The receptor deleted in colorectal cancer (DCC) mediates the attraction of growing axons to netrin-1 during brain development. In response to netrin-1 stimulation, DCC becomes a signaling platform to recruit proteins that promote axon outgrowth and guidance. The Ras GTPase-activating protein (GAP) p120RasGAP inhibits Ras activity and mediates neurite retraction and growth cone collapse in response to repulsive guidance cues. Here we show an interaction between p120RasGAP and DCC that positively regulates netrin-1-mediated axon outgrowth and guidance in embryonic cortical neurons. In response to netrin-1, p120RasGAP is recruited to DCC in growth cones and forms a multiprotein complex with focal adhesion kinase and ERK. We found that Ras/ERK activities are elevated aberrantly in p120RasGAP-deficient neurons. Moreover, the expression of p120RasGAP Src homology 2 (SH2)-SH3-SH2 domains, which interact with the C-terminal tail of DCC, is sufficient to restore netrin-1-dependent axon outgrowth in p120RasGAP-deficient neurons. We provide a novel mechanism that exploits the scaffolding properties of the N terminus of p120RasGAP to tightly regulate netrin-1/DCC-dependent axon outgrowth and guidance.

Netrin-1 is one of the many extracellular cues that guide axons to their target during development of the CNS (1–3). It has the ability to attract or repel axons via several transmembrane receptors (4). The netrin-1 receptor deleted in colorectal cancer (DCC)4 is expressed in the spinal cord and forebrain of vertebrates and mediates the netrin-1-dependent attraction of axons to netrin-1 in cortical neurons, and the N terminus of DCC C-terminal tail that regulates the recruitment of several proteins, including focal adhesion kinase (FAK), Src, Fyn, ezrin, and Myosin X (13–21).

The neuronal growth cone is found at the distal periphery of an extending axon where the signals from guidance cues are integrated. The signaling cascades initiated by the receptors expressed on the surface of the growth cone produce a coordinated cellular response by regulating cytoskeletal rearrangements (22, 23). Rho GTPases are also regulated by ephrins, semaphorins, and neurotrophins during neuronal development, but their role in netrin-1/DCC signaling has not been explored (24, 29). ERK is activated downstream of netrin-1 and DCC and is required for netrin-1-dependent axon outgrowth and guidance (30–32), but it remains unclear whether Ras mediates ERK activation downstream of netrin-1 and DCC. Until now, the Ras GTPase-activating protein (GAP) p120RasGAP was considered only to be an inhibitor of axon outgrowth and guidance because of the activity of its C-terminal RasGAP domain (33–35). In addition to its C-terminal GAP domain, the N terminus of p120RasGAP, comprising one Src homology (SH) 3 and two SH2 domains, interacts with a wide variety of proteins to regulate cell survival, proliferation, and migration (36, 37). Here we identified p120RasGAP in an SH2 domain screen for proteins that interact with the phosphorylated Tyr-1418 residue of DCC. We show that p120RasGAP forms a signaling complex with DCC in netrin-1-stimulated cortical neurons. p120RasGAP is required to control the basal levels of Ras and ERK activities in neurons. Moreover, p120RasGAP is essential for the attractive response of axons to netrin-1 in cortical neurons, and the N terminus of p120RasGAP was considered only to be an inhibitor of axon outgrowth and guidance because of the activity of its C-terminal RasGAP domain (33–35). 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p120RasGAP is sufficient to mediate netrin-1-mediated axon outgrowth. Together, these findings add another layer to the intricacy of the multiple and essential signaling pathways regulated by netrin-1 and DCC during axon extension and attraction.

**Experimental Procedures**

**Antibodies and Reagents**—The following antibodies were purchased: anti-GST, anti-RasGAP B4F8, and anti-DCC A-20 (Santa Cruz Biotechnology); anti-phosphotyrosine 4G10, anti-tubulin, and anti-DCC A5 (Millipore); anti-DCC G92-13 and anti-FAK (BD Biosciences); anti-phospho-p44/42 MAPK (Erk1/2) (Thr-202/Tyr-204) and anti-p44/42 MAPK (Erk1/2) (Cell Signaling Technology); anti-FAK (Tyr(P)-861) and anti-FAK (Tyr(P)-397) (Life Technologies, Novex); anti-active Ras (New-East Biosciences); anti-ezrin (provided by M. Arpin (38)); anti-DCC (Tyr(P)-1418) (polyclonal antibodies raised in rabbit against the peptide KPTEDPASVpYEQQDLDL (DCC-Tyr(P)-1418)); anti-mouse Alexa Fluor 488, anti-mouse and anti-rabbit Cy3, anti-rabbit Alexa Fluor 555 (Life Technologies, Molecular Probes); and anti-goat IgG Cy3 (Sigma). The following reagents were used. Recombinant chick netrin-1 and netrin-1 VI-V were produced and purified as described previously (39, 40). Glutamate was provided by D. Bowie (McGill University), and NGF was from Cedarlane.

**Plasmids, Sequence Alignment, and siRNAs**—The plasmids pRK5, pRK5-DCC, pRK5-DCC-Y1418F, pRK5-DCC-Y1361F, pRK5-DCC 1–1327, and pRK5-DCC 1–1421 have been described previously (13, 25, 41). The pCDNA3-GAP and pCDNA3-GAP-N (human) constructs were provided by T. Pawson (42). The plasmids encoding GST and GST-human p120RasGAP (N-SH2, C-SH2, and SH2-SH3-SH2) were provided by L. Larose. pmaxGFP was purchased from Lonza. The following reagents were transfected overnight using PEI (PolySciences) according to the instructions of the manufacturer (16, 43). Cortical neurons from embryonic day (E) 18 rat embryos (Charles River Laboratories) were dissociated mechanically and plated on dishes treated with poly-d-lysine (0.1 mg/ml, Sigma-Aldrich) or glass coverslips treated with poly-l-lysine (0.1 mg/ml, Sigma-Aldrich). Neurons were cultured in 10% FBS DMEM for 4 h, and the medium was replaced with Neurobasal-A medium supplemented with 2% B27 and 1% l-glutamine (Invitrogen) (16). The Amaxa rat neuron nucleofector kit (Lonza) was used according to the instructions of the manufacturer to electroporate siRNAs and plasmids. Neurons were transfected with the following reagents: purified recombinant netrin-1 or netrin-1 VI-V (200 or 500 ng/ml), glutamate (50 μM), and NGF (100 ng/ml).

**GST Pulldowns**—Transfected HEK293 cells were lysed in 1% Triton X-100 lysis buffer as described previously (28). Protein lysates (1 mg) were precleared with 30 μl of glutathione-agarose beads (Sigma-Aldrich) for 2 h at 4 °C and incubated with 10 or 20 μg of fresh GST or GST-p120RasGAP proteins coupled to glutathione-agarose beads for 3 h at 4 °C. Beads were washed three times in ice-cold lysis buffer and boiled in SDS sample buffer.

