

p190 Rho-GTPase activating protein associates with plexins and it is required for semaphorin signalling

Davide Barberis^{1,*}, Andrea Casazza^{1,*}, Raffaella Sordella², Simona Corso¹, Stefania Artigiani¹, Jeff Settleman², Paolo M. Comoglio¹ and Luca Tamagnone^{1,‡}

¹Institute for Cancer Research and Treatment (IRCC), University of Turin Medical School, Candiolo, Torino 10060, Italy

²Harvard Medical School, MGH Cancer Center, 149 13th Street, Charlestown, MA 02129, USA

*These authors contributed equally to this work

‡Author for correspondence (e-mail: luca.tamagnone@ircc.it)

Accepted 13 July 2005

Journal of Cell Science 118, 4689–4700 Published by The Company of Biologists 2005
doi:10.1242/jcs.02590

Summary

Plexins are transmembrane receptors for semaphorins, guiding cell migration and axon extension. Plexin activation leads to the disassembly of integrin-based focal adhesive structures and to actin cytoskeleton remodelling and inhibition of cell migration; however, the underlying molecular mechanisms are unclear. We consistently observe a transient decrease of cellular RhoA-GTP levels upon plexin activation in adherent cells. One of the main effectors of RhoA downregulation is p190, a ubiquitously expressed GTPase activating protein (GAP). We show that, in p190-deficient fibroblasts, the typical functional activities mediated by plexins (such as cell collapse and inhibition of integrin-based adhesion) are blocked or greatly impaired. Notably, the functional response can be rescued in these cells by re-expressing exogenous p190, but

not a mutant form specifically lacking RhoGAP activity. We furthermore demonstrate that semaphorin function is blocked in epithelial cells, primary endothelial cells and neuroblasts upon treatment with small interfering RNAs that knockdown p190 expression. Finally, we show that p190 transiently associates with plexins, and its RhoGAP activity is increased in response to semaphorin stimulation. We conclude that p190-RhoGAP is crucially involved in semaphorin signalling to the actin cytoskeleton, via interaction with plexins.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/118/20/4689/DC1>

Key words: GAP, p190, Plexin, Rho, Semaphorin

Introduction

Semaphorins constitute a large family of signalling molecules, conserved in evolution from invertebrates to humans, with over twenty members in mammals. Semaphorins were originally identified as axon repelling cues for the wiring of axonal connections in the developing nervous system (Kolodkin et al., 1993; Luo et al., 1993). Later it was shown that they can also mediate attractive cues, through mechanisms that are still unclear (Polleux et al., 2000; Moreno-Flores et al., 2003; Pasterkamp et al., 2003; Masuda et al., 2004). Beyond their established role in axon guidance, semaphorins are now known to provide regulatory signals for a variety of other functions, particularly cell migration (for a review, see Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2004). For instance, the major developmental defects reported thus far in semaphorin-deficient mice are probably due to the aberrant migration of neural crest cells and endothelial cells (Behar et al., 1996; Kawasaki et al., 1999; Brown et al., 2001; Feiner et al., 2001).

Plexins are high affinity semaphorin receptors that occur either alone or in complex with the neuropilins (Tamagnone et al., 1999; Takahashi et al., 1999). Moreover, plexins and neuropilins can form complexes on the cell surface with receptor tyrosine kinases (such as Met, Ron, Otk and KDR) and engage in multiple signalling pathways (Winberg et al., 2001; Giordano et al., 2002; Conrotto et al., 2004; Toyofuku

et al., 2004). Nine plexins are known in humans, divided into four subfamilies [plexins A to D (see Tamagnone et al., 1999)]. The cytoplasmic domain of plexins is highly conserved and it is responsible for the functional response elicited by semaphorins (Maestrini et al., 1996; Takahashi and Strittmatter, 2001; Barberis et al., 2004). Its sequence is unique and it shares no striking homology with other proteins or known functional domains. However, it includes motifs with moderate similarity to GTPase activating proteins (Rohm et al., 2000) and it was recently demonstrated that it can exert GAP activity on the small GTPase R-Ras (Oinuma et al., 2004). The latter belongs to the superfamily of monomeric G proteins, which are molecular switches shifting between an active GTP-bound state and an inactive GDP-bound state (Etienne-Manneville and Hall, 2002). While GTP/GDP exchange factors (GEFs) are responsible for the functional activation of GTPases, GTPase activating proteins (GAPs) serve as negative regulators and lead to their functional inactivation (Bernards and Settleman, 2004). Besides R-Ras, GTPases of the Rho family can associate with specific plexins (Hu et al., 2001; Driessens et al., 2001; Zanata et al., 2002), and recent reports suggest that these interactions have a role in regulating the recruitment of additional signal transducers to the receptor (Oinuma et al., 2003; Oinuma et al., 2004; Turner et al., 2004). Moreover, it has been shown that plexin B1 (PLXNB1) and plexin B2 (PLXNB2) can associate with exchange factors for

RhoA (Rho-GEFs) and induce their activation and Rho signalling pathways (Aurandt et al., 2002; Perrot et al., 2002; Swiercz et al., 2002). Intriguingly, these interactions are restricted to plexins of the B subfamily, and they are likely to be involved in specific functions mediated by this subset of semaphorin receptors.

In vitro, semaphorins have been mainly studied for their ability to induce axonal collapse or cellular collapse, associated with the retraction of a protruding leading edge [either the axonal growth cone or the cell lamellipodium, respectively (see Luo et al., 1993; Eickholt et al., 1999; Takahashi et al., 1999)]. The mechanisms leading to the cytoskeletal rearrangements that drive this process have not been fully elucidated. However, we have recently shown that plexin activation inhibits integrin function by uncoupling focal adhesive complexes from the actin cytoskeleton (Serini et al., 2003; Barberis et al., 2004). This leads to pseudopodia retraction; it inhibits the protrusion of the leading edge and negatively regulates directional cell migration. It is well known that the assembly and maintenance of focal adhesive structures depends on the interaction between extracellular matrix components and integrins, as well as on the regulatory role of molecules mediating the association between integrins and the actin cytoskeleton, including GTPases of the Rho family (for reviews, see Van Aelst and Symons, 2002; Ridley et al., 2003; Raftopoulou and Hall, 2004). In particular, the stability of integrin-based adhesive structures is regulated by RhoA signalling (Rottner et al., 1999; Schwartz and Shattil, 2000). When lamellipodia are formed at the leading edge, new integrin-based adhesion sites to the ECM are established. However, this process is very dynamic in order to allow for fast directional changes, and only a fraction of these adhesive structures matures further into focal contacts (Rottner et al., 1999). This latter step requires RhoA activation and the formation of effective connections between the adhesive complexes and contractile F-actin cables, which are required to generate the tensile forces mediating cell shape changes and cell body translocation. Two main RhoA effectors are involved in this process: the formin molecule diaphanous (Dia), which promotes actin polymerization, and Rho-dependent kinase (or ROCK), which promotes myosin-mediated actin contraction. However, RhoA inactivation is required to release the tension on adhesive structures, leading to their disassembly (Burgstaller and Gimona, 2004). Then, upon disruption of cell-substrate contacts, the actin cytoskeleton is rearranged and the migration machinery resets to respond to a new directional signal. RhoA is also known as a negative regulator of cellular protrusions, probably through the induction of a stiff cortical actin network. In fact, upon reduced RhoA activity, random cell protrusions are increased, without resulting in efficient directional cell movement (Worthyake and Burridge, 2003). This explains why inhibition of RhoA signalling promotes axonal outgrowth and counteracts growth cone repulsion (Bito et al., 2000), while it hampers directional cell migration.

