Cellular/Molecular

# Inactivation of Ras by p120GAP via Focal Adhesion Kinase Dephosphorylation Mediates RGMa-Induced Growth Cone Collapse

**Mitsuharu Endo and Toshihide Yamashita**
Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

**The repulsive guidance molecule RGMa performs several functions in the developing and adult CNSs. RGMa, through its receptor neogenin, induces growth cone collapse and neurite outgrowth inhibition. Here, we demonstrate that RGMa binding to neogenin leads to the inactivation of Ras, which is required for the RGMa-mediated repulsive function in cortical neurons. This signal transduction is mediated by the Ras-specific GTPase-activating protein (GAP) p120GAP. The SH2 domain of p120GAP interacts with focal adhesion kinase (FAK), which is phosphorylated at Tyr-397. When the cells are stimulated with RGMa, FAK undergoes dephosphorylation at Tyr-397 and is dissociated from p120GAP, and this dissociation is followed by an increase in the interaction between p120GAP and GTP-Ras. In addition, the knockdown of p120GAP prevents RGMa-induced growth cone collapse and neurite outgrowth inhibition. Furthermore, RGMa stimulation induces Akt inactivation through p120GAP, and the expression of the constitutively active Akt prevents RGMa-induced growth cone collapse. Thus, RGMa binding to neogenin regulates p120GAP activity through FAK Tyr-397 dephosphorylation, leading to the inactivation of Ras and its downstream effector Akt, and this signal transduction plays a role in the RGMa-mediated repulsive function.**

## Introduction

During development, growing axons reach their target cells by following specific paths. Repulsive guidance proteins play a role in axon pathfinding by preventing axons from growing along the wrong paths and connecting to inappropriate target cells. The repulsive guidance molecule (RGM) is a developmental repulsive guidance protein that plays a role in axon pathfinding in chicken temporal retina (Monnier et al., 2002). RGMa, a mammalian homolog of chicken RGM, also shows neurite-repulsive ability, and a previous report has indicated that RGMa restricts the axonal extension of developmental entorhinal cortical neurons, thereby facilitating appropriate connection with the hippocampal dentate gyrus (Brinks et al., 2004; Ohshima et al., 2008).

The Rho family small GTPases play essential roles in mediating neurite outgrowth and maintaining growth cone morphology by regulating cytoskeletal reorganization. The neogenin receptor has been shown to mediate the repulsive function of RGMa by activating a small GTPase RhoA (Rajagopalan et al., 2004; Hata et al., 2006). RhoA, its downstream effector Rho kinase, and myosin II have been implicated in RGMa-induced growth cone collapse and neurite outgrowth inhibition (Conrad et al., 2007; Kubo et al., 2008). Ras, another small GTPase that is abundantly distributed in neuronal axons and growth cones, promotes axonal extension during development (Yoshimura et al., 2006; Oinuma et al., 2007; Fivaz et al., 2008). The activity of small GTPases is upregulated by specific guanine nucleotide exchange factors (GEFs) and downregulated by GTPase-activating proteins (GAPs). Several repulsive proteins have been shown to decrease Ras activity by activating specific GAPs, thereby inducing growth cone collapse and neurite retraction (Elowe et al., 2001; Oinuma et al., 2004; Dail et al., 2006). However, the involvement of Ras activity regulation in the RGMa-neogenin signal transduction has not been confirmed. A Ras-specific GTPase-activating protein, p120GAP, has been shown to act as a mediator of an ephrin-dependent Ras inactivation pathway in neuronal cells (Elowe et al., 2001; Dail et al., 2006). A previous report showed that the activity of p120GAP is regulated by the interaction between the SH2 domain of p120GAP and focal adhesion kinase (FAK) phosphorylated at Tyr-397 (Hecker et al., 2004). However, the functional role of the interaction between p120GAP and FAK in neuronal cells has not been elucidated.

In this study, we show that RGMa binding to neogenin decreases Ras activity in N1E-115 neuroblastoma cells and primary cortical neurons. RGMa-induced growth cone collapse is inhibited in the neurons that express constitutively active RasV12. In addition, p120GAP, which is downstream of RGMa-neogenin, mediates Ras inactivation. RGMa stimulation decreases the interaction between p120GAP and FAK by influencing the dephos-

Received Feb. 23, 2009; revised April 7, 2009; accepted April 17, 2009.

This work was supported by National Institute of Biomedical Innovation Research Grant 05-12 and a Grant-in-Aid for Young Scientists (S) from Japan Society for the Promotion of Science. We thank Prof. K. Mizuno for providing pHA- and pMyc-C1 vectors, Prof. P. Mehlen and Prof. E. Fearon for providing a VSV-tagged neogenin construct, and Prof. U. Kikkawa for providing an Akt-myr construct. We thank Dr. Rieko Muramatsu for her assistance in culturing primary neurons.

Correspondence should be addressed to either of the following: Mitsuharu Endo, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan, E-mail: mendo@med.kobe-u.ac.jp; or Toshihide Yamashita, Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, E-mail: yamashita@molneu.med.osaka-u.ac.jp.

DOI:10.1523/JNEUROSCI.0927-09.2009

 0270-6474/09/296649-14$15.00/0

phorylation of FAK Tyr-397, and increases the interaction between p120GAP and GTP-Ras. Furthermore, RGMa-induced growth cone collapse and neurite outgrowth inhibition are inhibited by p120GAP knockdown in cortical neurons. Together, these results suggest that p120GAP mediates the RGMa-induced Ras inactivation through neogenin by decreasing the phosphorylation level of FAK at Tyr-397, thereby inducing growth cone collapse and neurite outgrowth inhibition.

## Materials and Methods

*Antibodies and reagents.* Mouse monoclonal antibody to Ras was obtained from Upstate. Mouse monoclonal antibodies to p120GAP, α-tubulin, and Myc (9E10) and rabbit polyclonal antibodies to neogenin, FAK, glutathione *S*-transferase (GST), and SHP2 were purchased from Santa Cruz Biotechnology. Antibodies to vesicular stomatitis virus (VSV) tag and hemagglutinin (HA) tag were purchased from Sigma-Aldrich. Antibodies to Akt and Erk and phosphospecific antibodies to Akt and Erk were obtained from Cell Signaling Technology. Phosphospecific antibodies to FAK (Tyr-397) and FAK (Tyr-861) were obtained from BioSource International. PP2, PP3, wortmannin, and 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene (U0126) were purchased from Calbiochem. Pervanadate stock solution (30 mM) was prepared immediately before use by mixing a 1 ml solution of 30 mM sodium orthovanadate and 6 μl of $H_2O_2$ (30% stock) for 15 min at room temperature. Residual $H_2O_2$ was removed by the addition of 50 μM catalase after incubation.

*Plasmid constructs and short interfering RNAs.* To generate a secreted form of RGMa, cDNA corresponding to human RGMa (aa 1–404), which lacks a C-terminal GPI (glycosylphosphatidylinositol) anchor region, was fused to human IgG Fc cDNA in the pcDNA5FRT vector (Invitrogen). The cDNA encoding human p120GAP was purchased from American Type Culture Collection (ATCC no. 7491867). To obtain the p120GAP-Myc and GAP-N-Myc expression vectors, cDNA sequences corresponding to the full-length p120GAP and the N-terminal domain of p120GAP (GAP-N) (aa 1–442) were obtained by PCR using specific primers and inserted into the *Nhe*I and *Xho*I sites of pAPtag-5 (GenHunter). For GST fusion constructs, cDNA sequences corresponding to GAP-N, N-terminal SH2 domain of p120GAP (SH2) (aa 181–272), and SH3 domain of 120GAP (SH3) (aa 273–350) were subcloned to the pGEX-6P vector. To obtain the HA-FAK and Myc-FRNK expression vectors, the cDNAs encoding full-length human FAK and FRNK (aa 693–1052) were obtained by PCR from the HEK293T cDNA library and cloned into the pHA- and pMyc-C1 vectors, respectively (provided by K. Mizuno, Tohoku University, Sendai, Japan). The cDNA encoding human H-Ras was also obtained by PCR from the HEK293T cDNA library and cloned into the pcDNA vector (Invitrogen). H-RasV12 was generated by site-directed mutagenesis. The neogenin-VSV expression vector was provided by P. Mehlen (Université de Lyon, Lyon, France) and E. Fearon (University of Michigan Medical School, Ann Arbor, MI). The Akt-myr expression vector was provided by U. Kikkawa (Kobe University, Kobe, Japan).

The neogenin shRNA plasmid that targets the mouse neogenin mRNA sequence (5′-ACCTAGAACCATCATAGTG-3′) and the p120GAP shRNA plasmid that targets the mouse and rat p120GAP mRNA sequence (5′-GATGAAGCCACTACCCTAT-3′) were constructed by using pSUPER vector (OligoEngine). As control short hairpin RNAs (shRNAs), the plasmid for the mutated neogenin shRNA (5′-ACCTCGAACCATAATAGTG-3′) and the plasmid for the mutated p120GAP shRNA (5′-GATGACGCCACTACACTAT-3′) were constructed. The p120GAP short interfering RNA (siRNA) (5′-GCAGGGAAATCTGGAAGCTACCTTA-3′), the SHP2 siRNA (5′-CCAATATTAAGTATTCCCTGGTGGA-3′), and the control siRNA (Medium GC Duplex 2) were purchased from Invitrogen.

*Preparation of recombinant proteins.* Stable CHO cell lines secreting human RGMa-Fc were generated by using a Flp-in system (Invitrogen) as previously described (Hata et al., 2006). RGMa-Fc was purified from the conditioned media that were collected from 2 d cultured RGMa-Fc-CHO by using protein A-Sepharose beads. Recombinant human IgG Fc was purchased from R&D Systems. Recombinant RGMa-Fc and Fc proteins were used at 2 μg/ml for each experiment. GST fusion proteins were expressed in *Escherichia coli* BL21 and purified by using glutathione-Sepharose 4B beads from the bacterial extracts.

*Cell culture and transfection.* HEK293T and N1E-115 cells were maintained in DME medium (Invitrogen) supplemented with 10% FBS and transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

*Neuronal culture.* Cortical neurons were obtained as follows: Whole brains from Wistar rat on embryonic day 18–19 were dissected out, and unwanted portions (brainstem, hindbrain, and hippocampus) were trimmed with fine forceps. The cerebral cortices were dissected and dissociated by incubation with 0.25% trypsin for 15 min at 37°C, followed by washing and trituration in DMEM containing 10% FBS. Dissociated neurons were cultured on 100 μg/ml poly-L-lysine- and 10 μg/ml fibronectin-coated dishes in DMEM containing 10% FBS. After 12 h, the medium was replaced with Neurobasal medium containing B27 supplement.

*Ras activity assay.* The activity of Ras was determined by affinity purification using GST-Raf-RBD. Briefly, cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 5% glycerol, 10 mM $MgCl_2$, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM DTT, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates were clarified by centrifugation at 15,000 rpm for 10 min and the supernatants were incubated with GST-Raf-RBD immobilized on glutathione-Sepharose 4B beads for 45 min at 4°C. The beads were washed three times with lysis buffer, and bound proteins were eluted in SDS-sample buffer. Proteins were separated by SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane. GTP-bound Ras and total Ras were detected by immunoblotting using an anti-Ras antibody.

*GST pull-down assay and immunoprecipitation.* Cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% NP-40, 5% glycerol, 1 mM $Na_3VO_4$, 10 mM NaF, 10 mM β-glycerophosphate, 5 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, and incubated for 30 min on ice, and the extracts were clarified by centrifugation at 15,000 rpm for 10 min. For GST pull-down assays, cleared cell lysates were incubated with GST fusion proteins immobilized on glutathione beads for 1 h at 4°C. For immunoprecipitation, cleared cell lysates were incubated with 2–4 μg/ml antibodies and protein G-Sepharose beads for 4 h at 4°C. Precipitated proteins were separated by SDS-PAGE and transferred to PVDF membrane followed by immunoblotting.

*Far Western blotting.* HEK293T cells were transfected with neogenin-VSV and lysed. The lysates were separated by SDS-PAGE and transferred to the PVDF membrane and blocked by 5% nonfat dry milk in PBS. GST-GAP-N was eluted from glutathione-Sepharose 4B beads using 10 mM reduced glutathione, and excess glutathione was removed by using a PD10 column (GE Healthcare). Purified GST-GAP-N (5 μg/ml) was preincubated with recombinant active FAK (5 μg/ml) (GenWay Biotech) in PBS. Then, the GST-GAP-N alone or the mixture of GST-GAP-N and FAK were incubated with the membranes overnight at 4°C, and binding was detected using an anti-GST antibody.

