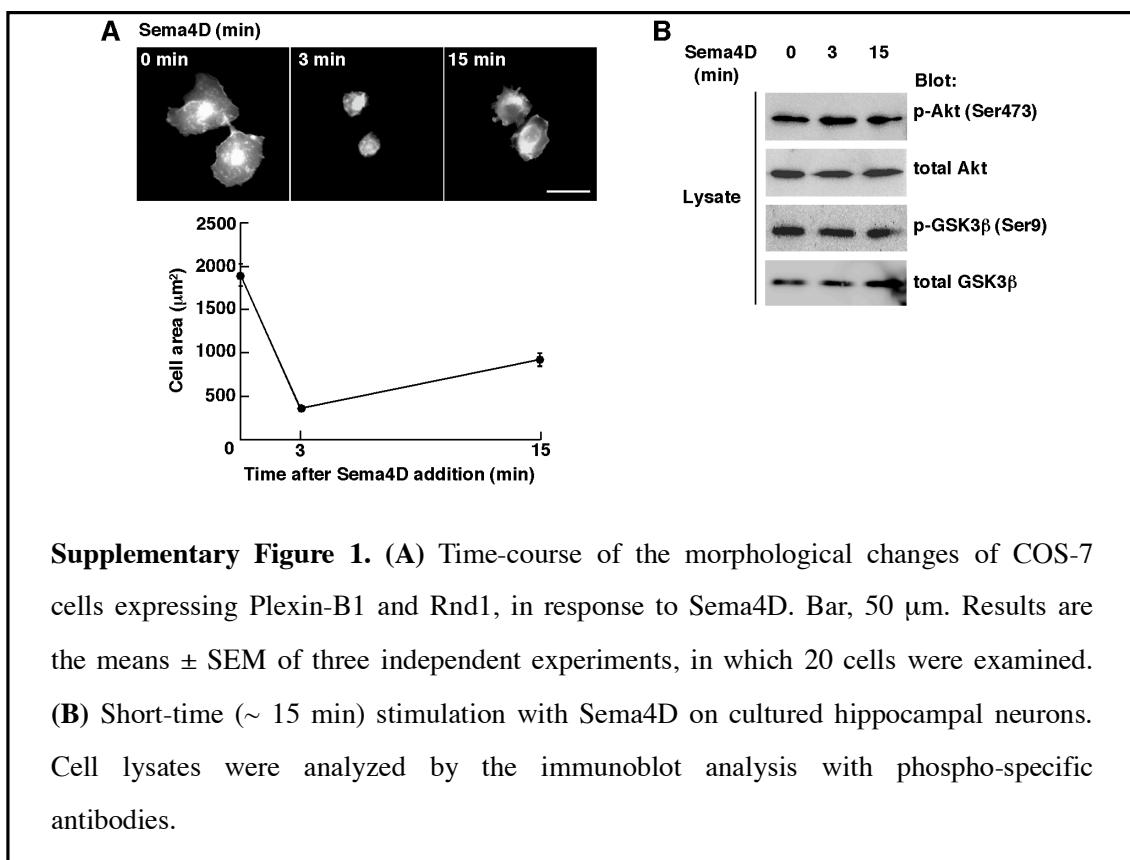


Supplementary information

Sema4D/Plexin-B1 activates GSK-3 β via R-Ras GAP activity, inducing growth cone collapse