The role of RhoA activation and signalling in semaphorin-dependent functions is very controversial. While it has been reported that this signalling pathway is required for Sema4D and B subfamily plexins (Hu et al., 2001; Aurandt et al., 2002; Swiercz et al., 2002; Oinuma et al., 2003), there is evidence that this is not the case for Sema3A and Sema3F (Jin and Strittmatter, 1997; Kuhn et al., 1999; Arimura et al., 2000;

Atwal et al., 2003; Turner et al., 2004). Moreover, we have recently shown that ROCK signalling is not required for plexin-mediated collapse response in adherent cells (Barberis et al., 2004) and in fact, upon its inhibition, the cells appeared to be more prone to semaphorin-induced collapse. This prompted us to further explore RhoA regulation in the pathway. Here we report that, unexpectedly, cellular Rho-GTP levels decrease upon plexin activation, consistent with the idea that the functional response to semaphorins may be driven by RhoA inactivation. One of the main effectors of localized Rho downregulation is p190, a ubiquitously expressed GTPase activating protein (GAP), under the control of plasma membrane receptors and tyrosine kinases (Brouns et al., 2000). p190 is also known as p190A, to distinguish it from p190B, an homologous protein with similar activities but different upstream regulatory mechanisms (Burbelo et al., 1998). Previous evidence indicated that p190A (simply referred to as p190 hereafter) has a crucial role in regulating cytoskeletal dynamics, by inhibiting focal adhesions and myosin-mediated contraction of F-actin cables (Vincent and Settleman, 1999; Ren et al., 2000; Burgstaller et al., 2004). In addition, it was reported that cells overexpressing p190 acquire a rounded phenotype with long, beaded extensions (Tatsis et al., 1998), which is similar to that observed upon semaphorin-induced cell collapse. We show here that p190-Rho-GAP is recruited to plexins and functionally activated upon ligand stimulation. We also show that, in the absence of p190 or in the presence of a mutant devoid of GAP activity, the functional response to semaphorins is abrogated. Therefore, p190-RhoGAP is identified here as a pivotal mediator of semaphorin signalling.

Materials and Methods

Cells

NIH-3T3 cells were grown in DMEM medium, supplemented with 10% heat-inactivated Colorado calf serum (Colorado Serum Company). PC12 rat neuroblasts were grown in presence of 10% horse serum plus 5% fetal bovine serum. Human umbilical vein endothelial cells (HUVEC) were isolated from cannulated human umbilical veins by treatment with collagenase (see Bussolino et al., 1992); they were seeded onto culture dishes coated with 1% gelatine, and maintained in complete endothelial growth medium containing 2% FBS and growth factors (EGMTM-2; Cambrex Bio Science Walkersville Inc.). Each culture was used only up to eight population doublings. All other cell lines were cultured in DMEM or RPMI media, supplemented with 10% fetal bovine serum (Gibco), in a humidified atmosphere of 5% CO₂ (or 10% for PC12). Fibroblasts derived from p190-deficient mice were previously described (Brouns et al., 2000) and wild-type counterparts were cultured similarly.

Antibodies

EC-6.9 monoclonal antibody recognizing the extracellular domain of PLXNB1 (and of a PLXNB1/A1 chimera) was used for immunofluorescence analysis (Barberis et al., 2004). In western blotting experiments, PLXNB1 was detected with IC-2 antiserum (Barberis et al., 2004). Mouse anti-VSV antibodies (clone P5D4) and anti-vinculin (clone VIN-1) were from Sigma. Anti-Myc (clone 9E10), anti-paxillin (clone 349), anti-Rho (26C4) and anti-actin antibodies were obtained from Santa Cruz. Anti-p190 monoclonal antibodies were from BD Transduction Laboratories. Anti-hCD31 (clone JC70A) was purchased from DAKO. Anti-Hsp90 antibodies (SPA-830) were from StressGene Biotech. HRP-conjugated anti-

mouse and anti-rabbit secondary antibodies were from Amersham (Amersham, UK).

Expression of plexins and semaphorins in mammalian cells

PLXNB1 and its mutated forms, as well as PLXNA1, all contained a VSV-G-tag at the N terminus. p190RhoGAP-GFP and p190RhoGAP^{R1283A}-GFP (expressed from pEGFP-C1 vector; Clontech) were kindly provided by K. Burridge. cDNA transfections in 293T cells were performed using the calcium phosphate method, according to a protocol described previously (De Palma and Naldini, 2002). All other cell types were efficiently transduced using lentiviral vectors carrying the specific cDNAs, subcloned into transfer plasmid pRRLsin.cPPT.hCMV.Wpre (kindly provided by L. Naldini). Lentiviral vector particles were generated in 293T cells by transient transfection, as previously described (De Palma et al., 2002; Barberis et al., 2004).

Pools of MDA-MB435 cells expressing myc-tagged forms of semaphorin 3A (Sema3A), semaphorin 3F (Sema3F), and the extracellular domain of semaphorin 4D (Sema4D) were obtained by lentiviral-mediated gene transfer. For all other functional experiments, a His-tagged version of the extracellular domain of Sema4D was affinity purified from the conditioned medium of transfected CHO producer cells, as previously described (Barberis et al., 2004).

Rho-GTP pull-down assays

Rho-GTP levels were measured with a modified version of the method described by Ren et al. (Ren et al., 1999). Briefly, cells were lysed for 5 minutes at 4°C in extraction buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 1 mM DTT), containing a cocktail of protease inhibitors (10 µg/ml each of aprotinin, leupeptin and pepstatin, plus 1 mM phenylmethylsulphonyl fluoride) and 200 µM sodium orthovanadate. Cleared lysates were incubated with agitation at 4°C for 60 minutes with 30 µg of GST-RBD-coated beads. GST-RBD, a GST fusion protein containing the RhoA-binding domain of rhotekin was previously purified from recombinant bacteria as described (Ren et al., 1999). Samples were then washed three times with 50 mM Tris, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 1% Triton X-100 and protease inhibitors (as above) and then submitted to immunoblot analysis with anti-RhoA monoclonal antibodies. Lysates used as input for pull-downs were equally analysed by immunoblotting, as loading controls. Band intensity was measured with Quantity One (BioRad).

Immunoprecipitation and western blotting

Cells were lysed with EB buffer (20 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) containing a cocktail of protease inhibitors (as above) and 1 mM sodium orthovanadate. Cleared extracts were incubated with the indicated antibodies in agitation for 2 hours at 4°C. The immunocomplexes were collected with protein A Sepharose (Pierce), extensively washed, and eluted in SDS-containing buffer. An aliquot of each lysate used as input for the immunoprecipitation (IP) was treated similarly. Samples were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Amersham). Western blot analysis using the indicated antibodies was performed according to standard protocols. Final detection was done with an ECL system (Amersham). Band intensity was quantified with Quantity One program (BioRad).

Immunofluorescence, immunocytochemistry and cell quantification

Cells were cultured overnight on glass coverslips, previously coated with 10 µg/µl fibronectin (Sigma). After the indicated treatments, cells were fixed with 4% paraformaldehyde (PAF) in PBS for 10

minutes on ice. Cells were then permeabilized for 5 minutes on ice with 0.2% Triton X-100 in PBS, washed and incubated with blocking buffer (PBS supplemented with 2% goat serum; Vector Laboratories Inc.) for 30 minutes at room temperature. Primary antibodies were incubated with cells for 30 minutes. F-actin was stained with fluorescein-labelled phalloidin (phalloidin-FITC; Sigma). Secondary anti-mouse-IgG antibodies were conjugated with Alexa Fluor 546 (red) or with Alexa Fluor 488 (green), both from Molecular Probes. Cells were examined and photographed using a Zeiss Axyoskope Confocal microscope and a Biorad Confocal Imaging System.

Immunocytochemical staining was performed using standard techniques. Briefly, after fixation with PAF and permeabilization, endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 minutes, followed by washing with PBS. After incubation with mouse monoclonal primary antibody, cells were washed and incubated with DAKOCytomation Envision[®] System labelled Polymer-HRP anti-mouse for 30 minutes at room temperature. Cell staining was eventually developed by incubation with 3,3'-diaminobenzidine/H₂O₂ (Dako).