**Immunoprecipitation**—Cortical neuron (2 days in vitro (DIV)) lysates were prepared as described previously (16). Protein lysates (1 mg) were incubated with 4 μg of anti-p120RasGAP with protein G-Sepharose beads (GE Healthcare) for 3 h at 4 °C.

**Immunoblotting and Quantitative Densitometry**—Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were stained with Ponceau S (Sigma-Aldrich), immunoblotted with the indicated antibodies, and visualized using enhanced chemiluminescence (Millipore). Optical density was measured using Quantity One software (Bio-Rad). The following optical density ratios were calculated: co-immunoprecipitated DCC and p120RasGAP over immunoprecipitated p120RasGAP, Tyr(P) (p120RasGAP) over p120RasGAP, and p120RasGAP over ezrin. The optical density -fold change was calculated by normalizing the ratio of each condition with the control ratio.

**Ras G-LISA Assay**—Cortical neuron (2 DIV) lysates were prepared and processed according to the instructions of the man-
Manufacturer (Cytoskeleton). Absorbance was read spectrophotometrically at 492 nm. The optical density -fold increase was calculated by normalizing each condition with the optical density of the control.

**Immunofluorescence**—Cortical neurons (2 DIV) were fixed for 30 min with 3.7% formaldehyde in PBS containing 20% sucrose at 37 °C, quenched for 5 min in 0.1M glycine at room temperature, permeabilized for 5 min in 0.25% Triton X-100, and blocked for 30 min with 3% BSA. Primary and secondary antibodies were incubated in 0.3% BSA. A 15-min fixation with 10% trichloroacetic acid in water was used for phospho-specific antibodies (44). Neurons were examined with an Olympus IX81 motorized inverted microscope (10). The DCC- Tyr(P)-1418 peptide was spotted onto nitrocellulose membranes with BSA as a control, and each membrane was incubated with either purified GST, GST-p120RasGAP N-SH2, or C-SH2 (100 ng/ml), followed by immunoblotting (IB) with anti-GST antibodies. One membrane was immunoblotted with phospho-specific anti-DCC-pY1418 (DCC-pY1418) antibodies.

**Axon Outgrowth and Dunn Chamber Assays**—The axon length of GFP-expressing cortical neurons (2 DIV) was measured with Metamorph software. Cortical neurons (2 DIV) were plated on coverslips used for Dunn chamber assembly as described previously (45). Gradients were generated with purified netrin-1 VI-V (200 ng/ml) or buffer containing PBS in the outer well. Cell images were acquired every 3–4 min for at least 90 min on a temperature-controlled stage. Neurites of at least 10 μm in length were tracked in GFP-expressing neurons. The final position of the growth cone was used to determine the angle turned over 90 min relative to the gradient position. Measurements are presented in rose histograms in bins of 10°, with the length of each segment representing the frequency of measurements in percent. Percentage distribution of turned angles, average turned angle, and average displacement are also represented.

**Statistical Analysis**—Statistical analysis was performed with GraphPad Prism 6. The data are presented as mean ± S.E.

**Results**

The N-terminal SH2 Domain of p120RasGAP Interacts with DCC via the Phosphorylated Tyr-1418 Residue in Vitro—To identify SH2-containing proteins that bind to the phosphorylated Tyr-1418 residue of DCC, we screened an SH2 domain array using as bait a 15-amino acid synthetic DCC peptide comprising Tyr(P)-1418 (DCC- Tyr(P)-1418) as absorbing PH domain relative to the absorbance obtained with a GST control. C2 and 5 μg of purified GST, GST-p120RasGAP N-SH2, and C-SH2 were resolved by SDS-PAGE, and the proteins were stained with Coomassie Blue. D, the DCC- Tyr(P)-1418 peptide was spotted onto nitrocellulose membranes with BSA as a control, and each membrane was incubated with either purified GST, GST-p120RasGAP N-SH2, or C-SH2 (100 ng/ml), followed by immunoblotting (IB) with anti-GST antibodies. One membrane was immunoblotted with phospho-specific anti-DCC-pY1418 (DCC-pY1418) antibodies.

**FIGURE 1.** The N-terminal SH2 domain of p120RasGAP interacts in vitro with DCC via phosphorylated Tyr-1418. A, p120RasGAP contains a proline-rich region (P) and N-SH2 and C-SH2, SH3, pleckstrin homology (PH), calcium-dependent phospholipid-binding (C2), and GAP domains. B, DCC- Tyr(P)-1418 was used as bait to screen a SH2 domain array by ELISA. Binding of p120RasGAP N-SH2 with 50 and 100 nM of DCC- Tyr(P)-1418 peptide is represented as the -fold increase in absorbance relative to the absorbance obtained with a GST control. C, 2 and 5 μg of purified GST, GST-p120RasGAP N-SH2, and C-SH2 were resolved by SDS-PAGE, and the proteins were stained with Coomassie Blue. D, the DCC- Tyr(P)-1418 peptide was spotted onto nitrocellulose membranes with BSA as a control, and each membrane was incubated with either purified GST, GST-p120RasGAP N-SH2, or C-SH2 (100 ng/ml), followed by immunoblotting (IB) with anti-GST antibodies. One membrane was immunoblotted with phospho-specific anti-DCC-pY1418 (DCC-pY1418) antibodies.
To validate the interaction between the N-SH2 domain of p120RasGAP and DCC- Tyr(P)-1418, purified GST fusion proteins of the individual N-SH2 and C-terminal SH2 (C-SH2) domains of p120RasGAP (Fig. 1, A and C) were tested for their ability to bind to immobilized DCC- Tyr(P)-1418 peptide in a dot blot assay (Fig. 1D). The N-SH2 domain was the sole domain capable of interacting with DCC- Tyr(P)-1418 and did not bind to a control spot of BSA (Fig. 1D). The GST control protein did not bind to the DCC peptide or to BSA (Fig. 1D), and tyrosine phosphorylation of DCC- Tyr(P)-1418 was confirmed with a phospho-specific antibody raised against DCC-Tyr(P)-1418 (Fig. 1D). Therefore, we conclude that the N-SH2 domain of p120RasGAP interacts directly with the synthetic DCC peptide via Tyr(P)-1418.