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Supplemental Figures



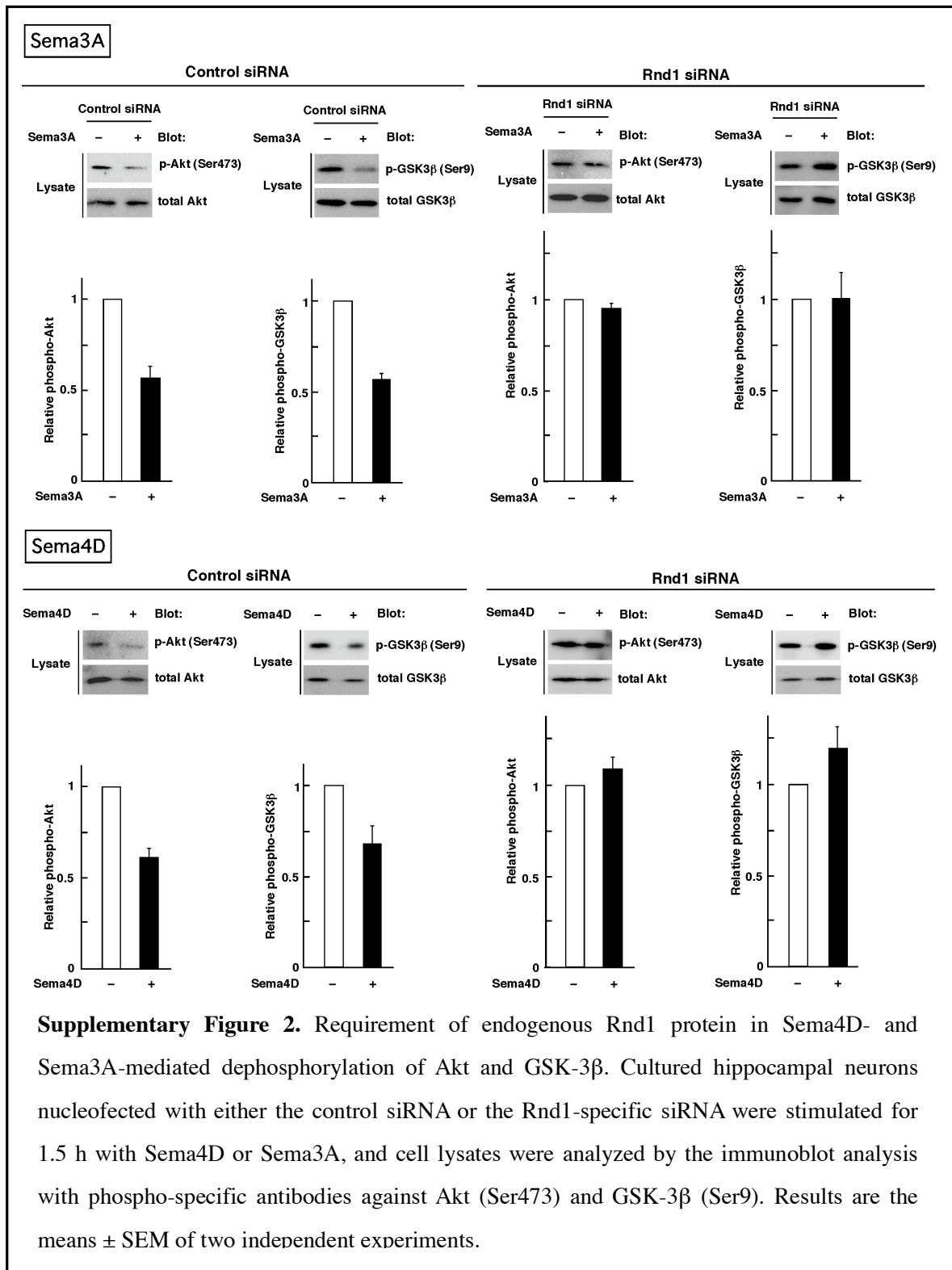
COS-7 cells and cultured hippocampal neurons take different time-courses of dephosphorylation of Akt and GSK-3 β in response to Sema4D

COS-7 cells expressing full-length, wild-type Plexin-B1 and Rnd1 show rapid and transient (within 3~5 min) inactivation of R-Ras in response to Sema4D (Oinuma et al., 2004a,b), and Akt and GSK-3 β dephosphorylation (Fig. 3A). These time-courses

were correlated with morphological changes, and Sema4D-treated COS-7 cells showed transient reduction in cell area within 3 min (supplementary Fig 1A). By contrast, short-time (~15 min) exposure to Sema4D had no effect on Akt and GSK-3 β phosphorylation in cultured hippocampal neurons (supplementary Fig 1B), and 1.5 h was required for full collapse of growth cones (data not shown) and dephosphorylation of Akt and GSK-3 β .

Requirement of endogenous Rnd1 protein in Sema4D- and Sema3A-mediated dephosphorylation of Akt and GSK-3 β in cultured hippocampal neurons

Both Sema4D- and Sema3A-mediated inactivation of R-Ras required Rnd1 binding to the cytoplasmic domains of plexins (Oinuma et al., 2004a, Toyofuku et al., 2005), and dephosphorylation of Akt and GSK-3 β in COS-7 cells also required Rnd1 (Fig. 3). We further confirmed requirement of endogenous Rnd1 protein in Sema4D- and Sema3A-mediated dephosphorylation of Akt and GSK-3 β in cultured hippocampal neurons by expression of the Rnd1-specific siRNA (Oinuma et al., 2004a) using nucleofection technology (supplementary Fig 2). Cultured hippocampal neurons nucleofected with the control siRNA showed dephosphorylation of Akt and GSK-3 β in response to Sema4D or Sema3A, whereas those transfected with the Rnd1-specific siRNA did not. These results suggest that endogenous Rnd1 protein is required for Sema4D- and Sema3A-induced dephosphorylation of Akt and GSK-3 β in cultured hippocampal neurons.