*Growth cone collapse assay.* Dissociated cortical neurons were centrifuged and resuspended in 100 μl of specified Nucleofection solution (Amaxa), mixed with a total of 4 μg of plasmid [1:3 ratio of green fluorescent protein (GFP) and RasV12, Akt-myr, or shRNA plasmid] or 3 μg of siRNA, and electroporated using the O-03 program of a Nucleofector Device (Amaxa). Electroporated neurons were cultured on poly-L-lysine- and fibronectin-coated dishes in DMEM containing 10% FBS and B27 supplement. After 12 h, the medium was replaced with Neurobasal medium containing B27 supplement, and cells were cultured for another 48 h. Then, neurons were treated with Fc or RGMa-Fc for 30 min, fixed with 4% paraformaldehyde containing 0.25% glutaraldehyde, and stained with rhodamin-phalloidin (Invitrogen). Collapsed growth cones were recognized by the absence of extending lamellipodia.

*Neurite outgrowth assay.* Dissociated cortical neurons were electroporated with 3 μg of siRNA using the O-03 program of the Nucleofector device, and cultured on poly-L-lysine-coated dishes in DMEM containing 10% FBS and B27 supplement. Twenty-four hours after electroporation, cells were detached with 1.5 mM EDTA in PBS, resuspended in

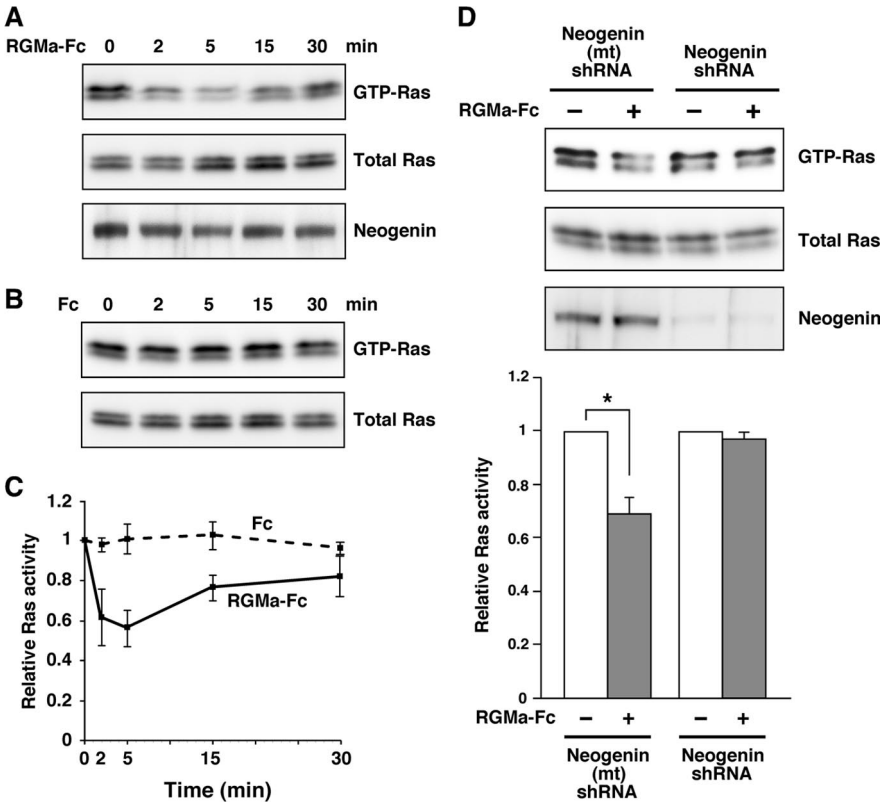


**Figure 1.** RGMa stimulation decreases the level of GTP-bound Ras through neogenin. ***A***, ***B***, Time course of Ras activity after RGMa-Fc (***A***) or Fc treatment (***B***) in N1E-115 cells. The N1E-115 cells were treated with RGMa-Fc or Fc for the indicated durations. Then, the cell lysates were incubated with GST-RBD, and bound GTP-Ras was detected using the anti-Ras antibody. The total Ras and neogenin expressions were detected by immunoblotting the cell lysates. ***C***, Relative Ras activities after RGM-Fc (solid line) or Fc (dashed line) treatment in the N1E-115 cells. The relative Ras activity was determined on the basis of the amount of Ras bound to GST-RBD, which was normalized to the total amount of Ras in the cell lysates. The results are represented as means ± SD of three independent experiments. ***D***, N1E-115 cells were transfected with neogenin shRNA or mutated neogenin shRNA, and the transfected cells were treated with RGMa-Fc for 5 min. The level of Ras activity was determined using the procedure described above. The relative Ras activities are expressed as the means ± SD of three independent experiments (graph). $*p < 0.05$ by Student's *t* test.

Neurobasal medium containing B27 supplement, replated on poly-L-lysine- and fibronectin-coated dishes, and cultured for another 24 h with Fc or RGMa-Fc. The coculture assay was performed as previously described (Hata et al., 2006). Briefly, siRNA-electroporated neurons were plated on confluent monolayers of either RGMa-CHO or control CHO cells in DMEM/F12 medium containing 10% FBS and B27 supplement for 36 h. Then, neurons were fixed with 4% paraformaldehyde containing 4% sucrose, and immunostained with an anti-βIII-tubulin antibody (Tuj1; Covance). The length of the longest neurite for each βIII-tubulin-positive neuron was then determined.

*Statistics.* Statistical analyses were performed using Student's *t* test or two-way ANOVA followed by Bonferroni's *post hoc* test.

## Results

### RGMa stimulation inhibits Ras activity through neogenin

We were interested in identifying a novel signal transduction pathway of RGMa-neogenin. To determine whether RGMa regulates the activity of the small GTPase Ras, we measured Ras activity by a pull-down assay using a GST-fused Ras-binding domain of Raf (Raf-RBD). N1E-115 neuroblastoma cells that expressed the RGMa receptor neogenin were treated with recombinant RGMa-Fc (Fig. 1*A*). The level of active GTP-bound Ras (GTP-Ras) decreased after the RGMa-Fc treatment (Fig. 1*A*), whereas the control Fc treatment did not induce Ras inactivation (Fig. 1*B*). We observed that the Ras activity in the N1E-115 cells reached a minimum at 5 min after the RGMa-Fc treatment and rapidly returned to the basal level (Fig. 1*C*).

To determine whether neogenin mediates RGMa-induced Ras inactivation, we performed knockdown experiments for endogenous neogenin by using siRNA. An siRNA target sequence that shows a strong knockdown effect on endogenous mouse neogenin expression has been reported previously (Kang et al., 2004). As shown in Figure 1*D*, neogenin expression decreased specifically in the N1E-115 cells expressing the neogenin shRNA. When the cells were treated with RGMa-Fc, there was no change in the Ras activity in these cells; in contrast, Ras activity was downregulated in the cells expressing the control shRNA, which had a mutated target sequence for neogenin (Fig. 1*D*). These results suggest that RGMa-neogenin binding leads to Ras inactivation.

### p120GAP mediates the RGMa-induced inactivation of Ras

We investigated whether Ras GAP, a negative regulator of Ras, plays a role in the Ras inactivation induced by RGMa-neogenin binding. A Ras-specific GAP, p120GAP, has two SH2 domains and one SH3 domain in the N-terminal region and a catalytic domain in the C-terminal region. Previous studies have shown that p120GAP hydrolyzes GTP-Ras to GDP-Ras by binding to GTP-Ras through the C-terminal catalytic domain (Trahey and McCormick, 1987; Marshall et al., 1989). In the N1E-115 cells, RGMa-Fc treatment increased the amount of p120GAP coprecipitated with GTP-Ras by GST-Raf-RBD (Fig. 2*A*). This result suggests that RGMa stimulation induces Ras inactivation by increasing the interaction between p120GAP and GTP-Ras.

Next, we inhibited p120GAP activity or p120GAP expression to assess the role of p120GAP in the RGMa-induced Ras inactivation. Since the SH2 and SH3 domains are required for protein–protein interaction, the expression of the N-terminal domain of p120GAP (GAP-N), which lacks the C-terminal catalytic domain but retains the N-terminal SH2 and SH3 domains, prevents endogenous p120GAP activity by acting as a dominant-negative form (McGlade et al., 1993; Elowe et al., 2001). When this GAP-N was transfected into the N1E-115 cells, the RGMa-induced inactivation of Ras was abolished (Fig. 2*B*). Then, we used a knockdown approach. We observed that a target siRNA sequence that had been used in transgenic mouse models and in rat cardiac myocytes showed a strong knockdown effect on endogenous p120GAP expression in N1E-115 cells (Kunath et al., 2003; Yue et al., 2004). The knockdown of p120GAP expression by shRNA effectively inhibited RGMa-induced Ras inactivation; in contrast, the expression of the control shRNA, which had a mutated target sequence for p120GAP, did not affect RGMa-induced Ras inactivation (Fig. 2*C*). These results indicate that RGMa promotes the hydrolysis of GTP-Ras to GDP-Ras by influencing p120GAP activity.