**Netrin-1 Promotes the Association of p120RasGAP with DCC in Embryonic Cortical Neurons**—We next examined the interaction of p120RasGAP and DCC in dissociated E18 rat cortical neurons, which are a good cellular model to investigate netrin-1/DCC-induced signaling pathways in the context of axon outgrowth and guidance (6, 16, 28, 43, 46, 47). DCC and p120RasGAP co-immunoprecipitated, and the interaction peaked after 10 min of stimulation with netrin-1 (Fig. 2, A and B). Then we evaluated the localization of DCC and p120RasGAP by immunostaining cortical neurons following stimulation with netrin-1 (Fig. 2, C and D). The correlation between DCC and p120RasGAP fluorescence intensities in whole cells (wc) and growth cones (gc) in three independent experiments (number of neurons = 65, 51, 54, and 40 from left to right; unpaired Student’s t test; ns, not significant; *, p = 0.028; n, netrin-1; –, unstimulated.)
netrin-1 stimulation. Visualization by confocal microscopy revealed that p120RasGAP and DCC were both present in the cell bodies, axons, and growth cones of cortical neurons (Fig. 2C). Netrin-1 increased the fluorescence intensity of p120RasGAP and DCC in the axons and growth cones (Fig. 2C). Quantification of the mean Pearson’s correlation coefficient (r) revealed that the correlation between p120RasGAP and DCC fluorescence intensity was increased significantly in growth cones after 10 min of netrin-1 stimulation (r = 0.42 versus 0.16, p = 0.028), whereas netrin-1 treatment resulted in no significant change of the co-association (r = 0.27 versus 0.24, p > 0.05) in whole cells (Fig. 2D). We identified p120RasGAP as a novel DCC binding partner in embryonic cortical neurons, and we demonstrated that netrin-1 promotes the recruitment of p120RasGAP to DCC preferentially in growth cones.

p120RasGAP Associates with a DCC Multiprotein Signaling Complex in Netrin-1-stimulated Cortical Neurons—We next examined whether p120RasGAP is tyrosine-phosphorylated in response to netrin-1. We observed that p120RasGAP was tyrosine-phosphorylated in cortical neurons after 10 min of stimulation with netrin-1, concomitant with its association with DCC (Fig. 3, A and B). Moreover, activated ERK (pERK) and FAK (FAK- Tyr(P)-397) co-immunoprecipitated with p120RasGAP and DCC in response to netrin-1 stimulation (Fig. 3A). Together, these results show that the assembly of a DCC-p120RasGAP protein complex with ERK and FAK is induced by netrin-1 in dissociated rat cortical neurons.

p120RasGAP Requires To Maintain Basal Ras and ERK Activities in Cortical Neurons—To further characterize the function of p120RasGAP in cortical neurons, endogenous p120RasGAP expression was down-regulated in E18 rat cortical neurons by electroporating synthetic siRNA targeting the 5’ end of p120RasGAP mRNA (RASA) (34), which led to a significant decrease of p120RasGAP expression compared with control siRNA (Fig. 4, A and B). Then we investigated the role of p120RasGAP, a negative regulator of Ras through its C-terminal GAP domain (37), in Ras activation in neurons by immunofluorescence using anti-Ras-GTP antibodies and confocal microscopy. To validate the anti-Ras-GTP antibodies, we first monitored the levels of Ras-GTP in cortical neurons stimulated with NGF, a well established activator of Ras in neurons (48, 49). In cortical neurons stimulated with NGF for 15 min, we observed a significant 2-fold (p < 0.05) increase in Ras-GTP fluorescence intensity (Fig. 4, C and D) that was comparable with the activation of Ras detected by G-LISA assay in NGF-treated cortical neuron lysates (Fig. 4E). In unstimulated p120RasGAP-deficient cortical neurons, Ras-GTP levels showed significant 1.56-fold (p < 0.005) and 2.03-fold (p < 0.001) increases in whole cells and growth cones, respectively (Fig. 4, F and G). Next we monitored the phosphorylation levels of ERK, a major signaling pathway activated downstream of Ras, by immunofluorescence and confocal microscopy in p120RasGAP-deficient neurons (Fig. 4F). We quantified the average fluorescence intensity of pERK at the plasma membrane of cortical neurons. In p120RasGAP-depleted neurons, pERK was increased significantly 1.35-fold (p < 0.05) and 1.53-fold (p < 0.05) in whole cells and growth cones, respectively (Fig. 4I). No change in total ERK fluorescence intensity was detected in p120RasGAP-deficient neurons (Fig. 4, J and K). Overall, p120RasGAP depletion in cortical neurons caused aberrant activation of Ras and ERK in whole cells and in neuronal growth cones. Therefore, these results demonstrate the involvement of p120RasGAP for the proper regulation of basal Ras and ERK activities in cortical neurons.

p120RasGAP Is Required for Netrin-1-dependent Attraction of Embryonic Cortical Neurons—To determine whether p120RasGAP regulates netrin-1-dependent chemotraction, we evaluated the effect of p120RasGAP depletion on cortical growth cone turning in response to a netrin-1 gradient using a Dunn chamber turning assay (45, 50, 51). The growth cones of cortical neurons electroporated with control siRNA turned randomly with no particular preference for any direction when exposed to a control PBS gradient (Fig. 5A) but were attracted to a netrin-1 gradient (Fig. 5B). The introduction of p120RasGAP siRNA inhibited the attractive response to netrin-1, and growth cones reverted to turning randomly (Fig. 5C).
**p120RasGAP Regulates Axon Outgrowth and Guidance**

**A.** CTL RASA siRNA

IB: RasGAP ezrin

**B.**

| siRNA      | p120RasGAP optical density (fold change) |
|------------|-----------------------------------------|
| CTL        | 1.0                                     |
| RASA       | 0.0                                     |

**C.**

IB: RasGAP ezrin

**D.**

| Time (min) | NGF Fluorescence intensity (fold increase) |
|------------|-------------------------------------------|
| 0          | 0.0                                       |
| 15         | 1.5                                       |

**E.**

| Time (min) | NGF Fluorescence intensity (fold increase) |
|------------|-------------------------------------------|
| 0          | 0.0                                       |
| 15         | 1.5                                       |

**F.**

control siRNA RASA siRNA

Ras-GTP

**G.**

| RASA siRNA | Ras-GTP Fluorescence intensity (AU) |
|------------|------------------------------------|
| w.c (CTL)  | 0                                  |
| w.c (RASA)| 2000                               |
| g.c (CTL)  | 0                                  |
| g.c (RASA)| 2000                               |

| RASA siRNA | Ras-GTP Fluorescence intensity (AU) |
|------------|------------------------------------|
| w.c (CTL)  | 0                                  |
| w.c (RASA)| 2000                               |
| g.c (CTL)  | 0                                  |
| g.c (RASA)| 2000                               |

**H.**

control siRNA RASA siRNA

pERK

**I.**

| RASA siRNA | pERK Fluorescence intensity (AU) |
|------------|----------------------------------|
| w.c (CTL)  | 0                                |
| w.c (RASA)| 750                              |
| g.c (CTL)  | 0                                |
| g.c (RASA)| 750                              |

| RASA siRNA | pERK Fluorescence intensity (AU) |
|------------|----------------------------------|
| w.c (CTL)  | 0                                |
| w.c (RASA)| 750                              |
| g.c (CTL)  | 0                                |
| g.c (RASA)| 750                              |

**J.**

control siRNA RASA siRNA

ERK

**K.**

| RASA siRNA | total ERK Fluorescence intensity (AU) |
|------------|--------------------------------------|
| w.c (CTL)  | 500                                  |
| w.c (RASA)| 500                                  |
| g.c (CTL)  | 500                                  |
| g.c (RASA)| 500                                  |

| RASA siRNA | total ERK Fluorescence intensity (AU) |
|------------|--------------------------------------|
| w.c (CTL)  | 500                                  |
| w.c (RASA)| 500                                  |
| g.c (CTL)  | 500                                  |
| g.c (RASA)| 500                                  |
p120RasGAP Regulates Axon Outgrowth and Guidance