Quantification involved counting at least 100 cells in at least two independent fields, per each experimental point. Experiments were performed in duplicate or triplicate and repeated at least three times with consistent results.

Cell-substrate adhesion and cell spreading assays

These assays were performed as described previously (Barberis et al., 2004). Briefly, 1-5 × 10⁴ cells harvested in 1 mM PBS/EDTA and resuspended in serum-free DMEM/0.2% BSA were dispensed into 96-microtiter wells (previously coated with 10 µg/ml fibronectin) and transferred to a cell culture incubator. After the indicated times, the wells were gently washed twice with PBS, and the adherent cells were fixed and subsequently stained with Crystal Violet and photographed with a Leica DMLB microscope coupled to a Leica DC300F camera. To quantify cell adhesion, the dye was eluted from adherent cells with 10% acetic acid, and the absorbance measured at 595 nm in a microplate reader.

Cell migration assay

Cell motility was assayed using Transwell[®] chamber inserts (Costar) with a porous polycarbonate membrane (8 µM pore size) as described previously (Barberis et al., 2004). The membrane was coated with 10 µg/ml fibronectin for 2 hours, and then blocked with 0.2% BSA. Cells were harvested from culture dishes by treatment with 1 mM EDTA (in PBS) and resuspended in 2% FBS-containing medium. Approximately 1 × 10⁵ cells were then allowed to migrate across the porous membrane towards the lower chamber containing the indicated factors (heregulinβ1, from Neomarkers Inc., and purified Sema4D) in medium containing 10% FBS. Cell migration was allowed to occur for 4-6 hours, in a cell culture incubator. Then, the cells adherent to the upper side of the filter were mechanically removed, while those that had migrated to the lower side were fixed with 11% glutaraldehyde and stained with crystal violet. Cells were photographed and the dye solubilized in 10% acetic acid to measure absorbance at 595 nm in a microplate reader.

RNA interference

The expression of p190RhoGAP was silenced by means of small interfering RNA (siRNA)-based technology, through lentiviral-mediated DNA transfer. To this end, the lentiviral transfer plasmid pCCLsin.PPT.hPGK.GFP.Wpre was used to express two independent siRNAs targeting p190RhoGAP transcripts (5'AAGGCAACCTG-GGAGAGTAAC-3' and 5'-AACCTGTCCATCTGCTTCTGG-3') or an unrelated sequence as negative control, under the transcriptional control of the H1 promoter derived from pSUPER plasmid

(Brummelkamp et al., 2002). Note that the vector carries an independent GFP expression cassette, to allow for the identification of transduced cells.

HUVEC chemorepulsion assay

HUVEC cell repulsion was measured as described previously (Bielenberg et al., 2004), with the following modifications. HUVEC cells were grown to confluence on gelatine-coated glass coverslips in 24-well cell culture dishes (Corning Inc.). The endothelial cell monolayer was washed twice and 6×10^3 MDA-MB435 tumour cells, transduced with different semaphorins, were seeded onto it in EGM-2 complete medium. Co-cultures were incubated for 48 hours, before analysis. Cells were then fixed with PAF and subjected to immunostaining. Semaphorin-secreting MDA-MB435 cells were detected with anti-Myc-tagged antibodies; endothelial cells were identified by GFP expression (associated with siRNA-transducing vectors) or alternatively by immunocytochemical staining with anti-CD31 antibodies. HUVEC-free areas were measured in at least three independent low magnification fields per each condition, by using ImageQuant software.

Neurite outgrowth in PC12 cells

For neural differentiation experiments, PC12 cells were grown in 48-well plates (precoated with poly-L-lysine) at a density of 5×10^3 cells/well in 10% FBS/DMEM. After 8 hours from seeding, purified Sema4D was added to the medium either in the presence or absence of NGF (7S, Roche Molecular Biochemicals). Neural differentiation was quantified after 48 hours by counting the cells bearing one or more neuritic processes of a length greater than its cell body diameter.

Measurement of p190-RhoGAP activation

Cells were washed twice with PBS and lysed in 50 mM Tris, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 10 μg/ml each of aprotinin and leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 200 μM ortovanadate. Lysates were clarified by

centrifugation for 10 minutes at 12,000 *g*, and the supernatants were incubated for 60 minutes with glutathione-Sepharose coated with a constitutively active form of Rho (Q63L) conjugated with GST, previously purified from bacteria (Noren et al., 2003). The samples were washed at least three times with lysis buffer, and bound proteins were eventually analysed by SDS-PAGE, followed by immunoblotting to detect p190-RhoGAP.

Results

While studying the molecular mechanisms mediating semaphorin functions, we had previously observed that an inhibitor of the RhoA effector kinase ROCK seemed to facilitate plexin-mediated cell collapse (Barberis et al., 2004). This was partly unexpected, considering other reports that had shown RhoA activation in response to PLXNB1 stimulation; however, it led us to investigate further whether a down-regulation of RhoA signalling could have a role in the plexin pathway. We noticed that, in non-serum-starved cells transfected with PLXNB1, Rho-GTP levels were basally increased, compared to controls. Moreover, as shown in Fig. 1A, Rho-GTP decreased remarkably upon treatment with the purified PLXNB1-ligand semaphorin 4D (Sema4D). RhoA inactivation was clearly detectable after 5 minutes of stimulation, consistent with the time when the collapse response started in these cells (Fig. 1A). Beyond 30 minutes of stimulation, Rho-GTP resumed basal levels or occasionally increased (not shown). Sema4D-induced RhoA inactivation was not observed in cells expressing the functionally-inactive mutated receptor PLXNB1-R1 (see Barberis et al., 2004) (data not shown). In addition, we studied RhoA regulation in the pathway downstream of the cytoplasmic domain of PLXNA1, by exploiting a previously described PLXNB1/A1 chimeric receptor (Barberis et al.,

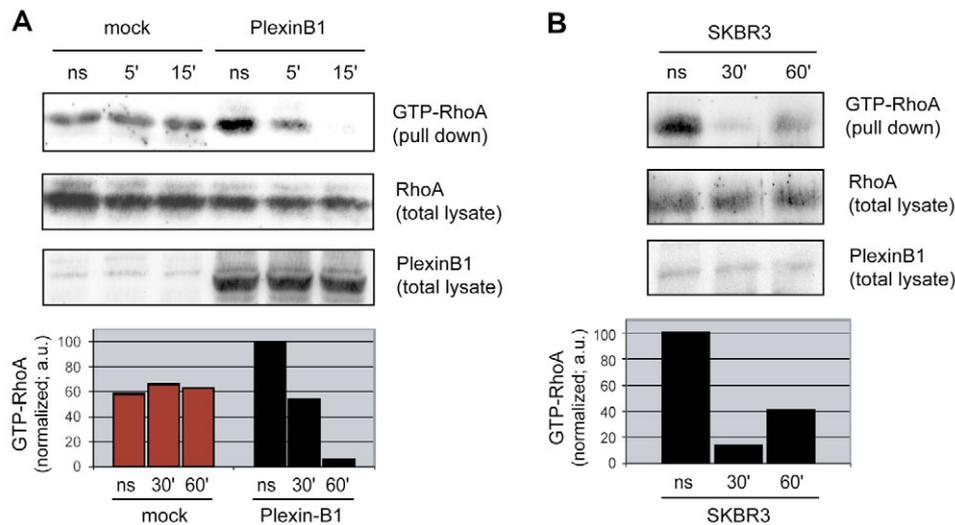


Fig. 1. Downregulation of RhoA-GTP in response to PLXNB1 activation. (A) NIH-3T3 fibroblasts either expressing PLXNB1 or mock transfected, were treated with the ligand Sema4D (5 nM) for the indicated times. RhoA-GTP levels were measured by pull-down with rhotekin-RBD-coated beads, followed by immunoblotting with anti-RhoA antibodies. Expression levels of RhoA and of the exogenous plexin in input lysates are shown below. The amount of pulled-down GTP-RhoA relative to total RhoA levels in each condition is reported at the bottom, based on band intensity quantification. (B) Measurement of RhoA-GTP levels by rhotekin-RBD pull-down, as above, in SKBR3 cells expressing endogenous PLXNB1 and treated with 10 nM Sema4D for the indicated times. Relative amounts of active RhoA were measured as above and are shown at the bottom.