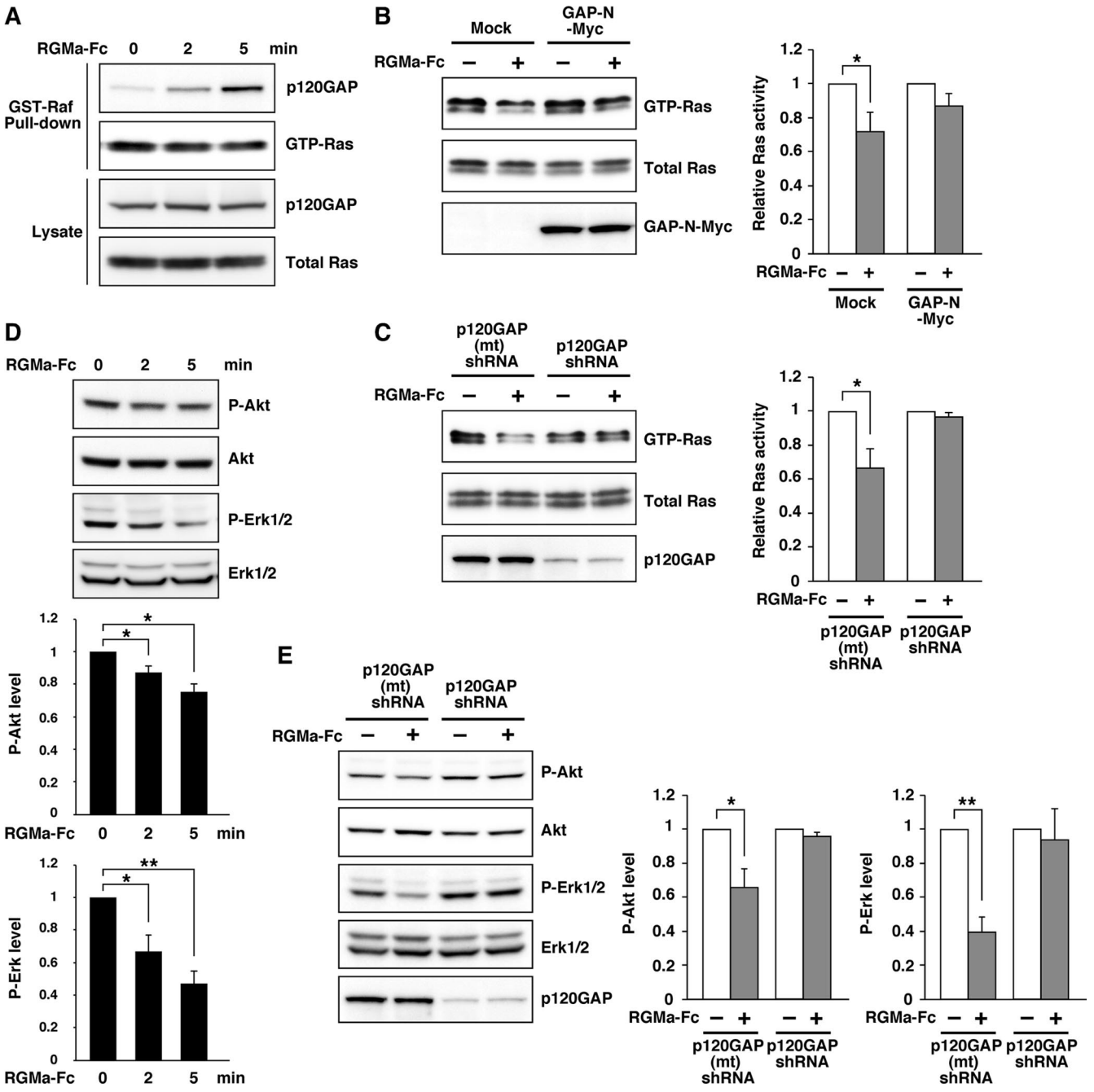


**Figure 2.** The involvement of p120GAP in the RGMa-induced inactivation of Ras, Akt, and Erk. ***A***, RGMa-Fc treatment increases the interaction between p120GAP and GTP-Ras. The N1E-115 cells were treated with RGMa-Fc for the indicated durations. The cell lysates were incubated with GST-RBD, and the coprecipitated p120GAP and GTP-Ras were detected using anti-p120GAP and anti-Ras antibodies. The total p120GAP and Ras were detected by immunoblotting the cell lysates. ***B***, ***C***, The expression of the dominant-negative p120GAP (GAP-N) and the RNA interference (RNAi) knockdown of p120GAP inhibited RGMa-induced Ras inactivation. The N1E-115 cells were transfected with GAP-N-Myc or a mock plasmid (***B***) or p120GAP shRNA or mutated p120GAP shRNA (***C***), and they were treated with RGMa-Fc for 5 min. The levels of Ras activity were determined as described in Figure 1. The relative Ras activities are represented as means ± SD of three independent experiments (graph). *$p < 0.05$ by Student's *t* test. ***D***, RGMa-Fc treatment decreased the phosphorylation of Akt and Erk in the N1E-115 cells. The N1E-115 cells were treated with RGMa-Fc for the indicated durations. The phosphorylation levels of Akt and Erk in the total cell lysates were analyzed by using anti-P-Akt and anti-P-Erk antibodies. The total Akt and Erk expressions were detected by anti-Akt and anti-Erk antibodies. The graphs indicate the relative P-Akt and P-Erk levels that were determined by the amount of P-Akt or P-Erk normalized to the total amounts of Akt or Erk, respectively, in the cell lysates. The results are represented as means ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ by Student's *t* test. ***E***, RNAi knockdown of p120GAP inhibited the RGMa-induced dephosphorylation of Akt and Erk. The N1E-115 cells were transfected with p120GAP shRNA or mutated p120GAP shRNA, and they were treated with RGMa-Fc for 5 min and lysed. The levels of P-Akt and P-Erk were determined as described in ***D***. The relative P-Akt and P-Erk levels are represented as means ± SD of three independent experiments (graph). *$p < 0.05$, **$p < 0.01$ by Student's *t* test.

We also analyzed the involvement of the two major signaling pathways downstream of Ras—PI3 kinase/Akt and Raf/Erk—by monitoring the phosphorylation levels of Akt and Erk, both of which are activated after phosphorylation. In the N1E-115 cells, the amount of phosphorylated Akt and Erk decreased within 5 min after the RGMa-Fc treatment (Fig. 2*D*). This RGMa-dependent dephosphorylation of Akt and Erk was not observed in the p120GAP-shRNA-expressing cells (Fig. 2*E*). These results

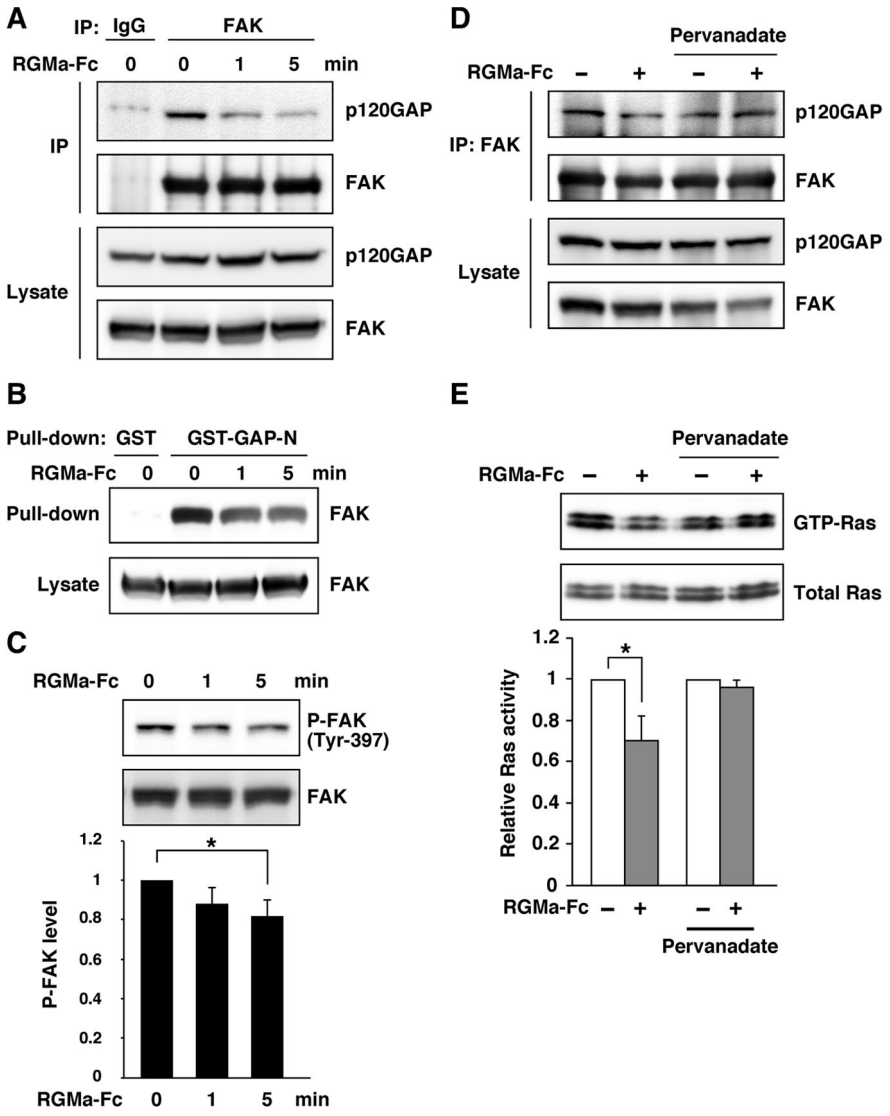


**Figure 3.** RGMa stimulation induces the dissociation of p120GAP from FAK through the dephosphorylation of FAK at Tyr-397. ***A***, RGMa-Fc treatment decreased the interaction between FAK and p120GAP. The N1E-115 cells were treated with RGMa-Fc for the indicated durations and lysed. Then, the cell lysates were immunoprecipitated with anti-FAK antibody or control IgG, and the coprecipitated p120GAP was detected using the anti-p120GAP antibody. ***B***, RGMa-Fc treatment decreased the interaction between FAK and GST-GAP-N. The N1E-115 cells were treated with RGMa-Fc for the indicated durations. Then, the cell lysates were incubated with GST-GAP-N, and the coprecipitated FAK was detected using an anti-FAK antibody. ***C***, RGMa-Fc treatment decreased the Tyr-397 phosphorylation of FAK. The N1E-115 cells were treated with RGMa-Fc for the indicated durations. The Tyr-397 phosphorylation level of FAK in the total cell lysates was analyzed by using the anti-P-FAK (Tyr-397) antibody. The graph indicates the relative P-FAK level determined by the amount of P-FAK normalized to the total amount of FAK in the cell lysates. The results are expressed as means ± SD of three independent experiments (graph). $*p < 0.05$ by Student's *t* test. ***D***, ***E***, treatment with tyrosine phosphatase inhibitor prevented the RGMa-induced dissociation of p120GAP from FAK and the Ras inactivation. The N1E-115 cells were pretreated with 30 $\mu$M pervanadate for 10 min, followed by treatment with RGMa-Fc for 5 min. The cell lysates were immunoprecipitated with anti-FAK antibody, and the coprecipitated p120GAP was detected using the anti-p120GAP antibody. Ras activity was also analyzed by the GST-RBD pull-down assay, as described in Figure 1. The relative Ras activities are represented as the means ± SD of three independent experiments (graph). $*p < 0.05$ by Student's *t* test.

indicate that the p120GAP-dependent Ras inactivation leads to inactivation of Akt and Erk in the N1E-115 cells.

### RGMa stimulation induces the dissociation of p120GAP from FAK by influencing the dephosphorylation of FAK at Tyr-397

It has been reported that p120GAP forms a complex with FAK in the developing rat brain, and this interaction is mediated through the SH2 domain of p120GAP (Serpente et al., 1996) (Fig. 4*B*). A previous study has shown that the interaction of p120GAP with FAK induces Ras activation in malignant tumor cells (Hecker et al., 2004). We observed the interaction between endogenous p120GAP and FAK in N1E-115 cells, and RGMa-Fc treatment decreased this interaction within 5 min (Fig. 3*A*); this time course is similar to that of RGMa-induced Ras inactivation. To examine whether RGMa regulates the interaction between FAK and the SH2 domain of p120GAP, FAK in the lysates of the N1E-115 cells was pulled down by a GST-fused p120GAP N-terminal domain (GST-GAP-N) that contained the SH2 domain. When the cells were treated with RGMa-Fc, the amount of FAK coprecipitated with GST-GAP-N decreased (Fig. 3*B*). This result suggests that RGMa stimulation negatively regulates the interaction between FAK and the SH2 domain of p120GAP.

The SH2 domains recognize and bind specific phosphorylated tyrosine residues in a sequence-specific manner (Pawson et al., 2001). The interaction between FAK and the SH2 domain of p120GAP is dependent on the Tyr-397 phosphorylation of FAK (Hecker et al., 2004). Corresponding to the RGMa-induced decrease in the interaction between FAK and the SH2 domain of p120GAP, the RGMa-Fc treatment resulted in a reduction in the phosphorylation of FAK at Tyr-397 (Fig. 3*C*). To confirm that the RGMa-induced decrease in the interaction between p120GAP and FAK depends on the tyrosine dephosphorylation of FAK, we used a tyrosine phosphatase inhibitor, pervanadate, to block the RGMa-induced dephosphorylation events. As shown in Figure 3*D*, pretreatment of the cells with pervanadate prevented the RGMa-induced dissociation of p120GAP from FAK. Consistent with this observation, the RGMa-induced Ras inactivation was also inhibited by pretreatment with pervanadate (Fig. 3*E*). These results suggest that the RGMa-induced Ras inactivation requires the dissociation of p120GAP from FAK, which occurs through the dephosphorylation of FAK at Tyr-397.