FIGURE 5. p120RasGAP is required for netrin-1-dependent attraction. Control (CTL) or p120RasGAP (RASA) siRNA was electroporated with pGFP as a transfection reporter plasmid. A, total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against p120RasGAP and ezrin as a loading control. B, quantitative densitometry of A represented as the -fold change (mean ± S.E.) relative to control siRNA measured in 10 independent experiments (unpaired Student’s t test; ****, p < 0.0001). C, cortical neurons were stimulated with NGF (100 ng/ml) or left unstimulated (–) for 15 min. Neurons were immunostained with anti-Ras-GTP antibodies. Scale bar = 10 µm. D, Ras-GTP fluorescence intensity and the -fold increase (mean ± S.E.) relative to unstimulated control neurons was measured in at least three independent experiments (n > 50 neurons/condition; unpaired Student’s t test; *, p < 0.05). E, the levels of Ras-GTP in each cell lysate were evaluated by G-LISA assay by measuring the absorbance at 492 nm, which was measured in whole cells and growth cones (w.c) in three independent experiments (n = 31, 32, 31, and 32 neurons; two-way ANOVA, Fisher’s least significant difference post test; *, p < 0.05). F, neurons were immunostained with antibodies against pERK. Arrows indicate cell bodies, and arrowheads indicate growth cones. Scale bar = 50 µm. G, the total ERK fluorescence intensity (mean ± S.E.) of GFP-expressing neurons in F was measured in whole cells and growth cones (w.c and growth cones (g.c) in three independent experiments (n = 35, 36, 30, and 35 neurons; two-way ANOVA; Fisher’s least significant difference post test; *, p < 0.05). J, neurons were immunostained with antibodies against ERK. Arrows and arrowheads indicate cell bodies and growth cones of GFP-expressing neurons, respectively, and squares indicate untransfected neurons. Scale bar = 50 µm. K, the total ERK fluorescence intensity (mean ± S.E.) of GFP-expressing neurons in J was measured in whole cells and growth cones (n = 41, 38, 41, and 38 neurons) in three independent experiments (two-way ANOVA, Tukey’s post test; ns, not significant).

FIGURE 4. p120RasGAP is required to maintain basal Ras and ERK activities in cortical neurons. Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in neurons at 0 DIV with pGFP as a transfection reporter plasmid. A, total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against p120RasGAP and ezrin as a loading control. B, quantitative densitometry of A represented as the -fold change (mean ± S.E.) relative to control siRNA measured in 10 independent experiments (unpaired Student’s t test; ****, p < 0.0001). C, cortical neurons were stimulated with NGF (100 ng/ml) or left unstimulated (–) for 15 min. Neurons were immunostained with anti-Ras-GTP antibodies. Scale bar = 10 µm. D, Ras-GTP fluorescence intensity and the -fold increase (mean ± S.E.) relative to unstimulated control neurons was measured in at least three independent experiments (n > 50 neurons/condition; unpaired Student’s t test; *, p < 0.05). E, the levels of Ras-GTP in each cell lysate were evaluated by G-LISA assay by measuring the absorbance at 492 nm, which was measured as the -fold change (mean ± S.E.) relative to the unstimulated lysate (0 min) in at least three independent experiments (unpaired Student’s t test; *, p < 0.05). F, neurons were immunostained with anti-Ras-GTP antibodies. Arrows indicate cell bodies, and arrowheads indicate growth cones of GFP-expressing neurons, and squares represent untransfected neurons. Scale bar = 50 µm. G, the Ras-GTP fluorescence intensity (arbitrary units (AU), mean ± S.E.) of GFP-expressing neurons in F was measured in whole cells (w.c) and growth cones (g.c) in three independent experiments (n = 31, 32, 31, and 32 neurons; two-way ANOVA, Fisher’s least significant difference post test; *, p < 0.05). J, neurons were immunostained with antibodies against pERK. Arrows indicate cell bodies, and arrowheads indicate growth cones. Scale bar = 50 µm. L, pERK fluorescence intensity (mean ± S.E.) of GFP-expressing neurons in H was measured in whole cells and growth cones in three independent experiments (n = 35, 36, 30, and 35 neurons; two-way ANOVA; Fisher’s least significant difference post test; **, p < 0.0004; ***. p < 0.0001). H, neurons were immunostained with antibodies against pERK. Arrows indicate cell bodies, and arrowheads indicate growth cones. Scale bar = 50 µm. L, pERK fluorescence intensity (mean ± S.E.) of GFP-expressing neurons in H was measured in whole cells and growth cones in three independent experiments (n = 35, 36, 30, and 35 neurons; two-way ANOVA; Fisher’s least significant difference post test; **, p < 0.0004; ***. p < 0.0001).
64% of the control growth cones were attracted to the netrin-1 gradient, whereas the percentage that turned toward the netrin-1 gradient in p120RasGAP-deficient neurons (46%) was similar to the percentage that turned toward PBS in control neurons (43%) (Fig. 5E). In fact, the turned angle of growth cones in response to netrin-1 (9.2° ± 4.0°, mean angle turned ± S.E.) was reduced significantly when p120RasGAP was depleted (−4.3° ± 3.8°) (Fig. 5F). p120RasGAP down-regulation did not have an effect on displacement rates during the time the growth cones were imaged (Fig. 5G). These results demonstrate that p120RasGAP is required for netrin-1-dependent chemoattraction.

The N Terminus of p120RasGAP Is Sufficient to Mediate Netrin-1-dependent Cortical Axon Outgrowth—We then explored the role of p120RasGAP in netrin-1-induced axon outgrowth in cortical neurons (16, 28). p120RasGAP siRNA was electroporated together with GFP cDNA as a reporter to visualize the neurons. We measured the average axon length of GFP-expressing neurons after 24 h of incubation with 200 ng/ml netrin-1 (n) or 50 μM glutamate (g) or left unstimulated (−) for 24 h, and axon outgrowth was assessed in GFP-expressing neurons. A, control vector (v), full-length (FL), and NT p120RasGAP were co-expressed with control or p120RasGAP siRNA and pGFP in cortical neurons. Scale bar = 50 μm. B and C, axon outgrowth was measured and expressed as the average axon length (micrometer, mean ± S.E.) in at least three independent experiments (n in B = 381, 224, 272, 191, 228, 180, 146, 162, 222, 165, 179, and 182 neurons, and n in C = 299, 210, 381, and 190 neurons; two-way ANOVA, Fisher’s least significant difference post test; ns, not significant; **, p < 0.005; ***, p < 0.001).