2004), and observed similar results to those for PLXNB1 (supplementary material Fig. S1).

We furthermore demonstrated that purified Sema4D elicited RhoA inactivation in SKBR3 cells, which express endogenous levels of PLXNB1 (Fig. 1B). Rho-GTP downregulation occurred with slower kinetics in these cells, consistent with our previous observation that the functional response peaks later in SKBR3 cells compared with fibroblasts overexpressing the receptor (Barberis et al., 2004).

Altogether, these data indicate that plexin signalling can elicit Rho inactivation. However, is this relevant for the functional response mediated by semaphorins? Indirect evidence of this came from the observation that NIH-3T3 fibroblasts expressing constitutively active RhoQ63L, or treated with lysophosphatidic acid (LPA) to increase Rho-GTP levels, do not undergo cell collapse in response to PLXNB1 stimulation (Barberis et al., 2004).

It is known that the downregulation of Rho-GTP levels is largely mediated by p190-RhoGAP [p190A (Vincent and Settleman, 1999)], an activator of the intrinsic GTPase activity of Rho, acting under control of receptor-mediated signals (referred to as p190 hereafter). Therefore, to directly assess whether p190 has a role in plexin signalling, we studied the previously described cell collapsing response (Barberis et al.,

2004) in cells deprived of p190 by expression of targeted small interfering RNA (siRNA) sequences. NIH-3T3 cells expressing PLXNB1 and undergoing collapse in response to Sema4D were engineered by means of lentiviral vectors to express siRNAs designed to selectively inactivate p190 transcripts or targeted to an unrelated sequence as control. Cells expressing p190-targeted siRNAs were found to have reduced levels of p190-RhoGAP (Fig. 2A) and were refractory to Sema4D-induced cell collapse (Fig. 2B), whereas cells expressing control siRNAs displayed an efficient functional response, indistinguishable from that of parental cells. Intriguingly, we found that p190 is also required downstream of the cytoplasmic domain of PLXNA1, since the functional response mediated by the chimeric receptor PLXNB1/A1 (Barberis et al., 2004) is impaired in p190-deficient fibroblasts (Fig. 2B, right panel). Therefore, our results indicate that p190-RhoGAP is required in the signalling pathway downstream of plexins of both the A and B subfamilies. Analogous results were obtained by testing semaphorin-induced collapse of COS cells similarly deprived of p190 by expression of targeted siRNA (data not shown).

We have previously shown that the inhibition of integrin-based adhesion is a pivotal step in semaphorin signalling. We therefore tested whether the regulatory function of plexins on integrin-mediated adhesion is dependent on p190. As shown in

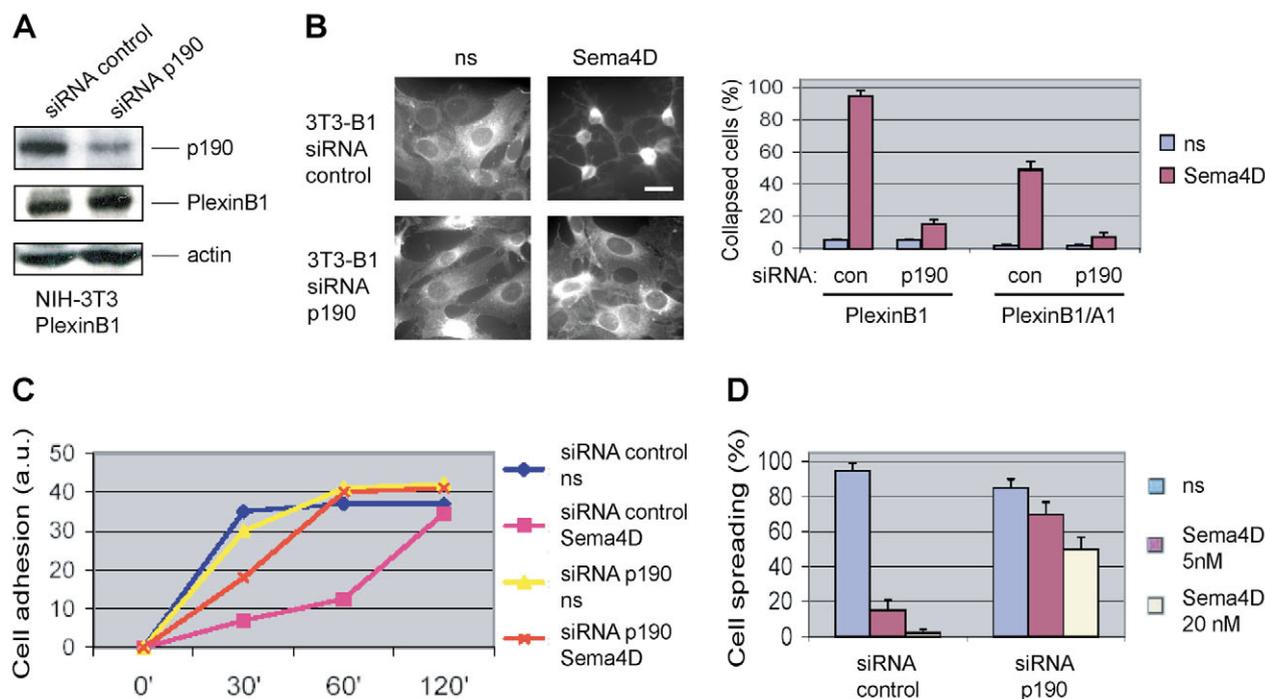


Fig. 2. p190 Rho-GAP is required to mediate plexin signalling in fibroblasts. (A) Immunoblots of total cell lysates of NIH-3T3 fibroblasts expressing PLXNB1 and engineered with siRNAs targeted to p190 transcript or to an unrelated sequence. The filter was probed with specific antibodies directed against p190, PLXNB1 and actin. The endogenous levels of p190 are greatly reduced in NIH-3T3 fibroblasts by expression of targeted siRNA, while plexin expression is unaffected. (B) NIH-3T3 fibroblasts expressing PLXNB1 (or PLXNB1/A1) and engineered with p190-targeted siRNAs (or controls) were grown on glass coverslips and subjected to treatment with 5 mM Sema4D for 15 minutes. Cells were then analysed by immunofluorescence with EC-6.9 antibodies directed against the extracellular domain of PLXNB1. Scale bar: 20 μm. The average fraction of collapsed cells was determined in each condition (Barberis et al., 2004) and it is shown on the right. Plexin-dependent collapse response is greatly impaired in the absence of p190. (C) The attachment of the same cells as above to fibronectin-coated wells was assayed, in the presence or absence of 5 nM Sema4D. After 30 and 120 minutes, adherent cells were fixed and stained with crystal violet. Cell adhesion was eventually quantified by eluting the dye and measuring the absorbance at 595 nm. (D) In a similar experiment as described in C, we scored the average fraction of fibroblasts spread on fibronectin, after 1 hour incubation with or without the indicated amounts of Sema4D (see Barberis et al., 2004).

Fig. 2C, while Semaphorin 4D significantly inhibited the adhesion to fibronectin of control cells, it was much less effective on cells expressing reduced levels of p190. More importantly, control cells could not extend lamellipodia and did not spread on the surface in the presence of the semaphorin, while the spreading of p190-deficient fibroblasts was significantly affected only at high ligand concentrations (Fig. 2D).