### The SH2 domain of p120GAP interacts with neogenin through the Tyr-397-phosphorylated FAK

The intracellular domain of neogenin has been shown to interact with FAK (Ren et al., 2004). We also observed the interaction between neogenin and FAK in N1E-115 cells by coimmunoprecipitation with an anti-neogenin antibody, and this interaction was not influenced by RGMa treatment (Fig. 4*A*). We further investigated the association between p120GAP and neogenin. Lysates from neogenin-transfected HEK293T cells were incubated with GST-fusion proteins containing GAP-N, the SH2 domain, or the SH3 domain of p120GAP. Neogenin was found to bind to

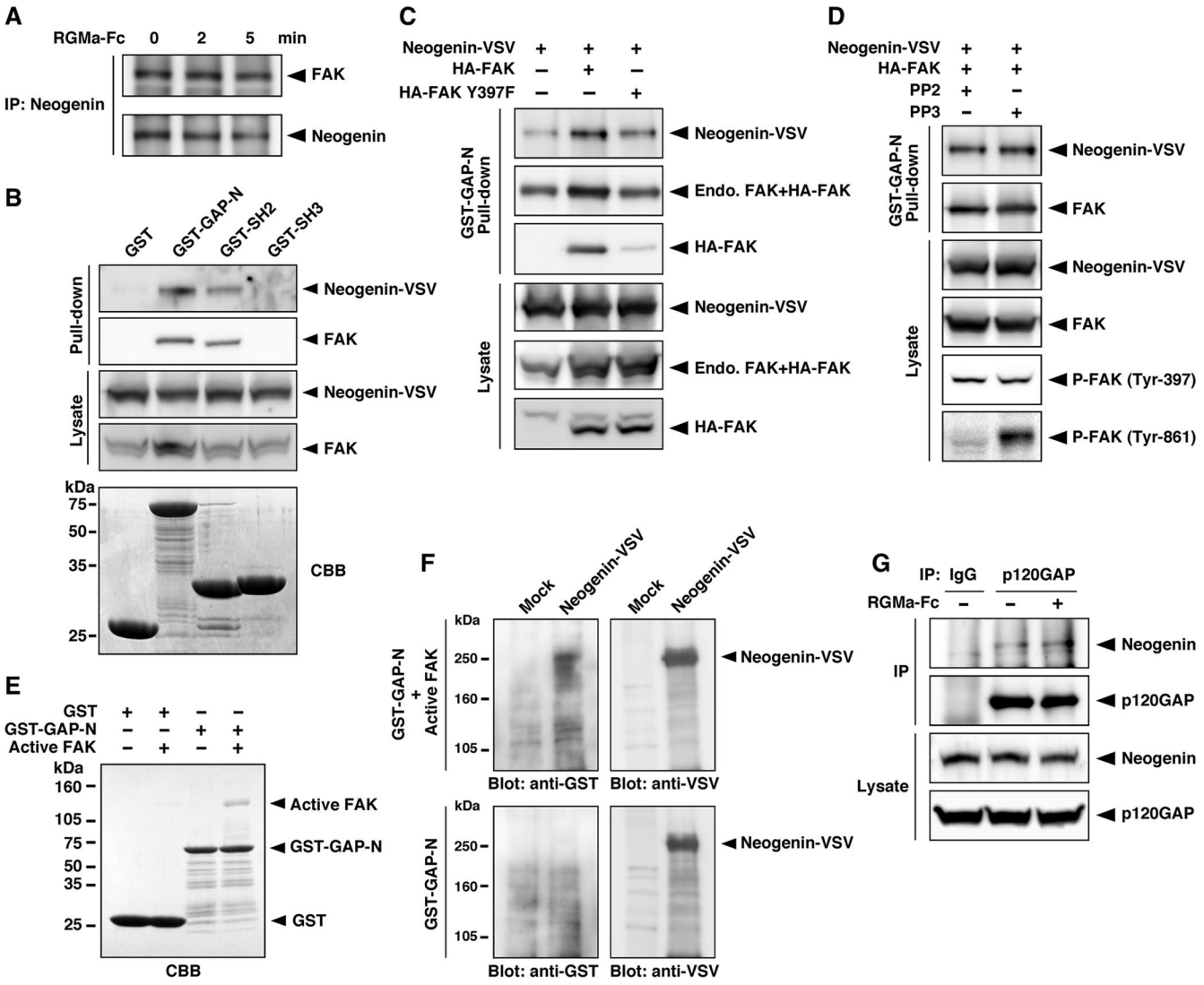


**Figure 4.** FAK mediates the interaction between p120GAP and neogenin. ***A***, RGMa-Fc treatment did not influence the interaction between FAK and neogenin. The N1E-115 cells were treated with RGMa-Fc for the indicated durations and lysed. Then, the cell lysates were immunoprecipitated with the anti-neogenin antibody, and the coprecipitated FAK was detected using the anti-FAK antibody. ***B***, SH2 domain of p120GAP interacts with neogenin and FAK. Cell lysates from the neogenin-VSV-expressing HEK293T cells were incubated with GST, GST-GAP-N, GST-SH2, or GST-SH3 bound to glutathione-Sepharose. The coprecipitated neogenin and FAK were analyzed by immunoblotting with the anti-VSV and anti-FAK antibodies. GST-fusion proteins were detected by Coomassie brilliant blue (CBB) staining. ***C***, Expression of FAK WT, but not of the Y397F mutant, increased the amounts of neogenin and FAK precipitated with GST-GAP-N. The HEK293T cells were transfected with the indicated plasmids and lysed. The cell lysates were incubated with GST-GAP-N bound to glutathione-Sepharose. The coprecipitated neogenin and FAK were analyzed by immunoblotting with anti-VSV, anti-FAK, and anti-HA antibodies. Endo. FAK, Endogenous FAK. ***D***, SFK inhibition did not influence the amounts of neogenin and FAK precipitated with GST-GAP-N. The HEK293T cells expressing neogenin-VSV and HA-FAK were treated with 5 $\mu$M PP2 or 5 $\mu$M PP3 for 30 min and lysed. The cell lysates were incubated with GST-GAP-N bound to glutathione-Sepharose. The coprecipitated neogenin and FAK were analyzed by immunoblotting with anti-VSV and anti-FAK antibodies. The phosphorylation levels of FAK at Tyr-397 and Tyr-861 in the total cell lysates were analyzed by anti-P-FAK (Tyr-397) and anti-P-FAK (Tyr-861) antibodies. ***E***, FAK directly interacted with GST-GAP-N. Recombinant active FAK was incubated with GST or GST-GAP-N bound to glutathione-Sepharose. The precipitated FAK, GST, and GST-GAP-N proteins were detected by CBB staining. ***F***, Neogenin, FAK, and GST-GAP-N form the triple complex. Cell lysates from the HEK293T cells transfected with neogenin-VSV or a mock plasmid were separated by SDS-PAGE, and subjected to Far Western blotting by using GST-GAP-N together with or without recombinant active FAK. Only the GST-GAP-N mixed with active FAK bound to neogenin-VSV. ***G***, RGMa-Fc does not influence the interaction between p120GAP and neogenin. The N1E-115 cells were treated with RGMa-Fc for 5 min and lysed. Then, the cell lysates were immunoprecipitated with the anti-p120GAP antibody, and the coprecipitated neogenin was detected using the anti-neogenin antibody.

GAP-N and the SH2 domain, but not to the control GST or the SH3 domain (Fig. 4*B*). We also detected FAK in the precipitates formed by GST-GAP-N and GST-SH2 (Fig. 4*B*), suggesting that the SH2 domain of p120GAP interacts with neogenin through FAK binding with neogenin. Therefore, we examined the involvement of FAK in the interaction between neogenin and the SH2 domain of p120GAP. As shown in Figure 4*C*, the coexpression of FAK wild type (WT) with neogenin increased the amount of neogenin coprecipitated with GST-GAP-N, whereas coexpression of FAK Y397F, which showed very weak binding to GST-GAP-N, did not increase neogenin coprecipitation. This result indicates that FAK mediates the interaction between neogenin and the SH2 domain of p120GAP, and this interaction is dependent on the phosphorylation of FAK at Tyr-397. Since the phosphorylation of FAK at Tyr-397 leads to additional phosphorylation of tyrosine residues in the C terminus of FAK through the activity of Src family kinase (SFK), the tyrosine phosphorylation at additional sites on FAK may mediate the interaction between FAK and the SH2 domain of p120GAP. To examine the involvement of SFK-dependent tyrosine phosphorylation of FAK in the interaction between the SH2 domain of p120GAP and FAK or neogenin, we used the SFK inhibitor PP2. The cells expressing

neogenin and FAK were treated with PP2, or an inactive analog PP3, and the cell lysates were subjected to the GST-GAP-N pull-down assay. As shown in Figure 4*D*, the FAK phosphorylation at Tyr-861, an SFK-dependent phosphorylation site (Calalb et al., 1996), in the PP2-treated cells was lower than that in the PP3-treated cells; however, PP2 did not have any effect on the phosphorylation of FAK at Tyr-397. Interestingly, there was no significant difference between the amounts of FAK and neogenin coprecipitated with GST-GAP-N in the PP2-treated cells and PP3-treated cells (Fig. 4*D*), suggesting that the SFK-dependent tyrosine phosphorylation of FAK is not required for the interaction between FAK and neogenin and the SH2 domain of p120GAP. In addition, we observed the interaction between the purified recombinant active FAK and GST-GAP-N (Fig. 4*E*), suggesting that FAK directly binds to the SH2 domain of p120GAP. This finding supports the notion that FAK directly binds to the C-terminal domain of neogenin to mediate the link between p120GAP and neogenin. Therefore, we next examined whether the SH2 domain of p120GAP interacts directly with neogenin or indirectly via FAK. To this end, we performed Far Western blotting using GST-GAP-N and recombinant active FAK. The lysates from neogenin-expressing HEK293T cells were separated using SDS-PAGE and transferred to PVDF membranes. The membranes were probed using GST-GAP-N together with or without recombinant active FAK. We observed that GST-GAP-N bound to neogenin on the PVDF membrane only in the presence of active FAK (Fig. 4*F*). This result indicates that FAK acts as the link in the interaction between p120GAP and neogenin. We determined whether RGMa stimulation mediates the interaction between p120GAP and neogenin. A detectable level of neogenin was immunoprecipitated with the anti-p120GAP antibody from the lysates of the N1E-115 cells; however, we did not detect any signals for neogenin when nonspecific IgG was used for immunoprecipitation (Fig. 4*G*). However, there was no significant difference between the amounts of neogenin immunoprecipitated with the anti-p120GAP antibody in the case of the RGMa-Fc-treated cells and nontreated cells (Fig. 4*G*).

**RGMa stimulation inhibits Ras activity in cortical neurons**

To investigate the functional roles of the inactivation of Ras in the RGMa-neogenin signaling pathways, we used cultured cortical neurons that had neogenin and were capable of responding to RGMa (Yoshida et al., 2008). The level of active GTP-Ras in the RGMa-Fc-treated neurons was lower than that in the control Fc-treated neurons (relative Ras activity: at 30 min after RGMa-Fc treatment, 0.77 ± 0.04 of control levels) (Fig. 5*A*). We observed that Ras activity decreased from 10 min to 1 h after the RGMa-Fc treatment (Fig. 5*B*). Consistent with this observation, Akt, a Ras-downstream factor, was dephosphorylated after RGMa-Fc treatment in the cortical neurons as well as in the N1E-115 cells; in contrast, we did not observe dephosphorylation of Erk, another Ras-downstream factor, in the RGMa-Fc-treated cortical neurons (Fig. 5*C*). Therefore, Akt, but not Erk, may be a downstream target of the RGMa-induced Ras inactivation. Furthermore, we observed that RGMa-Fc treatment increased the amount of p120GAP coprecipitated with GTP-Ras by GST-Raf-RBD in the cortical neurons, similar to the results for the N1E-115 cells (Fig. 5*D*). We also observed the interaction of endogenous p120GAP with FAK and neogenin in the cultured cortical neurons, although the interaction of p120GAP with neogenin was very weak compared with that with FAK (Fig. 5*E*), and RGMa-Fc treatment decreased the interaction between p120GAP and FAK in the cortical neurons (Fig. 5*F*) as well as in the N1E-115 cells. Next, we examined whether RGMa stimulation influences the Tyr-397 phosphorylation of FAK and the interaction between FAK and the SH2 domain of p120GAP. The amount of FAK coprecipitated with GST-GAP-N and the phosphorylation level of FAK at Tyr-397 decreased within 10 min after RGMa-Fc treatment (Fig. 5*G*,*H*); this time course is similar to that of RGMa-induced Ras inactivation. These results suggest that RGMa stimulation induces Ras inactivation by increasing the interaction between p120GAP and GTP-Ras in cortical neurons; this interaction may possibly occur through the dissociation of p120GAP from FAK, which is caused by the dephosphorylation of FAK at Tyr-397.

**The involvement of Ras inactivation via p120GAP in RGMa-induced growth cone collapse**

To examine the involvement of Ras inactivation in the repulsive function of RGMa, we transfected constitutively active H-RasV12 along with GFP in the cortical neurons. When the neurons that expressed only GFP were treated with RGMa-Fc, the axonal shafts became more slender and many growth cones exhibited a distorted shape (Fig. 6*A*). The rate of growth cone collapse of the RGMa-Fc-treated neurons significantly increased at 30 min, in comparison with the rate of growth cone collapse in the neurons treated with Fc as a control (Fig. 6*B*) (Fc, 27.4 ± 5.6%; RGMa-Fc, 44.7 ± 5.7%). In contrast, the neurons expressing H-RasV12 had robust axonal shafts and the growth cones exhibited a more expansive morphology (Fig. 6*A*). In the neurons expressing H-RasV12, there was no significant difference between the effects of RGMa-Fc and Fc treatments on the rate of the collapsed growth cones (Fig. 6*B*) (Fc, 22.1 ± 3.3%; RGMa-Fc, 25.4 ± 3.8%). This observation suggests that Ras inactivation is required for RGMa-induced growth cone collapse.