FIGURE 6. The N terminus of p120RasGAP is sufficient to mediate netrin-1-dependent cortical axon outgrowth. Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in E18 rat cortical neurons at 0 DIV with pGFP as a transfection reporter plasmid. Neurons at 1 DIV were incubated with 200 ng/ml netrin-1 (n) or 50 μM glutamate (g) or left unstimulated (−) for 24 h, and axon outgrowth was assessed in GFP-expressing neurons. A, control vector (v), full-length (FL), and NT p120RasGAP were co-expressed with control or p120RasGAP siRNA and pGFP in cortical neurons. Scale bar = 50 μm. B and C, axon outgrowth was measured and expressed as the average axon length (micrometer, mean ± S.E.) in at least three independent experiments (n in B = 381, 224, 272, 191, 228, 180, 146, 162, 222, 165, 179, and 182 neurons, and n in C = 299, 210, 381, and 190 neurons; two-way ANOVA, Fisher’s least significant difference post test; ns, not significant; **, p < 0.005; ***, p < 0.001).
A RasGAP-deficient neuron was specific to netrin-1, suggesting that p120RasGAP is required for netrin-1 to positively regulate axon extension. To determine the domains within p120RasGAP responsible for the response to netrin-1, we expressed siRNA-resistant human full-length or the N terminus (NT) domain of p120RasGAP in control or p120RasGAP-depleted neurons (Fig. 6, A and B, and supplemental Fig. S1). Re-expression of p120RasGAP full-length restored basal axon length in p120RasGAP-deficient neurons, whereas it inhibited netrin-1-induced axon outgrowth in both control and p120RasGAP-depleted neurons (Fig. 6, A and B). In contrast, re-expression of the NT of p120RasGAP lacking the GAP domain was sufficient to rescue netrin-1-induced cortical axon extension \((p < 0.001)\) in p120RasGAP-depleted neurons (Fig. 6, A and B). Therefore, the N-terminal scaffolding SH2-SH3-SH2 domains mediate the positive regulation of netrin-1-dependent axon outgrowth by p120RasGAP, whereas overexpression of p120RasGAP blocks the response of axons to netrin-1. This is in agreement with our findings showing that p120RasGAP is necessary to maintain basal Ras-GTP and pERK levels (Fig. 4), which is a requisite for axon extension (34).

The N Terminus of p120RasGAP Interacts with the C Termi-

Because the N terminus of p120RasGAP mediates netrin-1-induced axon outgrowth, we next investigated the molecular interaction between the SH2-SH3-SH2 domains of p120RasGAP and DCC. GST-p120RasGAP domains were incubated with protein lysates from HEK293 cells overexpressing either wild-type DCC or DCC mutant proteins (Fig. 7, A and B). DCC proteins did not bind to the GST

\[\text{FIGURE 7. The N terminus of p120RasGAP interacts with the C terminus of DCC.} \]

A, the intracellular domain of rat DCC (amino acids 1120–1445) contains three conserved regions (P1, P2, and P3). The conserved tyrosine residue in the phospho-deficient mutants DCC-Y1418F and DCC-Y1361F was substituted for a phenylalanine residue. The truncation mutants DCC 1–1421 and 1–1327 are truncated before P3 or P2, respectively. B, DCC, DCC-Y1418F, DCC-Y1361F, DCC 1–1421, and DCC 1–1327 were expressed in HEK293 cells. C–F, proteins from cell lysates were pulled down using purified GST control protein (C), GST-p120RasGAP N-SH2 (D), C-SH2 (E), or SH2-SH3-SH2 (NT) (F). Associated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against DCC, FAK-Tyr(P)-397, and tubulin. GST fusion proteins were stained with Ponceau S. G, the N-SH2 and C-SH2 domains of p120RasGAP interact with phosphorylated Tyr-1418 and Tyr-1361, respectively. Alternatively, the N-SH2 domain can also interact with FAK-Tyr(P)-397 and DCC independently of DCC-Tyr-1418.
protein control (Fig. 7 C). p120RasGAP N-SH2 interacted with DCC, DCC-Y1361F, and DCC 1–1421 lacking the P3 region (Fig. 7, A and D). Surprisingly, DCC-Y1418F was still able to interact with p120RasGAP N-SH2 (Fig. 7D). Because FAK interacts with both the P3 region of DCC and p120RasGAP (17, 52, 53), we suspected that DCC-Y1418F might be pulled down indirectly by p120RasGAP N-SH2 via an interaction with FAK (Fig. 7G). The phosphorylation of Tyr-397 on FAK has been shown to mediate its interaction with the N-SH2 domain of p120RasGAP (53). Indeed, GST-N-SH2 pulled down FAK-Tyr(P)-397 along with DCC and DCC mutant proteins (Fig. 7D), suggesting that p120RasGAP N-SH2 is also able to interact with DCC and FAK independently of DCC-Tyr-1418. Furthermore, the expression of either DCC 1–1421 or DCC 1–1327 severely impaired the phosphorylation of FAK on Tyr-397 in total cell lysates, confirming that the P3 region of DCC is important for the phosphorylation of FAK on Tyr-397 in HEK293 cells (Fig. 7B), as reported previously (14, 17). Consequently, the expression of DCC 1–1421 or DCC 1–1327 impaired or completely abolished the interaction of FAK-Tyr(P)-397 with GST-N-SH2, respectively. It also suggested that phosphorylated Tyr-1418, which is able to directly interact in vitro with p120RasGAP N-SH2 (Fig. 1), might mediate the binding of DCC 1–1421 lacking the P3 region with GST-N-SH2 (Fig. 7D).

It has been shown previously that the 2 SH2 domains of p120RasGAP bind simultaneously to two adjacent tyrosine residues on binding partners such as p190RhoGAP and tyrosine kinase receptors (54–57). Therefore, we examined whether p120RasGAP C-SH2 could also interact with a phosphorylated tyrosine residue in the intracellular domain of DCC. We selected Tyr-1361 as a candidate binding site for C-SH2 because it is the closest to DCC-Tyr-1418 (Fig. 7A). GST-C-SH2 interacted with DCC, DCC-Y1418F, and DCC 1–1421 proteins, whereas its interaction with DCC 1–1327 and DCC-Y1361F was impaired (Fig. 7E), demonstrating that p120RasGAP C-SH2 interacted preferentially with DCC via the phosphorylated Tyr-1361 residue. FAK-Tyr(P)-397 was also pulled down indirectly with p120RasGAP C-SH2 via a DCC or DCC-Y1418F interaction, but its interaction with DCC 1–1327, DCC-Y1361F, and DCC 1–1421 was reduced severely reduced (Fig. 7E). Finally, the SH2-SH3-SH2 domains of p120RasGAP interacted with FAK-Tyr(P)-397 and DCC, DCC-Y1418F, DCC-Y1361F, and DCC 1–1421 but much less with DCC 1–1327 (Fig. 7F). Together, these data demonstrate cooperative binding of the N- and C-SH2 domains of p120RasGAP with the C terminus region of DCC and FAK (Fig. 7G).

Discussion

In this study, we report a novel interaction between p120RasGAP and the netrin-1 receptor DCC in embryonic cortical neurons. We demonstrate that p120RasGAP is essential for netrin-1 to induce axon outgrowth and attraction of embryonic cortical neurons and that the N terminus of p120RasGAP serves as a scaffolding protein to mediate netrin-1-induced cortical axon outgrowth (Fig. 8). To our knowledge, this study is the first one to support a positive role for p120RasGAP during axon outgrowth and guidance.

