Supplementary information

Plexin-B1 is a GAP for M-Ras, remodeling dendrite morphology

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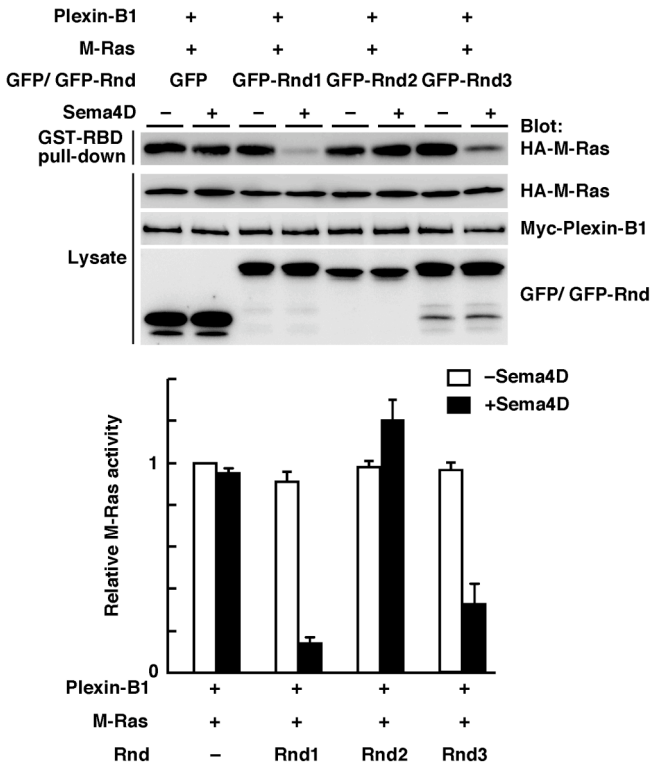
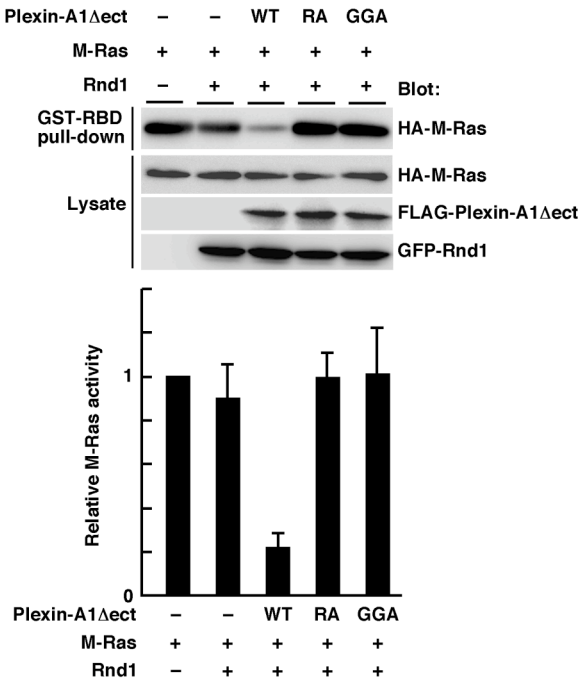
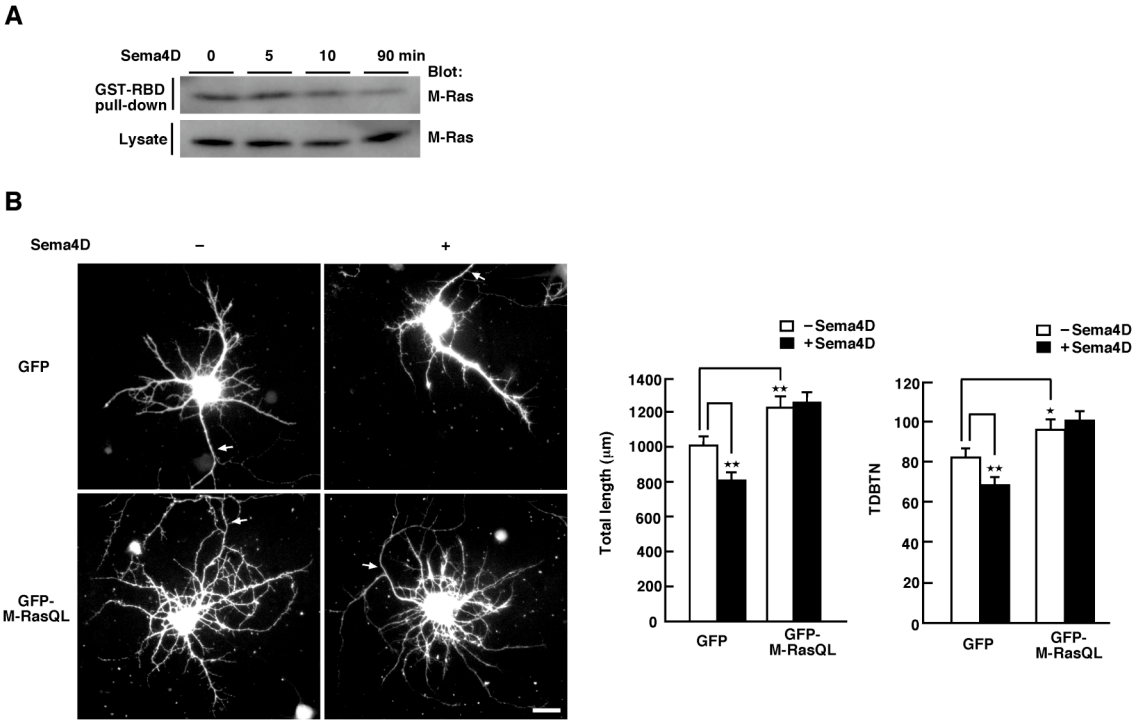
Supplementary Figures