We further examined whether p120GAP plays a role in the growth cone behavior downstream of RGMa-neogenin. We knocked down the expression of endogenous p120GAP by electroporation of a p120GAP shRNA plasmid in the cortical neurons. The expression of p120GAP in p120GAP-shRNA-transfected neurons decreased to ~60% of that in the control-shRNA-transfected neurons (Fig. 6*C*). This result indicates that the p120GAP expression in the p120GAP-shRNA-transfected neurons was almost entirely knocked down, because the estimated transfection efficiency in the GFP-transfected neurons was ~30–40%. When the control shRNAs, which had a mutated target sequence for p120GAP, were electroporated into the neurons, RGMa-Fc treatment enhanced the rate of growth cone collapse (Fig. 6*D*,*E*) (Fc, 28.7 ± 1.0%; RGMa-Fc, 49.6 ± 1.6%). In contrast, treatment with RGMa-Fc did not significantly influence the rate of growth cone collapse in the neurons expressing p120GAP shRNA, in comparison with the Fc treatment (Fig. 6*D*,*E*) (Fc, 32.1 ± 2.2%; RGMa-Fc, 34.2 ± 2.6%). Thus, p120GAP is required for RGMa-induced growth cone collapse.

**The involvement of p120GAP in RGMa-induced neurite outgrowth inhibition**

Previous reports have shown that RGMa inhibits mammalian CNS neurite outgrowth *in vitro* (Brinks et al., 2004; Hata et al., 2006; Kubo et al., 2008; Ohshima et al., 2008; Yoshida et al., 2008). To evaluate the involvement of p120GAP in the long-term effects of RGMa, we performed two types of *in vitro* neurite outgrowth assays. The electroporation of chemically synthesized siRNA resulted in almost complete knockdown of p120GAP expression in the cortical neurons (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). The introduc-

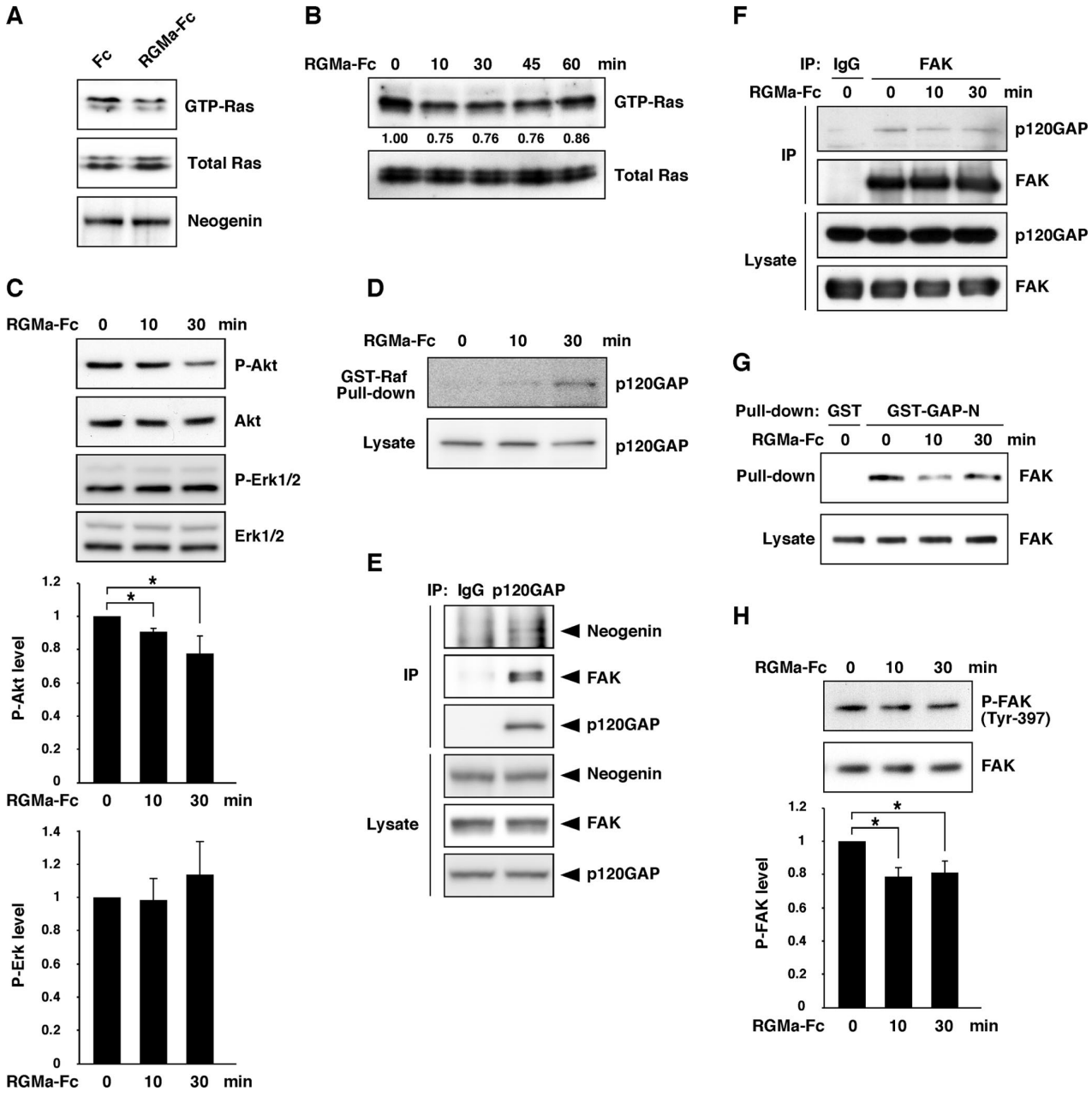


**Figure 5.** The involvement of p120GAP-mediated Ras inactivation in RGMa signaling in cortical neurons. ***A***, RGMa treatment induced inactivation of Ras. Cortical neurons were treated with RGMa-Fc or Fc for 30 min. The levels of Ras activity were determined by using the procedure described in Figure 1. ***B***, The time course of Ras activity after RGMa-Fc treatment in cortical neurons. The relative Ras activities are shown below the top panel. ***C***, RGMa-Fc treatment decreased the phosphorylation of Akt, but not of Erk. The cortical neurons were treated with RGMa-Fc for the indicated durations and lysed. The levels of P-Akt and P-Erk were determined as described in Figure 2*D*. The relative P-Akt and P-Erk levels are represented as means ± SD of three independent experiments (graph). *$p < 0.05$ by Student's *t* test. ***D***, RGMa-Fc treatment increased the interaction between p120GAP and GTP-Ras. The cortical neurons were treated with RGMa-Fc for the indicated durations. The cell lysates were incubated with GST-RBD, and the p120GAP coprecipitated with GTP-Ras was detected by using the anti-p120GAP antibody. The total p120GAP expression was detected by immunoblotting the cell lysates. ***E***, Endogenous p120GAP interacts with neogenin and FAK. The lysates from cortical neurons were used for immunoprecipitation with the anti-p120GAP antibody or control IgG. The whole-cell lysates and the immunoprecipitates were probed with anti-neogenin, anti-FAK, and anti-p120GAP antibodies, as indicated. ***F***, RGMa-Fc treatment decreased the interaction between FAK and p120GAP. The cortical neurons were treated with RGMa-Fc for the indicated durations and lysed. Then, the cell lysates were immunoprecipitated with anti-FAK antibody or control IgG, and the coprecipitated p120GAP was detected using the anti-p120GAP antibody. ***G***, RGMa-Fc treatment decreased the interaction between FAK and GST-GAP-N. The cortical neurons were treated with RGMa-Fc for the indicated durations. Then, the cell lysates were incubated with GST-GAP-N, and the coprecipitated FAK was detected using the anti-FAK antibody. ***H***, RGMa-Fc treatment decreased the phosphorylation of FAK at Tyr-397. The cortical neurons were treated with RGMa-Fc for the indicated durations. The phosphorylation level of FAK at Tyr-397 in the total cell lysates was analyzed by using the anti-P-FAK (Tyr-397) antibody. The graph indicates the relative P-FAK levels that were determined as described in Figure 3*C*. The results are expressed as means ± SD of three independent experiments (graph). *$p < 0.05$ by Student's *t* test.

tion of this siRNA as well as that of p120GAP shRNA prevented RGMa-induced growth cone collapse (supplemental Fig. 1*B*,*C*, available at www.jneurosci.org as supplemental material). Consistent with the effect of p120GAP knockdown on the RGMa-Fc-induced growth cone collapse, the inhibition of neurite outgrowth by RGMa-Fc was absent in the p120GAP-siRNA-electroporated neurons, whereas neurite outgrowth of the control siRNA-electroporated neurons was significantly reduced

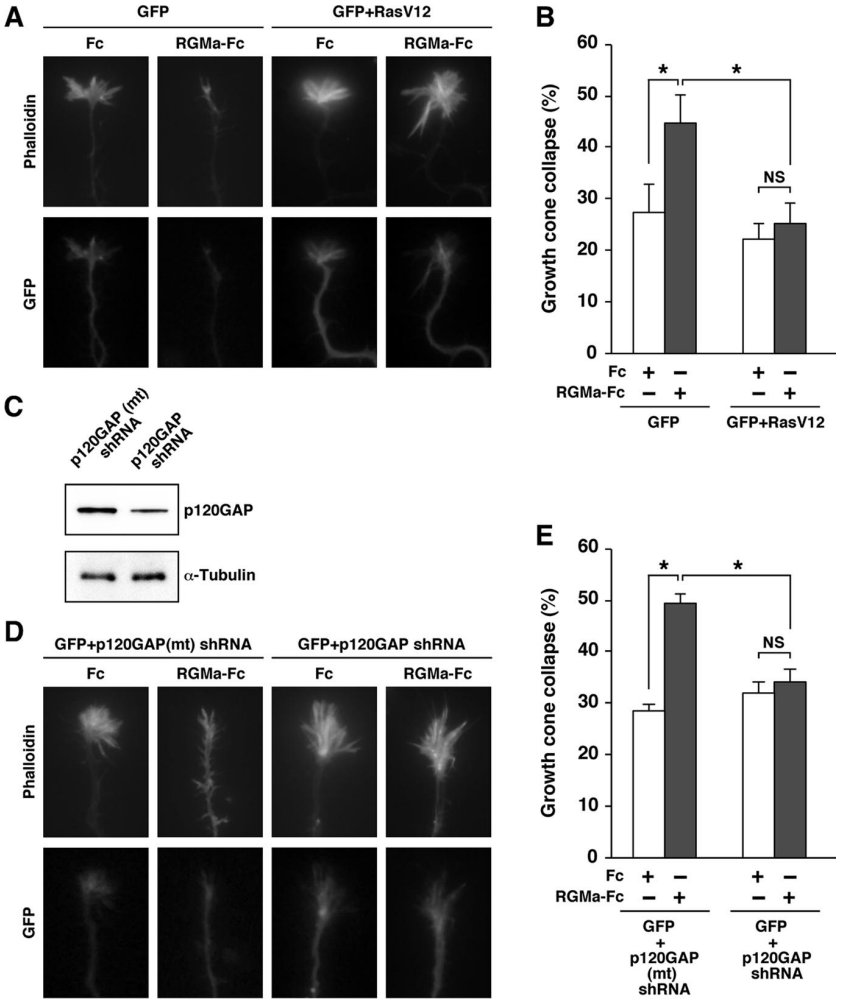


**Figure 6.** Ras inactivation mediated by p120GAP is required for RGMa-dependent growth cone collapse. ***A***, The expression of constitutively active Ras prevented RGMa-induced growth cone collapse. The cortical neurons were cotransfected with RasV12 or a mock plasmid along with GFP. At 60 h after the transfection, the neurons were treated with RGMa-Fc or Fc for 30 min, fixed, and stained with phalloidin for visualizing F-actin. ***B***, The percentages of the collapsed growth cones. The values are represented as the mean ± SD of three independent experiments (at least 70 growth cones in each experiment). *$p < 0.01$ by two-way ANOVA with Bonferroni's *post hoc* test. ***C***, The effect of p120GAP shRNA on the expression of p120GAP in cortical neurons. The cortical neurons transfected with p120GAP shRNA or mutated p120GAP shRNA were cultured for 60 h and lysed. The expressions of p120GAP and α-tubulin were detected with anti-p120GAP and anti-α-tubulin antibodies, respectively. ***D***, p120GAP knockdown prevented RGMa-induced growth cone collapse. The cortical neurons were cotransfected with p120GAP shRNA or mutated p120GAP shRNA along with GFP. At 60 h after the transfection, the neurons were treated with RGMa-Fc or Fc for 30 min, fixed, and stained with phalloidin for F-actin. ***E***, The percentages of the collapsed growth cones. The values are represented as the means ± SD of three independent experiments (at least 70 growth cones in each experiment). *$p < 0.01$ by two-way ANOVA with Bonferroni's *post hoc* test.

in the presence of RGMa-Fc (Fig. 7*A*,*B*) (control siRNA, Fc, 68.2 ± 2.8 μm; RGMa-Fc, 51.5 ± 2.3 μm; p120GAP siRNA, Fc, 66.2 ± 2.8 μm; RGMa-Fc, 67.7 ± 3.1 μm). We also obtained a similar result when the neurons were cultured on a confluent monolayer of RGMa-expressing CHO cells (Fig. 7*C*,*D*) (control siRNA, control CHO, 67.7 ± 3.3 μm; RGMa-CHO, 43.0 ± 2.1 μm; p120GAP siRNA, control CHO, 66.1 ± 3.4 μm; RGMa-CHO, 68.4 ± 3.4 μm). These results suggest that the effects of RGMa on growth cone morphology and neurite growth are regulated by the common signaling pathway involving p120GAP.