Notably, we could not obtain complete abrogation of p190 expression in NIH-3T3 fibroblasts; this may explain why the

functional response, although severely impaired, was not fully blocked in these cells. We therefore turned to immortalized fibroblasts derived from *p190*^{-/-} mice (Brouns et al., 2000) for further analysis. Notably, it was shown that these cells behave similarly to wild-type counterparts in assays of cell proliferation, cell adhesion to the ECM or cell migration (Brouns et al., 2000); moreover, Rho-GTP levels are not basally increased in these cells, presumably because of adaptation (Wennerberg et al., 2003). Fig. 3A shows that these

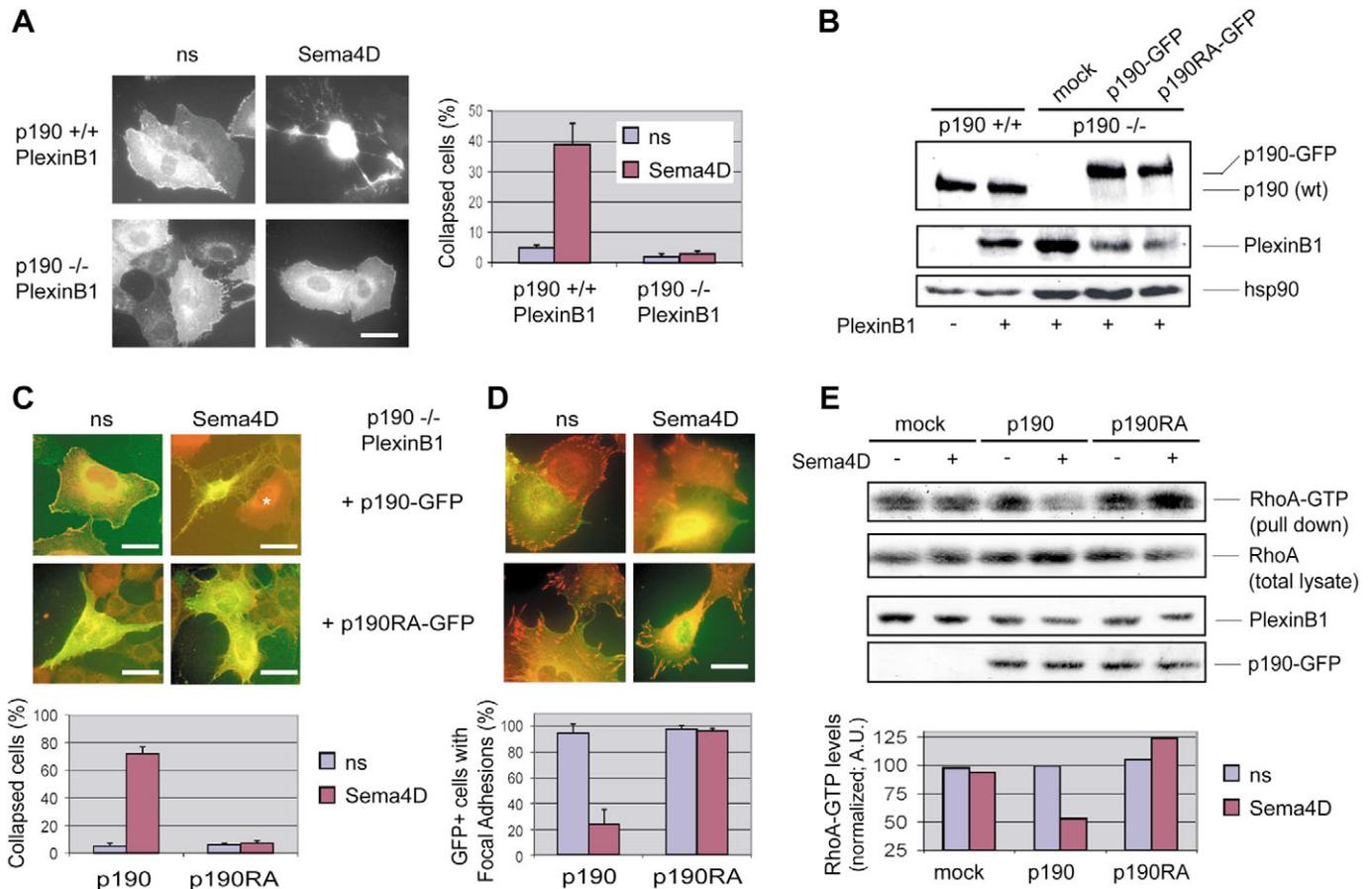


Fig. 3. Re-expression of p190 Rho-GAP in gene-deficient cells restores the functional response to plexin activation. (A) Fibroblasts derived from wild-type or from *p190*^{-/-} mouse embryos (Brouns et al., 2000) were engineered to express PLXNB1 (see B for protein expression analysis) and then treated with 10 nM Semaphorin 4D for 1 hour to test their collapse response (in analogy to Fig. 2B). Scale bar: 20 μ m. (B) PLXNB1-expressing *p190*^{-/-} knockout fibroblasts were engineered to express exogenous p190 or its inactive mutant p190RA (both conjugated with GFP). Protein expression levels were analysed by immunoblotting with specific antibodies. Hsp90 expression was determined as loading reference. (C) Merged fluorescence images of the cells described in B after treatment with 10 nM Semaphorin 4D for 30 minutes to induce cellular collapse: the red channel reveals PLXNB1, detected with specific antibodies (EC-6.9), while the green channel shows p190-GFP. Co-expression of PLXNB1 with p190, but not with its inactive mutant, is required and sufficient to rescue the functional response to Semaphorin 4D in these cells. The asterisk marks a cell expressing PLXNB1 but not p190, which is insensitive to the ligand. Scale bar: 20 μ m. The fraction of GFP-positive collapsed cells in each condition was counted and it is shown at the bottom. (D) *p190*-deficient fibroblasts expressing PLXNB1, and further transfected with p190-GFP or its inactive mutant p190RA-GFP (the same shown in A), were grown on fibronectin-coated coverslips and treated with 5 nM Semaphorin 4D for 5 minutes. Cells were then fixed and focal adhesions revealed in the red channel with anti-paxillin antibodies. After this short stimulation, very few cells underwent collapse [consistent with that described in 3T3 fibroblasts (see Barberis et al., 2004)], however, focal adhesions were disassembled in most of the cells expressing functionally competent p190-RhoGAP (identified by GFP expression). In contrast, semaphorin stimulation had no effect in cells lacking p190 or expressing the mutant devoid of RhoGAP activity (p190RA). Scale bar: 20 μ m. The fraction of GFP-positive cells containing focal adhesion was counted and it is shown at the bottom. (E) Affinity purification of RhoA-GTP (by rhokinin-RBD pull-down) from equal amounts of protein lysates of *p190*^{-/-} fibroblasts expressing PLXNB1 and the indicated exogenous p190 proteins, after treatment with 10 nM Semaphorin 4D for 15 minutes. Plexin-mediated Rho inactivation is abrogated in the absence of p190, but it is rescued by expression of exogenous p190-GFP in the gene-deficient cells. The mutated form of p190 (p190RA), lacking GAP activity, is unable to rescue the function. The effect was quantified by measuring the relative amounts of active RhoA in each condition (band intensity of RhoA-GTP versus total RhoA) and it is shown at the bottom.

cells failed to undergo collapse upon Sema4D stimulation, in contrast to embryo fibroblasts derived from wild-type animals and similarly engineered to express PLXNB1. Moreover, plexin activation in p190-deficient fibroblasts, unlike that seen in wild-type cells (Barberis et al., 2004), did not induce the disassembly of integrin-based focal adhesions, nor did it inhibit cell-spreading on fibronectin (supplementary material Fig. S2 and data not shown). A brief treatment with 10 μ M Y27632 (a selective ROCK inhibitor), however, could induce major actin remodelling in these cells, as expected, indicating that the downstream mechanisms regulating cytoskeletal dynamics are preserved (data not shown).