As a GAP, p120RasGAP stimulates the GTPase activity of Ras to inactivate the GTPase (58–60). Indeed, our findings demonstrate that p120RasGAP-deficient cortical neurons exhibit an aberrant increase in Ras/ERK activities, further supporting the hypothesis that p120RasGAP acts as a negative regulator of Ras activity in cortical neurons in the absence of...
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In conclusion, the depletion of p120RasGAP expression severely impairs netrin-1/DCC-mediated cellular functions in neurons. Netrin-1 cannot induce axon outgrowth or growth cone turning in p120RasGAP-deficient cortical neurons. In fact, netrin-1 decreases axon outgrowth in these neurons. It will be of great interest to address whether this reduction in axon outgrowth results from the loss of p190RhoGAP regulation in p120RasGAP-deficient cortical neurons. We propose that p120RasGAP acts as a molecular clutch and scaffold that primes and engages DCC in attractive netrin-1 signaling by recruiting key regulators (Fig. 8). Until now, the functions of the GAP domain have been studied separately from the functions of the N terminus. Studies that examine how the molecular interactions of p120RasGAP are integrated to initiate a unified cellular response will certainly be more successful at deciphering the intricacies of p120RasGAP functions in the future. The significance of these findings is not only limited to the mechanisms of axon growth and guidance because Ras/p120RasGAP activities and netrin-1/DCC signaling are implicated in the regulation of vascular development and the progression of cancer (36, 37, 82, 83). Notably, the autosomal dominant disorder capillary malformation-arteriovenous malformation is caused by heterozygous mutations in the p120RasGAP locus, RASA1 (84–87). The study of netrin-1 signal transduction outside of the nervous system will undoubtedly add to our understanding of the physiological functions of p120RasGAP.
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Author Contributions—J. A. B. and N. L. V. designed the study and wrote the article. J. A. B., N. L. V., and A. E. F. analyzed the data. J. A. B. performed the experiments shown in all figures. P. M. D. performed the experiments shown in Figs. 3 and 4, C–E, j, and K with J. A. B. R. A. and J. A. B. performed the experiments and analyzed the data shown in Fig. 5. T. E. K. provided purified netrin-1 VI-V for the turning assays.

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References

1. Bashaw, G. J., and Klein, R. (2010) Signaling from axon guidance receptors. Cold Spring Harb. Perspect. Biol. 2, a001941
2. Lykissas, M. G., Batistatou, A. K., Charalabopoulos, K. A., and Beris, A. E. (2007) The role of neurotrophins in axonal growth, guidance, and regeneration. Curr. Neurovasc. Res. 4, 143–151
3. Sánchez-Camacho, C., and Bovolenta, P. (2009) Emerging mechanisms in morphogen-mediated axon guidance. BioEssays 31, 1013–1025
4. Lai Wing Sun, K., Correia, J. P., and Kennedy, T. E. (2011) Netrins: versatile extracellular cues with diverse functions. Development 138, 2153–2169
5. Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G., and Tessier-Lavigne, M. (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell 87, 175–185
6. Shu, T., Valentino, K. M., Seaman, C., Cooper, H. M., and Richards, L. J. (2000) Expression of the netrin-1 receptor, deleted in colorectal cancer (DCC), is largely confined to projecting neurons in the developing forebrain. J. Comp. Neurol. 416, 201–212
7. Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Bedдинgton, R., Skarnes, W. C., and Tessier-Lavigne, M. (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87, 1001–1014
8. Fazeli, A., Dickinson, S. L., Hermiston, M. L., Tighe, R. V., Steen, R. G., Small, C. G., Stocekli, E. T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R. T., Gordon, J. I., Tessier-Lavigne, M., and Weinberg, R. A. (1997) Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 386, 796–804
9. Srour, M., Rivièere, J. B., Pham, J. M., Dubé, M. P., Girard, S., Morin, S., Han, M., Rao, Y., Hong, K., and Guan, K. L. (2004) Focal adhesion kinase in netrin-1 signaling. Nat. Neurosci. 7, 1204–1212
10. Liu, G., Beggs, H., Jürgensen, C., Park, H. T., Tang, H., Gorski, J., Jones, K. R., Reichardt, L. F., Wu, J., and Rao, Y. (2004) Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. Nat. Neurosci. 7, 1222–1232
11. Zhu, X. J., Wang, C. Z., Dai, P. G., Xie, Y., Song, N. N., Liu, Y., Du, Q. S., Mei, L., Ding, Y. Q., and Xiong, W. C. (2007) Myosin X regulates netrin receptors and functions in axonal path-finding. Neuron 54, 184–192
12. Wei, Z., Yan, J., Lu, Q., Pan, L., and Zhang, M. (2011) Cargo recognition mechanism of myosin X revealed by the structure of its tail MyTH4-FERM tandem in complex with the DCC P3 domain. Proc. Natl. Acad. Sci. U.S.A. 108, 3572–3577
13. Hirano, Y., Hatano, T., Takahashi, A., Torigiya, M., Inagaki, N., and Hoshkima, T. (2011) Structural basis of cargo recognition by the myosin-X MyTH4-FERM domain. EMBO J. 30, 2734–2747
14. Dent, E. W., Gupton, S. L., and Gertler, F. B. (2011) The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harb. Perspect. Biol. 3, a018180
15. Vitriol, A. E., and Zheng, J. Q. (2012) Growth cone travel in space and time: the cellular ensemble of cytoskeleton, adhesion, and membrane. Neuron 73, 1068–1081
16. Hall, A. and Lalli, G. (2010) Rho and Ras GTPases in axon growth, guidance, and branching. Cold Spring Harb. Perspect. Biol. 2, a018181
17. Li, X., Saint-Cyr-Proulx, E., Aktories, K., and Lamarche-Vane, N. (2002) Rac1 and Cdc42 but not RhoA or Rho kinase activities are required for neurite outgrowth induced by the Netrin-1 receptor DCC (deleted in colorectal cancer) in N1E-115 neuroblastoma cells. J. Biol. Chem. 277, 15207–15214
18. Shekarabi, M., and Kennedy, T. E. (2002) The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1. Mol. Cell Neurosci. 19, 1–17
19. Skaper, S. D. (2012) The neurotrophin family of neurotrophic factors: an overview. Methods Mol. Biol. 846, 1–12
20. Forcet, C., Stein, E., Pay, L., Corset, V., Llambi, F., Tessier-Lavigne, M., and Mehl, P. (2002) Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. Nature 417, 443–447
21. Campbell, D. S., and Holt, C. E. (2003) Apoptotic pathway and MAPKs overview.
22. Forcet, C., Stein, E., Pay, L., Corset, V., Llambi, F., Tessier-Lavigne, M., and Mehl, P. (2002) Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. Nature 417, 443–447
23. Campbell, D. S., and Holt, C. E. (2003) Apoptotic pathway and MAPKs overview.
24. Forcet, C., Stein, E., Pay, L., Corset, V., Llambi, F., Tessier-Lavigne, M., and Mehl, P. (2002) Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. Nature 417, 443–447
25. Li, X., Saint-Cyr-Proulx, E., Aktories, K., and Lamarche-Vane, N. (2002) Rac1 and Cdc42 but not RhoA or Rho kinase activities are required for neurite outgrowth induced by the Netrin-1 receptor DCC (deleted in colorectal cancer) in N1E-115 neuroblastoma cells. J. Biol. Chem. 277, 15207–15214
26. Shekarabi, M., and Kennedy, T. E. (2002) The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1. Mol. Cell Neurosci. 19, 1–17
27. Moore, S. W., Correia, J. P., Lai Wing Sun, K., Pool, M., Fournier, A. E., and Kennedy, T. E. (2008) Rho inhibition recruits DCC to the neuronal plasma membrane and enhances axon chemotraction to netrin 1. Development 135, 2855–2864
28. Briancon-Marjollet, A., Ghogha, A., Navabi, H., Triki, I., Auziol, C., Fromont, S., Piché, C., Enslen, H., Chehli, K., Cloutier, J. F., Castellani, V., Debant, A., and Lamarche-Vane, N. (2008) Trio mediates netrin-1-induced Rac1 activation in axon outgrowth and guidance. Mol. Cell Biol. 28, 2314–2323
29. Skaper, S. D. (2012) The neurotrophin family of neurotrophic factors: an overview. Methods Mol. Biol. 846, 1–12
30. Forcet, C., Stein, E., Pay, L., Corset, V., Llambi, F., Tessier-Lavigne, M., and Mehl, P. (2002) Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. Nature 417, 443–447
31. Campbell, D. S., and Holt, C. E. (2003) Apoptotic pathway and MAPKs differentially regulate chemotrophic responses of retinal growth cones. Neuron 37, 939–952
32. Ming, G. L., Wong, S. T., Henley, J., Yuan, X. B., Song, H. J., Spitzer, N. C., and Poo, M. M. (2002) Adaptation in the chemotactic guidance of nerve growth cones. Nature 417, 411–418
33. Elowe, S., Holland, S. J., Kulkarni, S., and 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
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Endo, M., and Yamashita, T. (2009) Inactivation of Ras by p120GAP via focal adhesion kinase dephosphorylation mediates RGMa-induced growth cone collapse. J. Neurosci. 29, 6649 –6662