### The involvement of Akt inactivation in RGMa-induced growth cone collapse

Next, we examined the involvement of Akt and Erk in the RGMa-induced growth cone collapse. As shown in Figure 8*A*, the treatment of the cortical neurons with wortmannin or U0126 resulted in specific inactivation of Akt or Erk, respectively. When the cultured cortical neurons were treated with wortmannin for 30 min, the rate of growth cone collapse increased significantly, whereas U0126 treatment did not have any effect on growth cone morphology within 30 min (Fig. 8*B*,*C*). To determine whether the inactivation of Akt is necessary for RGMa-induced growth cone collapse, we transfected constitutively active Akt-myr in the cortical neurons. The expression of Akt-myr increased the amount of phosphorylated Akt (Fig. 8*D*) and prevented the RGMa-induced growth cone collapse (Fig. 8*E*,*F*) (mock, Fc, 25.6 ± 2.6%; RGMa-Fc, 54.3 ± 4.9%; Akt-myr, Fc, 25.9 ± 6.1%; RGMa-Fc, 24.9 ± 3.6%), suggesting that RGMa stimulation induces growth cone collapse through the inactivation of Akt.

### The involvement of FAK dephosphorylation in RGMa-induced growth cone collapse

Next, we examined whether dephosphorylation of FAK at Tyr-397 is necessary for RGMa-induced growth cone collapse. To block the dephosphorylation of FAK in the cortical neurons, we treated cultured neurons with pervanadate, a potent tyrosine phosphatase inhibitor. However, pervanadate itself rapidly induced growth cone collapse (data not shown), and we substituted pervanadate with orthovanadate. We observed that RGMa-induced growth cone collapse was prevented by the orthovanadate treatment (Fig. 9*A*,*B*) (control, Fc, 32.5 ± 6.3%; RGMa-Fc, 53.6 ± 4.6%; orthovanadate, Fc, 33.1 ± 4.5%; RGMa-Fc, 33.1 ± 6.3%), although orthovanadate itself did not have any effect on growth cone morphology. In addition, we investigated the inhibitory activity of orthovanadate toward tyrosine phosphatase. The orthovanadate treatment increased the amount of Tyr-397-phosphorylated FAK and prevented RGMa-induced dephosphorylation of FAK (Fig. 9*C*).

Our findings suggest that RGMa stimulation induces dephosphorylation of FAK at Tyr-397 via an unidentified tyrosine phosphatase. Recently, neogenin has been shown to bind tyrosine phosphatase SHP2, depending on the tyrosine phosphorylation of neogenin (Ren et al., 2008). Since SHP2 can dephosphorylate FAK at Tyr-397 (Mañes et al., 1999; Rafiq et al., 2006), SHP2 is a candidate molecule for the regulation of FAK dephosphorylation and the repulsive function in RGMa signaling. However, SHP2 knockdown in cortical neurons did not have any effect on RGMa-induced growth cone collapse or neurite outgrowth inhibition (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

## Discussion

In this study, we found that, in neuronal cells, RGMa regulates the activity of the small GTPase Ras through neogenin. RGMa stimulation induces the inactivation of Ras and its downstream effector Akt, and this inactivation appears to be induced by an increase in the amount of p120GAP interacting with GTP-Ras. We demonstrated that the association between p120GAP and FAK plays a key role in regulating Ras activity in the RGMa signaling pathway. RGMa stimulation decreases the interaction between FAK and p120GAP, and this effect is mediated by the phosphorylation of FAK at Tyr-397. We further demonstrated that the Ras inactivation mediated through the p120GAP activity is important for RGMa-dependent growth cone collapse and neurite outgrowth inhibition. The proposed mechanism of action of p120GAP in RGMa signaling is summarized in Figure 10.

### p120GAP mediates RGMa signaling

The Ras-GAP-mediated Ras inactivation in neurite repulsion has been reported to be involved in ephrin-Eph signaling and semaphorin4D-Plexin-B1 signaling (Elowe et al., 2001; Oinuma et al., 2004; Dail et al., 2006). Similar to these findings, our study revealed that RGMa also induces Ras inactivation in the neogenin-expressing neuronal cells. The Ras inactivation most probably contributes to growth cone collapse, because the expression of constitutively active H-RasV12 prevents RGMa-induced growth cone collapse in cultured cortical neurons. Ras is activated by GEFs and inactivated by GAPs; therefore, inactivation of GEFs, activation of GAPs, or both of these phenomena lead to Ras inactivation (Lowy et al., 1991; Zheng and Quilliam, 2003; Iwashita and Song, 2008). We determined that p120GAP mediates Ras inactivation and repulsive function in the RGMa-signaling process on the basis of the following observations. The knockdown of p120GAP and expression of the dominant-negative form of p120GAP in N1E-115 cells inhibited RGMa-induced Ras inactivation, and the knockdown of p120GAP in the cortical neurons prevented RGMa-induced growth cone collapse and neurite outgrowth inhibition.

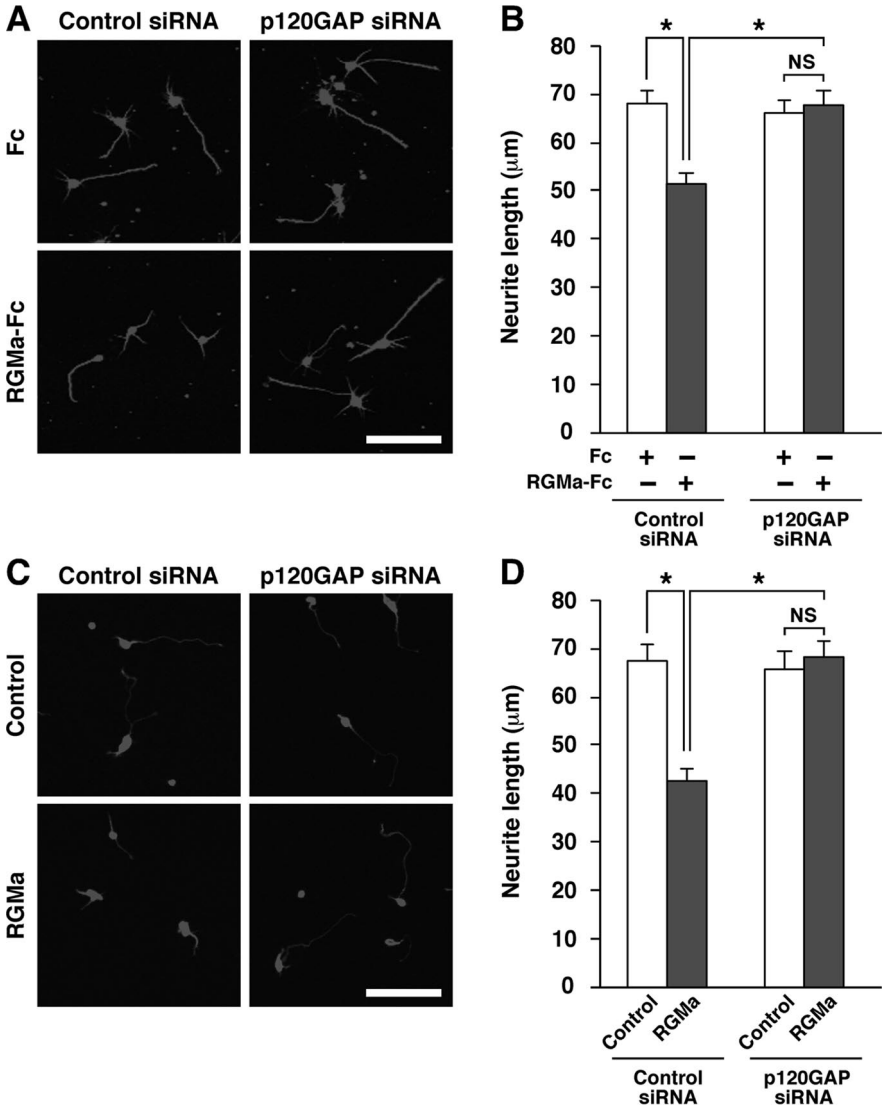


**Figure 7.** p120GAP is required for RGMa-induced neurite outgrowth inhibition. ***A***, p120GAP knockdown prevented RGMa-Fc-induced neurite outgrowth inhibition. The cortical neurons were transfected with p120GAP siRNA or control siRNA. At 24 h after the transfection, the neurons were replated and cultured with RGMa-Fc or Fc for 24 h. Then, the neurons were fixed and immunostained with the anti-βIII-tubulin antibody. Scale bar, 100 μm. ***B***, Mean length of the longest neurite per neuron. The values are represented as the means ± SEM from at least 100 neurons for each population. *$p < 0.01$ by Student's *t* test. ***C***, p120GAP knockdown prevented the neurite outgrowth inhibition induced by membrane-bound RGMa. The cortical neurons were transfected with p120GAP siRNA or control siRNA and cultured for 36 h on CHO cells (control) or RGMa-CHO cells (RGMa). Then, the neurons were fixed and immunostained with the anti-βIII-tubulin antibody. Scale bar, 100 μm. ***D***, Mean length of the longest neurite per neuron. The values are represented as the mean ± SEM for at least 100 neurons for each population. *$p < 0.01$ by Student's *t* test.

p120GAP can inactivate the major Ras isoforms (H-Ras, K-Ras, and N-Ras) and R-Ras, another member of the Ras subfamily (Boguski and McCormick, 1993; Rey et al., 1994; Li et al., 1997). Although H-Ras and R-Ras have highly homologous effector-binding domains and can bind common effectors such as PI3 kinase and Raf *in vitro*, in intact cells, H-Ras can activate both PI3 kinase/Akt and Raf/Erk pathways, whereas R-Ras can activate only the PI3 kinase/Akt pathway (Marte et al., 1997). We demonstrated that RGMa inactivates both Akt and Erk in N1E-115 cells, whereas RGMa inactivates Akt, but not Erk, in the cortical neurons. This difference in downstream signaling may be attributable to the different contributions of the Ras isoforms in each cell type. In addition, the inhibitory effect of H-RasV12 in RGMa-dependent growth cone collapse may be attributable to the inhibition of p120GAP binding to endogenous R-Ras as well as H-Ras. Therefore, additional studies are necessary to clarify the contribution of each Ras isoform to RGMa signaling.