To provide evidence that the lack of p190 is specifically responsible for abrogating plexin function in *p190*^{-/-} cells, we determined whether re-establishing its expression could rescue the functional responses. We therefore engineered gene-deficient fibroblasts to express GFP-fusion proteins of either wild-type p190 or its inactive form p190RA (Tatsis et al., 1998), carrying the R₁₂₈₃A point mutation in the GAP domain (Fig. 3B). The collapse response triggered by plexins was recovered by the exogenous expression of p190-RhoGAP; by contrast, the mutant form p190RA was ineffective, indicating that the Rho-GAP activity of p190 is required in the plexin signalling pathway (Fig. 3C). By immunostaining with anti-paxillin antibodies (Fig. 3D), we furthermore demonstrated that the expression of wild-type p190 (but not p190RA) in gene-deficient fibroblasts is sufficient to mediate the disassembly of integrin-based focal adhesive structures, which precedes the collapse response induced by plexin signalling (see Barberis et al., 2004). This strongly suggests that the RhoGAP activity of p190 mediates the inhibition of integrin function induced by semaphorins. Consistent with this conclusion, we also found that cellular Rho-GTP levels were unchanged upon plexin activation in p190-deficient cells, while this mechanism was clearly rescued upon re-expression of wild-type, but not mutated, p190 (Fig. 3E).

We have previously shown that a functional response to purified Sema4D is also observed in epithelial cells expressing endogenous levels of its receptor PLXNB1 (Barberis et al., 2004). Therefore, we assayed the functional requirement for p190-RhoGAP in SKBR3 mammary carcinoma cells. To this end, the downregulation of p190 protein was achieved by lentiviral-mediated expression of small interfering RNAs (siRNA) selectively targeted to the corresponding transcript (Fig. 4A). Fig. 4B shows that p190-depleted SKBR3 cells cannot undergo collapse in response to Sema4D, unlike parental cells (Barberis et al., 2004). In addition, the previously described disassembly of adhesive structures and inhibition of chemotaxis elicited by Sema4D in these cells (Barberis et al., 2004) was severely impaired upon siRNA-mediated knockdown of p190 (Fig. 4C). The expression of control siRNA, directed against an unrelated sequence, did not have any effect.

Semaphorins are known to regulate endothelial cell migration and angiogenesis. In particular, it has been shown that Sema3A and Sema3F mediate repulsion of primary endothelial cells (HUVEC) and inhibit angiogenesis (Serini et al., 2003;

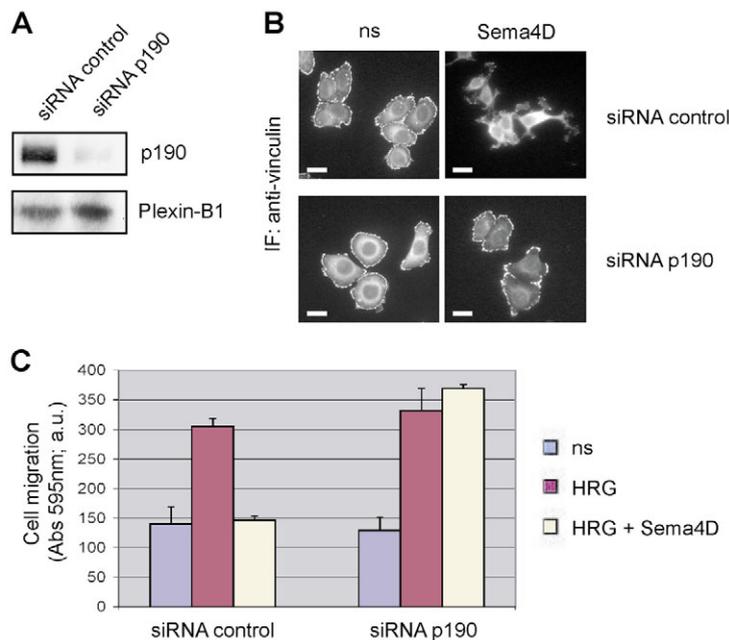
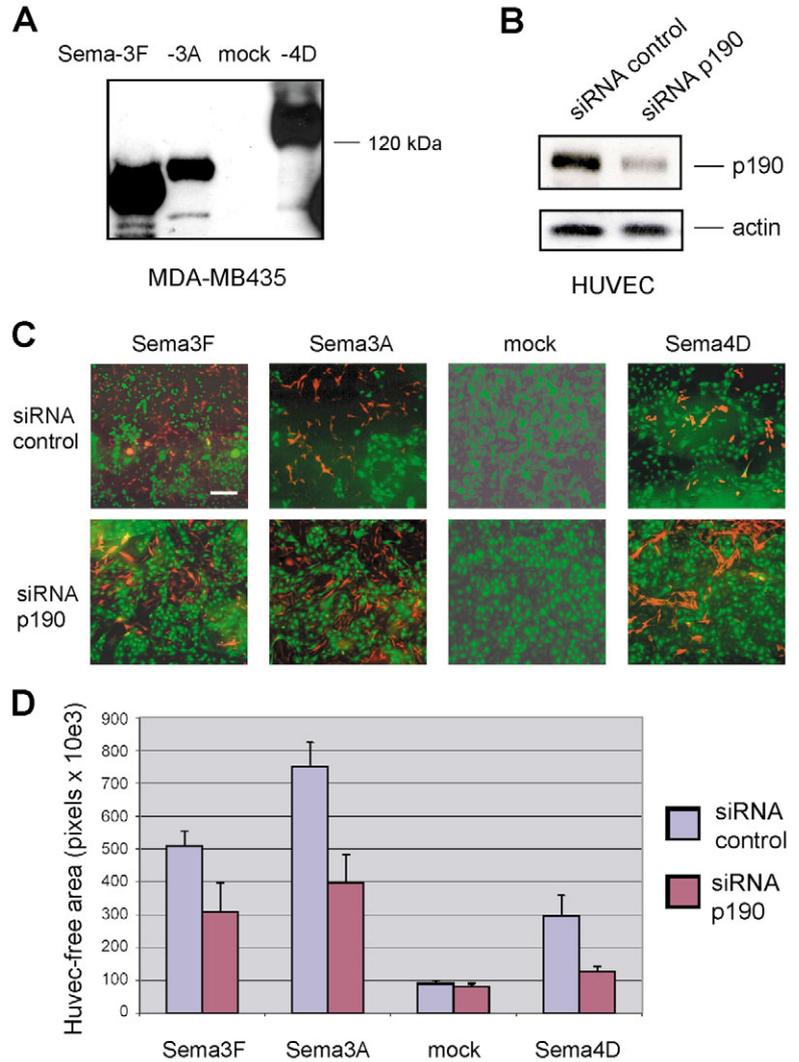


Fig. 4. p190 is required for multiple functional responses mediated by Sema4D in epithelial cells expressing endogenous PLXNB1. (A) Selective downregulation of p190 protein in SKBR3 cells by siRNA-mediated technology, demonstrated by immunoblotting with specific antibodies. The expression of endogenous PLXNB1 is unchanged. (B) Focal complex disassembly (revealed by vinculin immunostaining) and cellular collapse mediated by 1 hour treatment with 10 nM Sema4D was abrogated in p190-depleted SKBR3 cells. The functional response was unaffected by siRNAs targeting an unrelated sequence. The micrographs show results representative of at least three independent experiments. Scale bar: 40 μ m. (C) Chemotactic migration of SKBR3 treated with siRNAs (as above) was assessed in Transwell[®] inserts, in the presence of 0.2 nM heregulin- β 1 (HRG), with or without 5 nM Sema4D. After 6 hours, the cells that had migrated across the porous membrane were stained with Crystal Violet. Cell migration was quantified by eluting the dye and measuring absorbance at 595 nm. Results shown are the average of two independent experiments, performed in duplicate. The expected inhibition of directional cell migration is lost in p190-deficient cells.