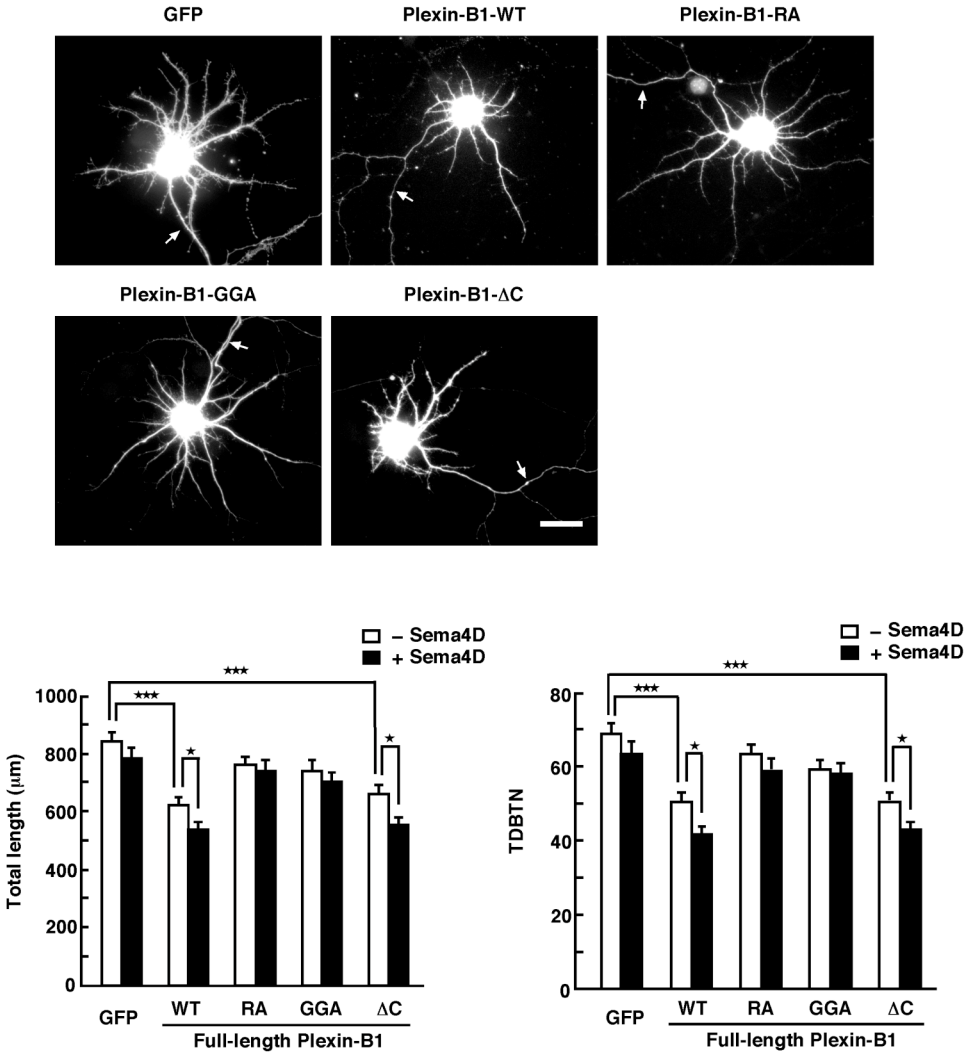
Supplementary Fig 1 Rnd1 and Rnd3 but not Rnd2 support M-Ras GAP activity of Plexin-B1. COS-7 cells transfected with the indicated plasmids were stimulated with Sema4D. The cell lysates were incubated with GST-RBD, and bound M-Ras and total cell lysates were analyzed by immunoblotting. Relative M-Ras activity was determined by the amount of M-Ras bound to GST-RBD normalized to the amount of M-Ras in cell lysates analyzed by NIH Image software (N = 3).