### Mechanisms of p120GAP activation in RGMa signal transduction

The SH2 domain of p120GAP interacts with FAK through the phosphorylation of FAK at Tyr-397. We found that endogenous

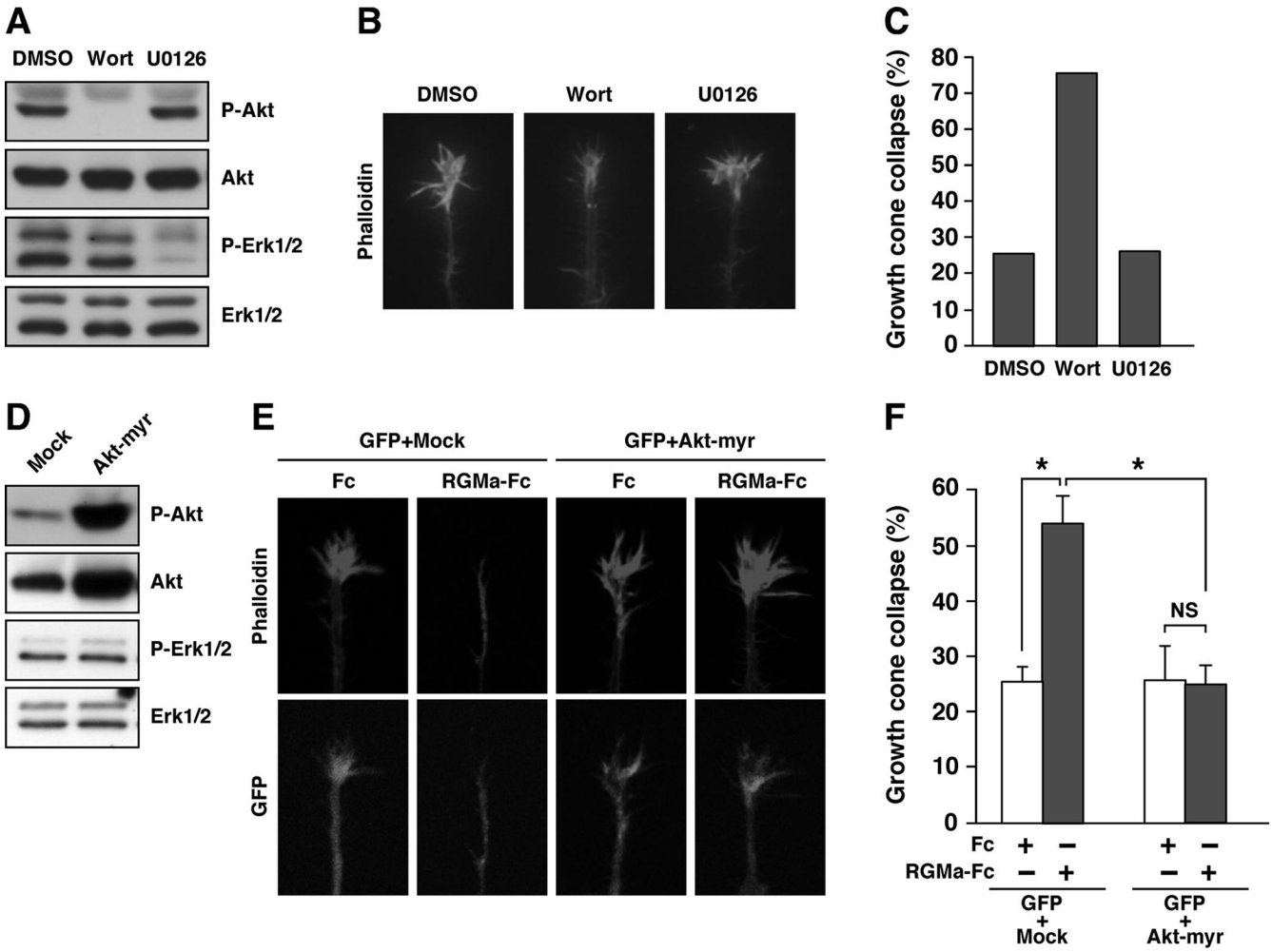


**Figure 8.** Akt, but not Erk, mediates the RGMa-induced growth cone collapse. ***A***, The effects of the inhibition of PI3 kinase or mitogen-activated protein kinase kinase (MEK) on the phosphorylation levels of Akt or Erk in the cortical neurons. The cortical neurons were treated with 100 nM wortmannin (Wort), 10 $\mu$M U0126, or DMSO for 30 min and lysed. The phosphorylation levels of Akt and Erk in the total cell lysates were analyzed by using anti-P-Akt and P-Erk antibodies. The total expressions of Akt and Erk were detected by the anti-Akt and anti-Erk antibodies. ***B***, PI3 kinase inhibition, but not MEK inhibition, induced growth cone collapse in cortical neurons. The cortical neurons were cultured for 48 h and treated with 100 nM wortmannin, 10 $\mu$M U0126, or DMSO for 30 min. Then, the neurons were fixed and stained with phalloidin for detecting F-actin. ***C***, The percentages of the collapsed growth cones after treatment with wortmannin, U0126, or DMSO. ***D***, Expression of constitutively active Akt (Akt-myr) increases the amount of P-Akt, but it does not influence the amount of P-Erk. The cortical neurons were transfected with Akt-myr or a mock plasmid and cultured for 60 h. The levels of P-Akt and P-Erk were analyzed as described in ***A***. ***E***, Expression of Akt-myr prevents RGMa-induced growth cone collapse. The cortical neurons were cotransfected with Akt-myr or a mock plasmid along with GFP. At 60 h after the transfection, the neurons were treated with RGMa-Fc or Fc for 30 min, fixed, and stained with phalloidin. ***F***, The percentages of collapsed growth cones. The values are represented as the means ± SD of three independent experiments (at least 70 growth cones in each experiment). *$p < 0.01$ by two-way ANOVA with Bonferroni's *post hoc* test.

p120GAP interacts with FAK in the cultured embryonic cortical neurons as well as in the N1E-115 cells, which is in agreement with a previous finding for an embryonic rat brain (Serpente et al., 1996). Our study is the first to explore the functional role of this interaction in neuronal cells. The interaction between p120GAP and FAK decreased downstream of RGMa signaling, and this decrease most probably led to the activation of p120GAP for GTP-Ras. However, it is uncertain how the FAK–p120GAP interaction regulates the activity of p120GAP. It has been reported that the overexpression of FAK can increase the level of GTP-Ras by decreasing the interaction between p120GAP and GTP-Ras, which occurs through the interaction between FAK and p120GAP (Hecker et al., 2004). In our experiments, RGMa stimulation induced dephosphorylation of FAK at Tyr-397 and caused dissociation of p120GAP from FAK. Concurrently, after RGMa stimulation, the interaction between p120GAP and GTP-Ras increased. Thus, FAK seems to be involved in sequestering p120GAP from GTP-Ras, and RGMa promotes the release of p120GAP from FAK by influencing the dephosphorylation of FAK at Tyr-397, thereby enabling the interaction of p120GAP with GTP-Ras. In this model, it is necessary to recruit the released p120GAP to the plasma membrane to access the GTP-Ras. Therefore, RGMa-induced Ras inactivation may involve another regulator such as annexin A6 that binds p120GAP and regulates the distribution of p120GAP to the plasma membrane, depending on the intracellular $Ca^{2+}$ concentration (Davis et al., 1996; Grewal et al., 2005; Grewal and Enrich, 2006). Furthermore, our findings suggest that RGMa stimulation induces dephosphorylation of FAK at Tyr-397 via an unidentified tyrosine phosphatase. Although we hypothesized that SHP2 was the candidate tyrosine phosphatase, SHP2 is not required for the repulsive function of RGMa, suggesting that other tyrosine phosphatases mediate FAK dephosphorylation and repulsive function in RGMa signaling.

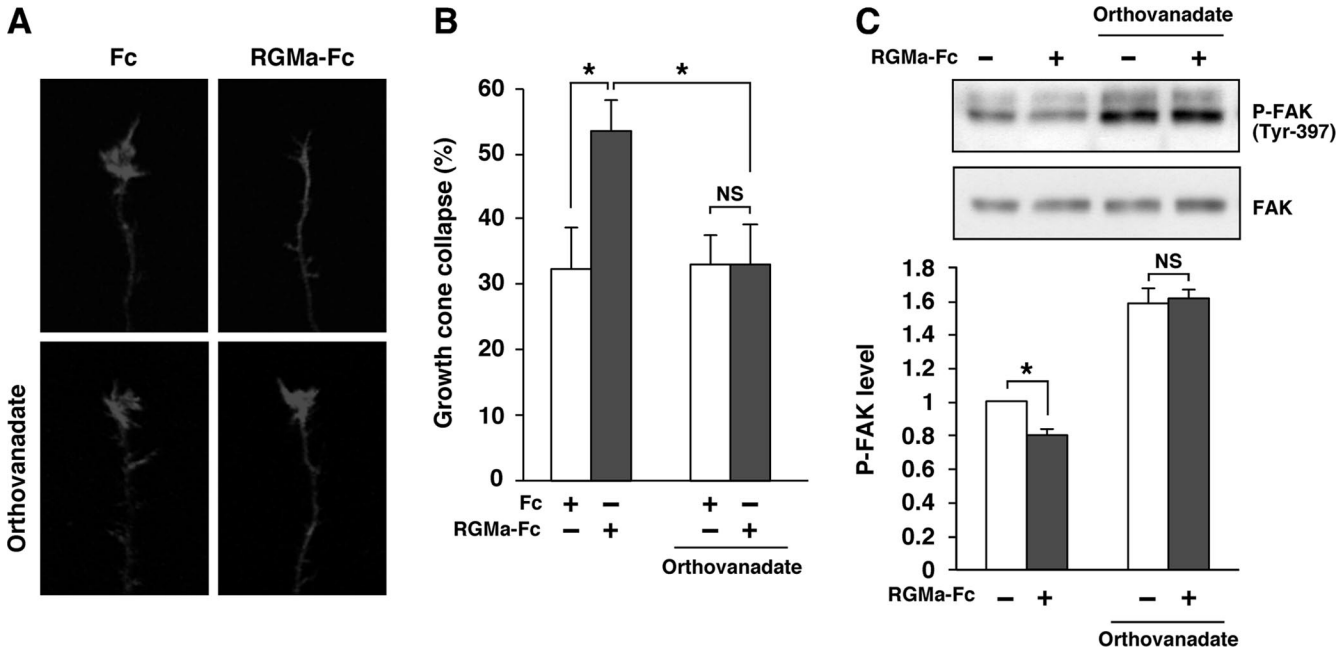


**Figure 9.** Treatment with tyrosine phosphatase inhibitor prevents RGMa-induced growth cone collapse and FAK dephosphorylation. ***A***, Cortical neurons were cultured for 48 h and pretreated with 1 mM orthovanadate for 30 min, which was followed by treatment with RGMa-Fc or Fc for 30 min. Then, the neurons were fixed and stained with phalloidin. ***B***, The percentages of the collapsed growth cones. The values are represented as the means ± SD of three independent experiments (at least 70 growth cones in each experiment). *$p < 0.01$ by two-way ANOVA with Bonferroni's *post hoc* test. ***C***, Cortical neurons were cultured for 48 h and pretreated with 1 mM orthovanadate for 30 min, followed by treatment with RGMa-Fc for 10 min. The Tyr-397 phosphorylation level of FAK in the total cell lysates was analyzed by using the anti-P-FAK (Tyr-397) antibody. The graph indicates the relative P-FAK level that was determined by using the procedure described in Figure 3*C*. The results are represented as the means ± SD of three independent experiments (graph). *$p < 0.05$ by Student's *t* test.

### FAK links p120GAP to neogenin

We also demonstrated that the SH2 domain of p120GAP could interact with neogenin through the Tyr-397-phosphorylated FAK. Recently, several SH2-containing proteins, including SHIP1, SHP2, and Fyn, have been shown to be associated with tyrosine-phosphorylated neogenin (Ren et al., 2008). Tyrosine phosphorylation of neogenin is dependent on FAK activation, which occurs via phosphorylation at Tyr-397, and additional activation of the SFK that binds to the Tyr-397-phosphorylated FAK (Ren et al., 2008). However, in our study, an SFK inhibitor PP2 did not influence the FAK-dependent interaction of the SH2 domain of p120GAP with neogenin. This result suggests that SFK-dependent tyrosine phosphorylation of neogenin is not involved in the interaction between p120GAP and neogenin. The results of our study, which was performed using purified proteins, support the idea that p120GAP may show indirect interaction with neogenin through FAK binding to neogenin rather than direct interaction with the tyrosine-phosphorylated neogenin.

