enhancing Lpd-Ena/VASP interaction. Axon morphogenesis requires c-Abl, which does not affect the localization of Lpd (Michael et al., 2010). In this study, we have shown that the M-Ras-binding and membrane translocation of Lpd are required for Lpd to induce dendritic outgrowth. Furthermore, overexpression of Lpd-ΔRA-CAAX, but not Lpd-ΔRA, is efficient to induce dendritic outgrowth even in M-Ras-deficient neurons. Thus, we propose that the major role of M-Ras may be recruitment of Lpd to the membrane, and the other upstream molecules such as c-Abl may be involved in Lpd activation after translocation to induce localized promotion of F-actin polymerization.

In conclusion, we have, for the first time, identified an Ena/VASP ligand, Lpd, as a novel effector of M-Ras in dendrites, and we demonstrate a novel mechanism in that Sema/Plexin-mediated M-Ras GAP activity regulates actin-based dendrite remodeling through Lpd. In addition to the nervous system, semaphorins and plexins are widely expressed in embryonic and adult tissues and mediate diverse biological processes, such as cardiac and skeletal development (Behar et al., 1996), tumor growth, metastasis (Christensen et al., 1998), and the immune response (Kumanogoh and Kikutani, 2003). Con-

sidering that GAP activity of plexins for R-Ras and M-Ras are conserved among different plexin subfamilies (Toyofuku et al., 2005, Uesugi et al., 2009) and that Lpd is a ubiquitously expressed protein (Krause et al., 2004), it will be intriguing to investigate whether M-Ras-Lpd-mediated F-actin regulation is a major system for a wide range of cellular morphogenesis mediated by Sema/Plexin.

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