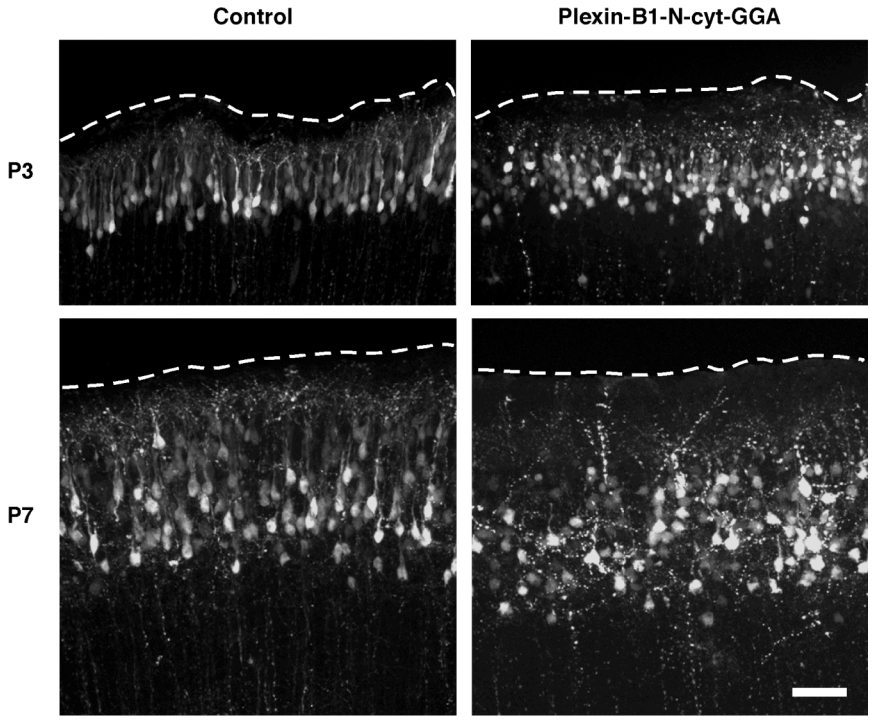
Supplementary Fig 2 Plexin-A1 shows a GAP activity for M-Ras. COS-7 cells were transfected with Plexin-A1Δect (wild-type, RA, or GGA mutant), HA-M-Ras, in the presence or absence of GFP-Rnd1. The cell lysates were incubated with GST-RBD, and bound M-Ras and total cell lysates were analyzed by immunoblotting. Relative M-Ras activity was determined by the amount of M-Ras bound to GST-RBD normalized to the amount of M-Ras in cell lysates analyzed by NIH Image software (N = 3).