Bielenberg et al., 2004), via the function of neuropilins and plexins of subfamily A and their regulatory activity on integrin-mediated adhesion. To test whether these semaphorin-mediated functions also depend on p190, we exploited a modified version of the chemorepulsion assay in co-culture, described by Bielenberg and coworkers (Bielenberg et al., 2004). We seeded MDA-MB435 tumour cells, which had been engineered to secrete different semaphorins (see Fig. 5A), onto confluent monolayers of HUVEC cells that were either wild-type or defective for p190-RhoGAP (due to expression of p190-targeted siRNAs, see Fig. 5B). Tumour cells expressing Sema3F induced repulsion of wild-type endothelial cells [consistent with that reported by Bielenberg et al. (Bielenberg et al., 2004)], as well as Sema3A-expressing cell, which were extremely effective in our experiments (Fig. 5C, see also supplementary material Fig. S3). Sema4D-expressing cells induced a weaker repulsion of HUVECs, whereas mock-transfected cells intercalated with endothelial cells without inducing any substantial repulsion. Importantly, the down-expression of p190 significantly reduced the retraction of

Fig. 5. p190 is required for semaphorin-induced repulsion of primary endothelial cells. (A) MDA-MB435 mammary carcinoma cells were engineered to express myc-tagged Sema3F, Sema3A, Sema4D or mock transfected. The secretion of the semaphorins in the conditioned medium as demonstrated by immunoblotting with anti-myc antibodies. (B) HUVEC cells were engineered to express siRNAs targeted to p190 transcript or to an unrelated sequence. The expression levels of p190 in cell lysates were then analysed by immunoblotting with specific antibodies. (C) siRNA-expressing HUVECs analysed as in B were grown to confluence on glass coverslips. 6×10^3 MDA-MB435 tumour cells engineered to express the indicated semaphorins (as shown in A) were then seeded onto the monolayer of endothelial cells. After 48 hours of co-culture, the cells were fixed and analysed by immunofluorescence with anti-myc antibodies to reveal semaphorin-expressing cells. HUVECs were identified by GFP expression (associated with siRNA-expression vectors). Scale bar: 200 μ m. In other experiments, the endothelial cell monolayers were revealed by immunocytochemistry with anti-CD31 antibodies (supplementary material Fig. S3). (D) HUVEC-free areas were identified by GFP expression or CD31 positivity (as described above) and measured by ImageQuant software. At least three independent low magnification fields were analysed, by two separate investigators, for each experimental point. The table shows average values.



endothelial cells in the presence of any of the semaphorins. We quantified this effect by measuring the HUVEC-free surface (Fig. 5D; see Materials and Methods for quantification method). Our results suggest that the directional retraction of endothelial cells from areas containing semaphorins depends on the activity of p190-RhoGAP, potentially mediating a localized release of stable cell-substrate adhesions.

Beyond its role in cell migration, the RhoGAP activity of p190 has been implicated in neurite outgrowth in neuroblasts in vitro (Brouns et al., 2001; Troller et al., 2004). Intriguingly, among the functions mediated by semaphorins (including Sema4D) is the ability to promote neurite outgrowth

from PC12 neural cells (Fujioka et al., 2003). We therefore analysed whether p190 might be required to mediate this response to Sema4D, by knocking down its expression through siRNA-mediated technology. Notably, we did not observe

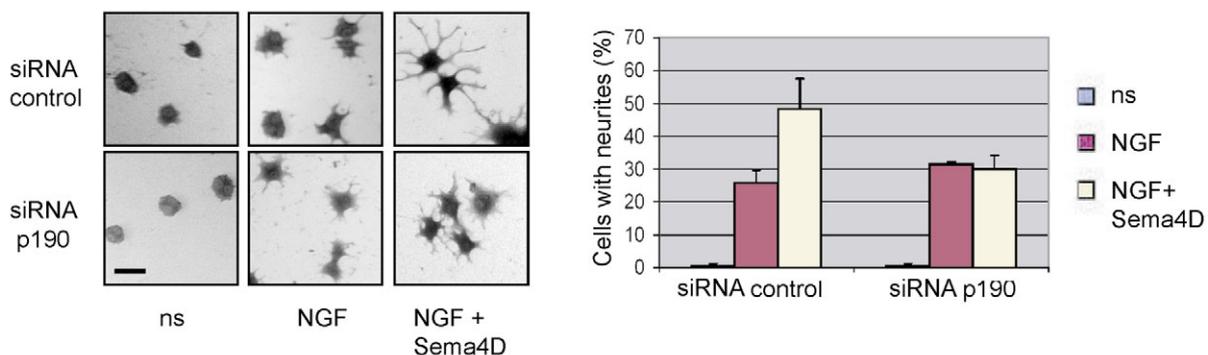
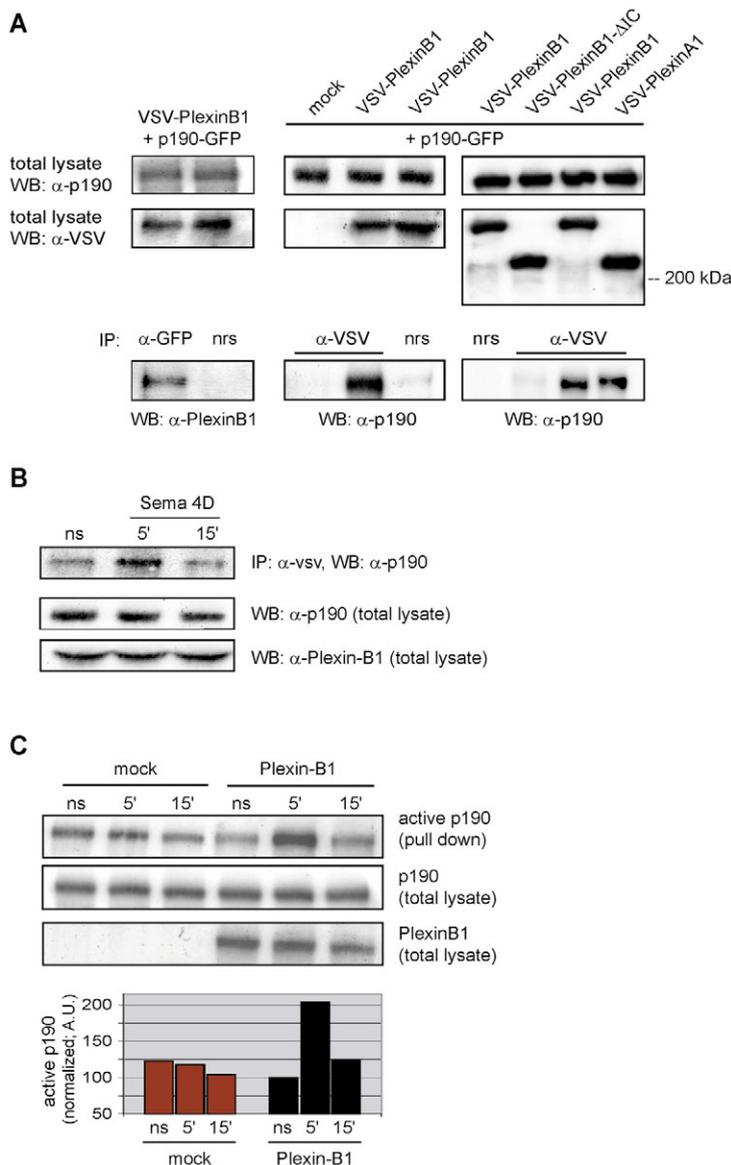


Fig. 6. p190 is required for Sema4D-induced neurite outgrowth. PC12 neuroblasts were grown for 48 hours in the presence of 50 ng/ml NGF, with or without 1 nM Sema4D, and then fixed and photographed. Scale bar: 20 μ m. The bar chart shows the mean percentage of cells with neurites (extending for at least one cell diameter) counted in three separate fields. Results are representative of two independent experiments. As discussed in the text, PC12 neuroblasts displayed minimal (if any) functional response to treatment Sema4D only (not shown). However, Sema4D-dependent synergism with NGF-induced neurite outgrowth is abrogated in cells depleted of p190 by targeted expression of siRNAs.