Supplemental methods

DNA constructs and site-directed mutagenesis

Hamagglutinin (HA)-tagged Rnd1, HA- and green fluorescent protein (GFP)-tagged human R-Ras and R-Ras-QL, GST-fused Ras-binding domain of c-Raf-1, N-terminal HA-tagged myristoylated form of R-RasGAP, and Myc-tagged Plexin-B1 constructs and the specific siRNA for Rnd1 and the control siRNA have been described previously (Oinuma et al., 2004a, Oinuma et al., 2004b). An effector loop mutant of R-Ras (D64A) was generated by PCR-mediated mutagenesis. PCR-amplified fragment (amino acids 1217-1895) were ligated into a vector containing an Ig κ -chain leader secretion signal sequence at the NH₂-terminus to express Plexin-A1 Δ ect. Plexin-A1-GGA (L1598G, V1599G, P1600A) and Plexin-A1-RA (R1429A, R1430A, R1746A) were generated by PCR-mediated mutagenesis. Akt, GSK-3 β , and CRMP-2 were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from HeLa, rat brain, mouse brain respectively and subcloned to pcDNA3 (Invitrogen). Src myristoylation signal (MGSSKS) were fused to Akt to generate a constitutively active form of Akt, and a constitutively active mutant of GSK-3 β (S9A) was generated by PCR-mediated mutagenesis.

Antibodies and reagents

We used the following antibodies: a mouse monoclonal antibody against Myc (Upstate Biotechnology); mouse monoclonal antibodies against α -tubulin and Flag (Sigma); a

mouse monoclonal antibody against GFP and a rabbit polyclonal antibody against R-Ras (Santa Cruz Biotechnology); a rabbit polyclonal antibody against GSK-3 β (Chemicon); rabbit polyclonal antibodies against p-Akt and p-GSK-3 β (Cell Signaling); a rat monoclonal antibody against HA (Roche); Rhodamine or Alexa 594-conjugated phalloidin and secondary antibodies (Molecular Probes); and secondary antibodies conjugated to horseradish peroxidase (DAKO). We also used the following antibodies: a rabbit polyclonal antibody against CRMP-2 (IBL); a rabbit polyclonal antibody against p-CRMP-2 (Thr 514; Yoshimura et al., 2005).

Immunoblotting

Proteins were separated by 7% or 12.5% SDS-PAGE, and were electrophoretically transferred onto polyvinylidene difluoride membrane (Millipore Corporation). The membrane was blocked with 3% low fat milk in TBS, and then incubated with primary antibodies. The primary antibodies were detected by using HRP-conjugated secondary antibodies and a chemiluminescence detection kit (Chemi-Lumi One, Nacalai Tesque).

Nucleofection

We used an electroporation-based gene transfer method that shows low toxicity but high efficiency, which relies on the direct introduction of the DNA into the nucleus, ‘nucleofection’ (Chandborn et al., 2006). Hippocampal neurons from E18.5 rat embryos (1.5×10^6 cells) were suspended in 100 μ l nucleofector solution (Amaxa Biosystems),

and were mixed with total 3 μg DNA (GFP : siRNA = 1 : 2) and nucleofected (program o-003, Nucleofector, Amaxa Biosystems) prior to plating. Three days after nucleofection, cells were stimulated with Sema4D or Sema3A. Nucleofection efficiency was above 75% as verified by the counting of GFP-positive cells (data not shown).

Measurement of COS-7 cell area

Measurement of cell area of COS-7 cells was performed as described previously (Oinuma et al., 2003). Briefly, 2×10^4 cells were seeded onto cover slips (circular, 13 mm in diameter), and cells were transfected with Myc-tagged full-length Plexin-B1 and GFP-tagged Rnd1. Sixteen h after transfection, cells were stimulated with Sema4D-Fc for the indicated times and fixed with 4% PFA in PBS. Cell areas of GFP- and Myc-staining double positive cells were determined from digital images acquired at x 40 magnification by using a Leica DC350F digital camera system equipped with a Nikon Eclipse E800 microscope and an Image-Pro Plus image analysis software.

Measurement of R-Ras activity in cultured hippocampal neurons

Hippocampal neurons (5×10^5 cells) at 3 d.i.v. were stimulated 1.5 h with Sema4D-Fc, lysed directly on dishes with ice-cold cell lysis buffer (25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5% Na-deoxycholate, 10% glycerol, 10 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 25 mM NaF, 1 mM orthovanadate, 10 $\mu\text{g}/\text{ml}$ pepstatin) containing 50 μg of GST-fused Ras binding domain of c-Raf-1 (GST-RBD).