Hancock, M. L., Prentner, N., Quan, J., and Flanagan, J. G. (2014) MicroRNA-132 is enriched in developing axons, locally regulates Rasa1 mRNA, and promotes axon extension. J. Neurosci. 34, 66 –78

Pamonsinlampatham, P., Hadi-Slimane, R., Lepeltetier, Y., Allain, B., Toccafondi, M., Garbay, C., and Raynaud, F. (2009) p120-Ras GAP forms a multi-interacting protein in downstream signaling. Biochimie 91, 320 –328

King, P. D., Lubeck, B. A., and Lapinski, P. E. (2013) Nonredundant functions for Ras GTP-activating proteins in tissue homeostasis. Sci. Signal. 6, re1

Algrain, M., Turunen, O., Vaheri, A., Louvard, D., and Arpin, M. (1993) Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. J. Cell Biol. 120, 129 –139

Serafini, T., Kennedy, T. E., Galko, M. I., Mizrayan, C., Jessell, T. M., and Tessler-Lavige, M. (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell 78, 409 –424

Kennedy, T. E., Wang, H., Marshall, W., and Tessler-Lavige, M. (2006) Axon guidance by diffusible chemoattractants: a gradient of netrin protein in the developing spinal cord. J. Neurosci. 26, 8866 –8874

Tcherkezian, J., Britts, P. A., Thomas, F., Roux, P. P., and Flanagan, J. G. (2010) Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation. Cell 141, 632 –644

Kulkarni, S. V., Gish, G. van der Geer, P., Hanks, S. K., and Pawson, T. (2000) Role of p120 Ras-GAP in directed cell movement. J. Cell Biol. 149, 457 –470

DeGeer, J., Boudreau, J., Schmidt, S., Bedford, F., Lamarche-Vane, N., and Debant, A. (2013) Tyrosine phosphorylation of the Rho guanine nucleotide-exchange factor Trio regulates netrin-1/DCC-mediated cortical axon outgrowth. Mol. Cell. Biol. 33, 739 –751

Hayashi, K., Tonemura, S., Matsu, T., and Tsukita, S. (1999) Immunofluorescence detection of ezrin/radixin/moesin (ERM) proteins with their carboxyl-terminal threonine phosphorylated in cultured cells and tissues. J. Cell Sci. 112, 119 –1158

Yam, P. T., Langlois, S. D., Morin, S., and Charron, F. (2009) Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. Neuron 62, 349 –362

Métin, C., Deléglise, D., Serafini, T., Kennedy, T. E., and Tessler-Lavige, M. (1997) A role for netrin-1 in the guidance of cortical efferents. Development 124, 5063 –5074

Richards, L. I., Koster, S. E., Tuttle, R., and O’Leary, D. D. (1997) Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. J. Neurosci. 17, 2445 –2458

Wu, C., Lai, C. F., and Mobley, W. C. (2001) Nerve growth factor activates from wildtype but not Nf1 lacking p120-Gap. Neuron 23, 66 –78

O’Leary, D. D., Tessler-Lavige, M., Henkemeyer, M., Yancopoulos, G. D., and Pawson, T. (1997) Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. EMBO J. 16, 3877 –3888

Hu, K. Q., and Settleman, J. (1997) Tandem SH2 binding sites mediate the RasGAP-RhoGAP interaction: a conformational mechanism for SH3 domain regulation. EMBO J. 16, 473 –483

Kazlauskas, A., Ellis, C., Pawson, T., and Cooper, J. A. (1990) Binding of GAP to activated PDGF receptors. Science 247, 1578 –1581

Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., and Schlessinger, J. (1990) The tyrosine phosphorylated carboxyterminal of the EGF receptor is a binding site for GAP and PLC-γ. EMBO J. 9, 4375 –4380

Gideon, P., John, J., Lautwein, A., Clark, R., Scheffler, J. E., and Wittinghofer, A. (1992) Mutational and kinetic analyses of the GTPase-activating protein (GAP)-p21 interaction: the C-terminal domain of GAP is not sufficient for full activity. Mol. Cell. Biol. 12, 2050 –2056

Marshall, M. S., Hill, W. S., Ng, A. S., Vogel, U. S., Schaber, M. S., Scolnick, E. M., Dixon, R. A., Sigal, I. S., and Gibbs, J. B. (1989) A C-terminal domain of GAP is sufficient to stimulate ras p21 GTPase activity. EMBO J. 8, 1105 –1110

Vogel, U. S., Dixon, R. A., Schaber, M. S., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs, J. B. (1988) Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature 335, 90 –93

Moran, M. F., Polakis, P., McCormick, F., Pawson, T., and 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

Shang, X., Moon, S. Y., and Zheng, Y. (2007) p200 RhoGAP promotes cell proliferation by mediating cross-talk between Ras and Rho signaling pathways. J. Biol. Chem. 282, 8801 –8811