Supplementary Fig 3 Sema4D/Plexin-B1 down-regulates M-Ras activity and suppresses dendritic growth in hippocampal neurons. (A) Hippocampal neurons at 7 DIV were treated with Sema4D for the indicated times. GTP-bound M-Ras isolated with GST-RBD and total M-Ras were detected with anti-M-Ras antibody. (B) Hippocampal neurons at 5 DIV were transfected with GFP or GFP-M-Ras(Q71L), and were fixed at 7 DIV after stimulation with Sema4D for 1.5 h. Transfected cells are shown by GFP fluorescence. An arrow indicates an axon. Bar, 20 μm. Total length of dendrites and TDBTN were measured. The results are the means ± SEM of three independent experiments ($n = 40$; $^{*}P < 0.05$; $^{**}P < 0.01$).



Supplementary Fig 4 Full-length Plexin-B1 suppresses dendrite growth. Cortical neurons at 5 DIV were co-transfected with GFP and indicated full-length Plexin-B1 constructs, and fixed at 7 DIV after stimulation with or without human Sema4D for 1.5 h. Transfected cells are shown by GFP fluorescence. An arrow indicates an axon. Bar, 20 μm. Total length of dendrites and TDBTN were measured. The results are the means ± SEM of four independent experiments (n = 60; *P < 0.05; ***P < 0.005).



Supplementary Fig 5 In vivo expression of Plexin-B1-N-Cyt-GGA promotes morphological complexity of cortical neurons. pCAG-EYFP-CAG expression vector, encoding Plexin-B1-N-Cyt-GGA, was in vivo electroporated into the neocortical ventricular zone of mouse embryos at E15, and the brains were fixed at P3 and P7. Coronal sections of the cerebral cortex were stained with Alexa 488-conjugated anti-GFP antibody. Scale bar: 50 μm.

Supplementary Methods

DNA constructs and site-directed mutagenesis.

Green fluorescent protein (GFP)-tagged Rnd1, GFP-Rnd2, GFP-Rnd3, hemagglutinin (HA)-tagged human R-Ras, GST-fused Ras-binding domain of c-Raf-1, Myc-tagged Plexin-B1, Plexin-B1-GGA (L1849G, V1850G, P1851A), Plexin-B1-RA (R1677A, R1678A, R1984A), Plexin-B1-ΔC (lacking the last seven carboxyl terminal amino acids), Plexin-B1Δect (deletion of amino acids 1-1306), Plexin-B1-Cyt (amino acids 1511-2136), and the specific shRNAs for R-Ras have been described previously (Oinuma et al, 2004a, Oinuma et al, 2004b, Oinuma et al, 2006). Plexin-A1Δect (deletion of amino acids 1-1217), Plexin-A1Δect-RA (R1429A, R1430A, R1746A), and Plexin-A1Δect-GGA (L1598G, V1599G, P1600A) have been described previously (Ito et al, 2006). M-Ras and TC21 were obtained by reverse-transcription polymerase reaction (PCR) from rat brain and mouse fibroblast, respectively, and they were subcloned into pGEX-6P-1 (GE Healthcare UK Ltd., Buckinghamshire, England), pEFBOS, and pEGFP-C2 (Clontech Laboratories Inc., Palo Alto, CA, USA). Constitutively active mutant of M-Ras (Q71L) and MEK1 (S218D/S222D) were

generated by PCR-mediated mutagenesis. MEK1 was obtained by PCR from HeLa cells. The shRNAs for M-Ras were designed to target 19 nucleotides at nucleotides 78-96 (5'-gagtgctctcactattcag-3') and nucleotides 142-160 (5'-gactcctacctgaagcata-3') of the rat M-Ras transcript and the control shRNA was designed to target 19 nucleotides of luciferase (5'-cgtacgcggaatacttcga-3) with no significant homology to any mammalian gene sequence; and they were expressed using an shRNA expression vector (Ambion Inc., Austin, TX) as described previously (Oinuma et al, 2004a). Plexin-B1-N-Cyt-GGA (Oinuma et al, 2006) was inserted into pCAG-EYFP-CAG vector (Saito and Nakatsuji, 2001).

Antibodies and reagents.