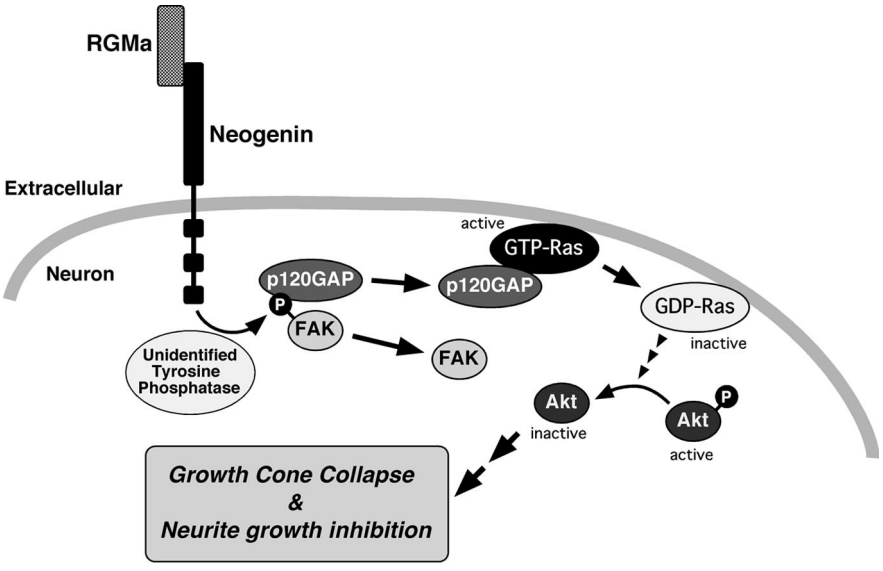


**Figure 10.** A diagram of the proposed mechanism of action of p120GAP in RGMa signaling. RGMa binding to neogenin accelerates the dissociation of p120GAP from FAK by dephosphorylating FAK at Tyr-397 via unidentified protein tyrosine phosphatase(s). The interaction between GTP-Ras and the p120GAP released from FAK mediates the inactivation of Ras and its downstream effector Akt. The inactivation of Ras and Akt contributes to RGMa-mediated repulsive function, including growth cone collapse and neurite outgrowth inhibition.

We detected the interaction between endogenous p120GAP and neogenin in neuronal cells, although the interaction was very weak. Neogenin is necessary for RGMa-induced inactivation of Ras, and RGMa stimulation induces the dissociation of p120GAP from FAK, which occurs through the dephosphorylation of FAK at Tyr-397. Neogenin also directly interacts with FAK (Ren et al., 2004), and this interaction was not influenced by RGMa stimulation in our experiment. These findings suggest that RGMa stimulation may dephosphorylate FAK binding to neogenin to reduce the interaction between p120GAP and neogenin. However, we did not observe significant changes in the interaction between p120GAP and neogenin after RGMa stimulation. Therefore, we

were not able to clarify the role of the interaction between p120GAP and neogenin in the RGMa-induced inactivation of Ras.

### Cross talk between Ras and Rho in the RGMa signaling pathway

RhoA and its downstream effector Rho kinase have been implicated in RGMa-mediated repulsive function (Hata et al., 2006; Conrad et al., 2007). Therefore, both Ras inactivation and RhoA activation seem to cooperatively regulate the repulsive function of RGMa. Although it has not been determined whether these two pathways are related to each other downstream of RGMa and neogenin, p120GAP can bind to p190RhoGAP, which has also been implicated in axon outgrowth (Moran et al., 1991; Settleman et al., 1992; Brouns et al., 2001). Therefore, RGMa might regulate p190RhoGAP via p120GAP, thereby inducing the activation of RhoA. Additional studies are necessary to elucidate the cross talk between the Ras and Rho signaling pathways in the repulsive activities of RGMa.

### Roles of the RGMa-mediated Ras inactivation in neuronal network formation

It has been demonstrated that RGMa is expressed in the inner molecular layer of the hippocampal dentate gyrus and that it restricts the axonal extension of developmental entorhinal cortical neurons, thereby facilitating appropriate connection with the dentate gyrus (Brinks et al., 2004; Ohshima et al., 2008). Furthermore, a recent study reported that RGMa suppresses neurite outgrowth, branching, and synapse formation in cultured cortical neurons (Yoshida et al., 2008), suggesting that RGMa may contribute to the formation of a precise wiring pattern, such as the entorhino-hippocampal connection, by inhibiting both synapse formation in the inner molecular layer of the dentate gyrus and axonal extension of the entorhinal cortical neurons. However, the molecular mechanisms of these inhibitory activities have not been determined, except for the involvement of Rho and Rho kinase in neurite outgrowth inhibition. Our observations in cortical neurons suggest that p120GAP-mediated Ras inactivation in RGMa signaling is one of the mechanisms of such a precise network connection, at least when considering the inhibition of axonal extension. As all the data were obtained by *in vitro* experiments, the relevance of the observations obtained in the present study must be assessed *in vivo* in the future studies.

## References


Boguski MS, McCormick F (1993) Proteins regulating Ras and its relatives. Nature 366:643–654.

Brinks H, Conrad S, Vogt J, Oldekamp J, Sierra A, Deitinghoff L, Bechmann I, Alvarez-Bolado G, Heimrich B, Monnier PP, Mueller BK, Skutella T (2004) The repulsive guidance molecule RGMa is involved in the formation of afferent connections in the dentate gyrus. J Neurosci 24:3862–3869.

Brouns MR, Matheson SF, Settleman J (2001) p190 RhoGAP is the principal Src substrate in brain and regulates axon outgrowth, guidance and fasciculation. Nat Cell Biol 3:361–367.

Calalb MB, Zhang X, Polte TR, Hanks SK (1996) Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. Biochem Biophys Res Commun 228:662–668.

Conrad S, Genth H, Hofmann F, Just I, Skutella T (2007) Neogenin-RGMa signaling at the growth cone is bone morphogenetic protein-independent and involves RhoA, ROCK, and PKC. J Biol Chem 7:784–792.

Dail M, Richter M, Godement P, Pasquale EB (2006) Eph receptors inactivate R-Ras through different mechanisms to achieve cell repulsion. J Cell Sci 119:1244–1254.

Davis AJ, Butt JT, Walker JH, Moss SE, Gawler DJ (1996) The $Ca^{2+}$-dependent lipid binding domain of P120GAP mediates protein-protein interactions with $Ca^{2+}$-dependent membrane-binding proteins. Evidence for a direct interaction between annexin VI and P120GAP. J Biol Chem 271:24333–24336.

Elowe S, Holland SJ, Kulkarni S, Pawson T (2001) Downregulation of the Ras-mitogen-activated protein kinase pathway by the EphB2 receptor tyrosine kinase is required for ephrin-induced neurite retraction. Mol Cell Biol 21:7429–7441.

Fivaz M, Bandara S, Inoue T, Meyer T (2008) Robust neuronal symmetry breaking by Ras-triggered local positive feedback. Curr Biol 18:44–50.

Grewal T, Enrich C (2006) Molecular mechanisms involved in Ras inactivation: the annexin A6–p120GAP complex. Bioessays 28:1211–1220.

Grewal T, Evans R, Rentero C, Tebar F, Cubells L, de Diego I, Kirchhoff MF, Hughes WE, Heeren J, Rye KA, Rinninger F, Daly RJ, Pol A, Enrich C (2005) Annexin A6 stimulates the membrane recruitment of p120GAP to modulate Ras and Raf-1 activity. Oncogene 24:5809–5820.

Hata K, Fujitani M, Yasuda Y, Doya H, Saito T, Yamagishi S, Mueller BK, Yamashita T (2006) RGMa inhibition promotes axonal growth and recovery after spinal cord injury. J Cell Biol 173:47–58.

Hecker TP, Ding Q, Rege TA, Hanks SK, Gladson CL (2004) Overexpression of FAK promotes Ras activity through the formation of a FAK/p120RasGAP complex in malignant astrocytoma cells. Oncogene 23:3962–3971.

Iwashita S, Song SY (2008) RasGAPs: a crucial regulator of extracellular stimuli for homeostasis of cellular functions. Mol Biosyst 4:213–222.

Kang JS, Yi MJ, Zhang W, Feinleib JL, Cole F, Krauss RS (2004) Netrins and neogenin promote myotube formation. J Cell Biol 167:493–504.

Kubo T, Endo M, Hata K, Taniguchi J, Kitajo K, Tomura S, Yamaguchi A, Mueller BK, Yamashita T (2008) Myosin IIA is required for neurite outgrowth inhibition produced by repulsive guidance molecule. J Neurochem 105:113–126.

Kunath T, Gish G, Lickert H, Jones N, Pawson T, Rossant J (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. Nat Biotechnol 21:559–561.

Li S, Nakamura S, Hattori S (1997) Activation of R-Ras GTPase by GTPase-activating proteins for Ras, Gap1(m), and p120GAP. J Biol Chem 272:19328–19332.

Lowy DR, Zhang K, DeClue JE, Willumsen BM (1991) Regulation of p21ras activity. Trends Genet 7:346–351.

Mañes S, Mira E, Gómez-Mouton C, Zhao ZJ, Lacalle RA, Martínez-A C (1999) Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. Mol Cell Biol 19:3125–3135.

Marshall MS, Hill WS, Ng AS, Vogel US, Schaber MD, Scolnick EM, Dixon RA, Sigal IS, Gibbs JB (1989) A C-terminal domain of GAP is sufficient to stimulate ras p21 GTPase activity. EMBO J 8:1105–1110.

Marte BM, Rodriguez-Viciana P, Wennström S, Warne PH, Downward J (1997) R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. Curr Biol 7:63–70.

McGlade J, Brunkhorst B, Anderson D, Mbamalu G, Settleman J, Dedhar S, Rozakis-Adcock M, Chen LB, Pawson T (1993) The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J 12:3073–3081.

Monnier PP, Sierra A, Macchi P, Deitinghoff L, Andersen JS, Mann M, Flad M, Hornberger MR, Stahl B, Bonhoeffer F, Mueller BK (2002) RGM is a repulsive guidance molecule for retinal axons. Nature 419:392–395.

Moran MF, Polakis P, McCormick F, Pawson T, Ellis C (1991) Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21ras GTPase-activating protein. Mol Cell Biol 11:1804–1812.

Ohshima Y, Kubo T, Koyama R, Ueno M, Nakagawa M, Yamashita T (2008) Regulation of axonal elongation and pathfinding from the entorhinal cortex to the dentate gyrus in the hippocampus by the chemokine stromal cell-derived factor 1 alpha. J Neurosci 28:8344–8353.

Oinuma I, Ishikawa Y, Katoh H, Negishi M (2004) The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. Science 305:862–865.

Oinuma I, Katoh H, Negishi M (2007) R-Ras controls axon specification upstream of glycogen synthase kinase-3beta through integrin-linked kinase. J Biol Chem 282:303–318.

Pawson T, Gish GD, Nash P (2001) SH2 domains, interaction modules and cellular wiring. Trends Cell Biol 11:504–511.

Rafiq K, Kolpakov MA, Abdelfettah M, Streblow DN, Hassid A, Dell'Italia LJ,

Sabri A (2006) Role of protein-tyrosine phosphatase SHP2 in focal adhesion kinase down-regulation during neutrophil cathepsin G-induced cardiomyocytes anoikis. J Biol Chem 281:19781–19792.

Rajagopalan S, Deitinghoff L, Davis D, Conrad S, Skutella T, Chedotal A, Mueller BK, Strittmatter SM (2004) Neogenin mediates the action of repulsive guidance molecule. Nat Cell Biol 6:756–762.

Ren XR, Ming GL, Xie Y, Hong Y, Sun DM, Zhao ZQ, Feng Z, Wang Q, Shim S, Chen ZF, Song HJ, Mei L, Xiong WC (2004) Focal adhesion kinase in netrin-1 signaling. Nat Neurosci 7:1204–1212.

Ren XR, Hong Y, Feng Z, Yang HM, Mei L, Xiong WC (2008) Tyrosine phosphorylation of netrin receptors in netrin-1 signaling. Neurosignals 16:235–245.

Rey I, Taylor-Harris P, van Erp H, Hall A (1994) R-ras interacts with ras-GAP, neurofibromin and c-raf but does not regulate cell growth or differentiation. Oncogene 9:685–692.

Serpente N, Birling MC, Price J (1996) The regulation of the expression, phosphorylation, and protein associations of pp125FAK during rat brain development. Mol Cell Neurosci 7:391–403.

Settleman J, Albright CF, Foster LC, Weinberg RA (1992) Association between GTPase activators for Rho and Ras families. Nature 359:153–154.

Trahey M, McCormick F (1987) A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science 238:542–545.

Yoshida J, Kubo T, Yamashita T (2008) Inhibition of branching and spine maturation by repulsive guidance molecule in cultured cortical neurons. Biochem Biophys Res Commun 372:725–729.

Yoshimura T, Arimura N, Kawano Y, Kawabata S, Wang S, Kaibuchi K (2006) Ras regulates neuronal polarity via the PI3-kinase/Akt/GSK-3beta/CRMP-2 pathway. Biochem Biophys Res Commun 340:62–68.

Yue Y, Lypowy J, Hedhli N, Abdellatif M (2004) Ras GTPase-activating protein binds to Akt and is required for its activation. J Biol Chem 279:12883–12889.

Zheng Y, Quilliam LA (2003) Activation of the Ras superfamily of small GTPases. EMBO Rep 4:463–468.