Cacalano, N. A., Sanden, D., and Johnston, J. A. (2001) Tyrosine-phosphorylated SOCS-3 inhibits STAT activation but binds to p120 RasGAP and activates Ras. Nat. Cell Biol. 3, 460 –465

van der Geer, P., Henkemeyer, M., Jacks, T., and Pawson, T. (1997) Averant Ras regulation and reduced p190 tyrosine phosphorylation in cells lacking p120-Gap. Mol. Cell. Biol. 17, 1840 –1847

Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B., and Aaronson, S. A. (1989) PDGF induction of tyrosine phosphorylation of GTPase-activating protein. Nature 342, 711 –714

Ellis, C., Moran, M., McCormick, F., and Pawson, T. (1990) Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature 343, 377 –381

Kaplan, D. R., Morrison, D. K., Wong, G., McCormick, F., and Williams, L. T. (1990) PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell 61, 125 –133

Liu, X. Q., and Pawson, T. (1991) The epidermal growth factor receptor phosphates GTPase-activating protein (GAP) at Tyr-460, adjacent to the GAP SH2 domains. Mol. Cell. Biol. 11, 2511 –2516

Woodcock, S. A., and Hughes, D. A. (2004) p120 Ras GTPase-activating protein associates with fibroblast growth factor receptors in Drosophila. Biochem. J. 380, 767 –774

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

Oinuma, I., Ishikawa, Y., Katoh, H., and Negishi, M. (2004) The Sema-5A receptor Plexin-B1 is a GTPase activating protein for R-Ras. Science 305, 862 –865

Leblanc, V., Tocque, B., and Delumeau, I. (1998) Ras-GAP controls Rho-mediated cytoskeletal reorganization through its SH3 domain. Mol. Cell. Biol. 18, 5567 –5578

Nakata, H., and Watanabe, Y. (1996) Proliferation and differentiation of PC12 cells were affected by p21ras GTPase activating proteins and its deletion mutant proteins. Biochem. Biophys. Res. Commun. 218, 538 –543

Tocque, B., Delumeau, I., Parker, F., Maurier, F., Multon, M. C., and Schweighoffer, F. (1997) Ras-GTPase activating protein (GAP): a putative effector for Ras. Cell Signal. 9, 153 –158

Yatani, A., Okabe, K., Polakis, P., Halenbeck, R., McCormick, F., and
Brown, A. M. (1990) ras p21 and GAP inhibit coupling of muscarinic receptors to atrial K⁺ channels. Cell 61, 769–776
76. McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcock, M., Chen, L. B., and Pawson, T. (1993) The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J. 12, 3073–3081
77. Tomar, A., Lim, S. T., Lim, Y., and Schlaepfer, D. D. (2009) A FAK-p120RasGAP-p190RhoGAP complex regulates polarity in migrating cells. J. Cell Sci. 122, 1852–1862
78. Viswanatha, R., Wayt, J., Ohouo, P. Y., Smolka, M. B., and Bretscher, A. (2013) Interactome analysis reveals ezrin can adopt multiple conformational states. J. Biol. Chem. 288, 35437–35451
79. Ger, M., Zitkus, Z., and Valius, M. (2011) Adaptor protein Nck1 interacts with p120 Ras GTPase-activating protein and regulates its activity. Cell Signal. 23, 1651–1658
80. Li, X., Meriane, M., Triki, I., Shekarabi, M., Kennedy, T. E., Larose, L., and Lamarche-Vane, N. (2002) The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism. J. Biol. Chem. 277, 37788–37797
81. Park, S., Liu, X., Pawson, T., and Jove, R. (1992) Activated Src tyrosine kinase phosphorylates Tyr-457 of bovine GTpase-activating protein (GAP) in vitro and the corresponding residue of rat GAP in vivo. J. Biol. Chem. 267, 17194–17200
82. Larrieu-Lahargue, F., Thomas, K. R., and Li, D. Y. (2012) Netrin ligands and receptors: lessons from neurons to the endothelium. Trends Cardiovasc. Med. 22, 44–47
83. Mehlen, P., and Guenebeaud, C. (2010) Netrin-1 and its dependence receptors as original targets for cancer therapy. Curr. Opin. Oncol. 22, 46–54
84. Bayrak-Toydemir, P., and Stevenson, D. (2014) in GeneReviews (Internet) (Pagon, R. A., Adam, M. P., Ardinger, H. H., Bird T. D., Dolan C. R., Fong, C.-T., Smith, R. J. H., and Stephens, K. eds.) December 19, 2013 Ed., University of Washington, Seattle
85. Eerola, I., Boon, L. M., Mulliken, J. B., Burrows, P. E., Dompmartin, A., Watanabe, S., Vanwijck, R., and Vikkula, M. (2003) Capillary malformation-arteriovenous malformation, a new clinical and genetic disorder caused by RASA1 mutations. Am. J. Hum. Genet. 73, 1240–1249
86. Revencu, N., Boon, L. M., Mendola, A., Cordisco, M. R., Dubois, J., Clapuyt, P., Hammer, F., Amor, D. I., Irvine, A. D., Basela, E., Dompmartin, A., Syed, S., Martin-Santiago, A., Ades, L., Collins, F., Smith, J., Sanradura, S., Barrio, V. R., Burrows, P. E., Blei, F., Cozzolino, M., Brunetti-Pierri, N., Vicente, A., Abramowicz, M., Désir, J., Vilain, C., Chung, W. K., Wilson, A., Gardiner, C. A., Dwight, Y., Lord, D. J., Fishman, L., Cytrynbaum, C., Chamilin, S., Ghali, F., Gilaberte, Y., Joss, S., Boente Medel, C., Léauté-Labrèze, C., Dürre, M. A., Bayliss, S., Martorell, L., González-Enseñat, M. A., Mazereeuw-Hautier, J., O’Donnell, B., Bessis, D., Pyeritz, R. E., Salhi, A., Tan, O. T., Wargen, O., Mulliken, J. B., and Vikkula, M. (2013) RASA1 mutations and associated phenotypes in 68 families with capillary malformation-arteriovenous malformation. Hum. Mutat. 34, 1632–1641
87. Revencu, N., Boon, L. M., Mulliken, J. B., Enjolras, O., Cordisco, M. R., Burrows, P. E., Clapuyt, P., Hammer, F., Dubois, J., Basela, E., Brancati, F., Carder, R., Quintal, J. M., Dallapiccola, B., Fischer, G., Frieden, I. J., Garzon, M., Harper, J., Johnson-Patel, J., Labrèze, C., Martorell, L., Paltiel, H. J., Pohl, A., Prendiville, J., Quere, I., Siegel, D. H., Valente, E. M., Van Hagen, A., Van Hest, L., Vaux, K. K., Vicente, A., Weibel, L., Chitayat, D., and Vikkula, M. (2008) Parkes Weber syndrome, vein of Galen aneurysmal malformation, and other fast-flow vascular anomalies are caused by RASAI mutations. Hum. Mutat. 29, 959–965