We used the following antibodies: a mouse monoclonal antibody against Myc (Upstate Biotech, Lake Placid, NY); mouse monoclonal antibodies against α-tubulin, β-actin and MAP2 (2a+2b) (Sigma-Aldrich, St. Louis, MO); a mouse monoclonal antibody against GFP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit polyclonal antibodies against phospho-p44/42 MAP kinase (Thr202/Thr204) and p44/42 MAP kinase (Cell Signaling Technology, Beverly, MA); a rabbit polyclonal antibody against Rnd1

(Oinuma et al., 2004a), a rat monoclonal antibody against HA (clone 3F10; Roche Applied Science, Basel, Switzerland); a goat polyclonal antibody against M-Ras (R&D Systems, Minneapolis, MN); a mouse monoclonal antibody against the extracellular ligand binding region (raised against amino acids 771-1070 of human origin) of Plexin-B1 (Santa Cruz Biotechnology, Inc.); Alexa 594 conjugated secondary antibodies (Molecular Probes, Inc., Junction City, OR); and secondary antibodies conjugated to horseradish peroxidase (Dako Cytomation, Cambridgeshire, UK). The pharmacological MEK1/2 inhibitor UO126 was purchased from Calbiochem (La Jolla, CA) and dissolved in DMSO. Laminin and poly-L-lysine were purchased from Sigma-Aldrich and used at 10 μg and 25 μg/ml, respectively.

Nucleofection.

Cortical neurons from E18 rats ($2x10^6$ cells) were suspended in 100 μl of Nucleofector solution (Amaxa Biosystems Inc., Cologne, Germany), mixed with total 4 μg of plasmid DNA for knockdown of M-Ras (GFP : shRNA = 1:4), or total 2 μg (GFP) and 4μg (GFP-M-RasQL) of plasmid DNA for overexpression of M-Ras, and nucleofected (program O-003) prior to plating using Nucleofector device (Amaxa). At 7 DIV, cells

were directly lysed on dishes with 2x Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting. Nucleofection efficiency was greater than 75% (Oinuma et al, 2006).

Pull-down assay and immunoprecipitation.

Recombinant GST-fusion proteins were purified from *E. coli* as described (Oinuma et al., 2004a). 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 100 mM GTP-gS or GDP in loading buffer (20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 20 mM EDTA, and 10% glycerol) at 30 ˚C for 15 min. The reaction was stopped by addition of $MgCl_2$ to final concentration of 20 mM. *In vitro* binding assays and immunoprecipitation assays were performed as described (Oinuma et al., 2004a). To examine the direct interaction between Plexin-B1 and M-Ras, 1 mg of Myc-Plexin-B1-Cyt and GST-M-Ras, and 10 mg of Rnd1 were mixed in binding buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM GTP, and 1 mM phenylmethylsulphonyl fluoride (PMSF)). For immunoprecipitation studies, COS-7 cells were lysed in lysis buffer (20

mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 1% Nonidet P-40 (NP-40), 0.25% Na-deoxycholate, 10% glycerol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM PMSF, and 1 mM DTT).

In vivo electroporation.

Pregnant ICR mice were purchased from Japan SLC (Shizuoka, Japan), and treated in accordance with the guidelines for the Animal Care and Use Committee of the Graduate School of Biostudies of Kyoto University. In vivo electroporation was performed as described previously (Saito and Nakatsuji, 2001). In brief, timed pregnant ICR mice were deeply anesthetized and the uterine horns carrying embryos were exposed through a midline abdominal incision. Two micro litters of plasmid solusions (0.5 μg/μl diluted with saline) were injected into the lateral ventricle of the embryos using a micropipette made from a glass capillary. Electric pulses (50 msec at 950 msec intervals) were delivered 5 times with forceps-type electrodes (CUY650P5, Nepagene) and an electroporator (CUY21EDIT, Nepagene) at 50 V for E15. The uterine horns were then placed back into the abdominal cavity and the abdominal wall was sutured.

Immunohistochemistry

P3 or P7 mice 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 40-μm-thick coronal sections using a cryostat (CM3050S, Leica). The sections were stained with Alexa 488-conjugated anti-GFP antibody (Invitrogen). Images were obtained a confocal microscope (Nikon) and several confocal Z-stack images of sections were merged using EZ-C1 software (Nikon).

Supplementary References

Oinuma I, Katoh H, Negishi M (2006) Semaphorin 4D/Plexin-B1-mediated R-Ras GAP activity inhibits cell migration by regulating β1 integrin activity. *J Cell Biol* **173**: 601-613

Saito T, Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev Biol* **240**: 237-246