Fig. 7. p190 is recruited to plexins, and its GAP activity is induced upon receptor activation. (A) p190-GFP and either PLXNB1 or PLXNA1 (VSV-tagged) specifically co-precipitate from lysates of co-transfected 293T cells, whereas a truncated form of PLXNB1 lacking the cytoplasmic domain (PlexinB1- Δ IC) does not associate with p190. Immunoprecipitations were performed using either anti-tag antibodies (GFP for p190 and anti-VSV for plexins, respectively) or non-related serum (nrs). Western blots were probed with the indicated anti-tag or protein-specific antibodies. Note that PLXNB1 is larger than other plexins (approx. 300 kDa), while PlexinB1- Δ IC and PLXNA1 are almost identical in size (approx. 220 kDa). (B) Co-immunopurification of endogenous p190 with PLXNB1 transfected in 293T cells is induced after 5 minutes stimulation with 5 nM Sema4D. The expression levels of p190 and PLXNB1 in cell lysates are shown at the bottom. (C) Functionally active p190 was pulled-down (by means of constitutive active RhoQ63L-GST coated beads) from lysates of 3T3 fibroblasts expressing PLXNB1 and treated with 5 nM Sema4D for the indicated times. Immunoblotting with specific antibodies was used to detect p190 in pull-downs and in total lysates (included as loading controls). As measured by band density quantification, the level of activated p190 transiently increases upon plexin activation.



significant phenotypic changes in PC12 cells expressing siRNAs targeted to p190 transcript, compared with cells expressing control siRNAs. Consistent with findings reported by Fujioka and coworkers, control PC12 neuroblasts displayed minimal (if any) functional response to treatment with Sema4D only (not shown), while purified Sema4D synergized with nerve growth factor (NGF) induced neurite outgrowth in PC12 cells expressing control siRNAs. In contrast, as shown in Fig. 6, semaphorin activity was completely lost in cells undergoing siRNA-mediated p190 down-expression. Altogether, these results point to p190-RhoGAP activation as a general mechanism required for plexin signalling in different cells.

We then asked whether p190 is physically associated with semaphorin receptor complexes. In co-immunoprecipitation experiments, we demonstrated that both PLXNA1 and PLXNB1 specifically associate with p190-RhoGAP, and this requires the cytoplasmic domain of the receptor (Fig. 7A). The association appears to be constitutive upon protein overexpression. We thus tested the association of PLXNB1 with endogenous p190 and found that, while basally low, it was transiently increased upon stimulation with the ligand Sema4D (Fig. 7B).

It was reported previously that the functional activation of p190-RhoGAP is indicated by the increased ability to associate with its substrate Rho-GTP (Noren et al., 2003). Therefore, we used Sepharose beads coated with Rho-Q63L (a constitutively active form always bound to GTP) to pull down functionally activated p190-RhoGAP from lysates of cells treated with Sema4D. As shown in Fig. 7C, upon plexin activation, the association between p190 and its substrate was significantly increased. This is consistent with evidence that p190 mediates the down-regulation of Rho-GTP induced by plexins.

Discussion

Semaphorins control a wide range of biological functions. Moreover, some of them can induce antagonistic responses in

different cellular populations or experimental settings. For example, the same semaphorin can either repel neuronal processes (axons or dendrites) or attract them and induce their outgrowth (e.g. Kolodkin et al., 1993; Polleux et al., 2000; Swiercz et al., 2002; Schwamborn et al., 2004; Masuda et al., 2004). In a similar manner, the same semaphorin can either inhibit directional cell migration and induce apoptosis or promote cell migration and cell survival (e.g. Giordano et al., 2002; Toyofuku et al., 2004; Barberis et al., 2004). This is consistent with an ability of semaphorins to trigger a range of intracellular pathways. For most described functional responses, it has been shown that plexins are a required component of the receptor complex. However, other signalling receptors can associate with plexins and have been implicated in various biological outcomes.

The signalling pathways mediated by the conserved cytoplasmic domain of plexins have not been fully elucidated. In fact, although several putative signal transducers can interact with plexins, for only a few of them has a ligand-dependent

regulation been shown (Pasterkamp and Kolodkin, 2003). While this manuscript was in preparation, it was reported that the cytoplasmic domain of PLXNB1 associates with the activated form of the small GTPase R-Ras, and promotes its inactivation through an intrinsic R-Ras-GAP activity (Oinuma et al., 2004). This is quite unique, since all other known GAP proteins are recruited from the cytoplasm to protein complexes in the plasma membrane. In this paper, we provide strong evidence that the functional response mediated by plexins requires p190-RhoGAP activity. The evidence that Rho-GTP downregulation by p190 is required to mediate plexin function is at least three-fold: (1) multiple functional responses to plexin signalling, elicited in fibroblasts, tumour epithelial and primary endothelial cells, and related to control of cell adhesion and cell motility are abrogated in the absence of p190-RhoGAP; (2) neurite outgrowth mediated by semaphorin stimulation of PC12 neuroblasts is abrogated upon knockdown of p190-RhoGAP; (3) the functional response to semaphorins is rescued by re-expressing p190 in gene-deficient cells, but not by re-expressing a mutated form devoid of GAP activity. Furthermore, we provided evidence that functional responses mediated by different semaphorins, as well as by both cytoplasmic domains of PLXNB1 and PLXNA1, require p190 activity, strongly suggesting that this signalling pathway is shared by all family members. Our evidence that p190 associates with plexins in semaphorin receptor complexes clearly points to a direct involvement of p190-RhoGAP activity in plexin signalling. We also showed that, upon plexin activation, the association between p190 and its substrate Rho-GTP is increased, and accounts for the observed downregulation of active Rho. Moreover, the kinetics of Rho-GTP downregulation is consistent with that of the functional response observed in fibroblasts engineered with plexins (approx. 10 minutes) and in SKBR3 cells expressing endogenous PLXNB1 (approx. 30 minutes). It should be noted that, intriguingly, one of the molecules associated with activated p190 is p120-RasGAP, which can also use R-Ras as a substrate (Kinbara et al., 2003); this could provide an additional mechanism to mediate R-Ras inactivation upon plexin signalling.

It was reported that Rnd1/Rnd3 GTPases interact with p190 *in vitro* (in pull-down assays and in yeast two-hybrid assays) and induce its GAP activity (Wennerberg et al., 2003). Intriguingly, Rnd1 also associates with the cytoplasmic domain of plexins (Rohm et al., 2000), and this seems to be a prerequisite for the recruitment of additional plexin effector molecules, such as R-Ras or Rho GEFs (Oinuma et al., 2003; Oinuma et al., 2004). Nevertheless, we could not observe changes in the association of p190 to PLXNB1 upon Rnd1 overexpression (not shown). Therefore, at the present time, we are not able to determine whether ligand-induced association of p190 with plexins is direct or depends on additional intermediary molecules.

In apparent contradiction to our data, others have previously reported increased Rho-GTP levels upon activation of plexins of the B-subfamily. This effect is mediated by Rho Exchange Factors associated with plexins (PDZ-Rho-GEFs), and requires the tyrosine kinase activity of ErbB receptors, induced by semaphorin treatment (Aurandt et al., 2002; Swiercz et al., 2002; Swiercz et al., 2004). In fact, we found that the cytosolic levels of active Rho are often increased in cells expressing high levels of PLXNB1, which may be accounted by kinase-

dependent basal activation of PDZ-RhoGEFs. We have shown here that soon after receptor stimulation, and consistent with the time when the collapse response arises, RhoGTP levels decrease. This effect is transient, and at later time points active Rho resumes its initial levels (or even increases). Notably, to reveal semaphorin-dependent activation of Rho-GEFs (and Rho-GTP upregulation), cells must be subjected to extensive serum-starvation in order to silence tyrosine kinase activities and reduce Rho-GTP basal levels below detection threshold (Swiercz et al., 2002). In these conditions, a further downregulation of RhoGTP induced by the ligand cannot be detected. Notably, increased levels of active Rho were observed following one hour in presence of Sema4D (Swiercz et al., 2002), which is long after we observed Rho-GTP downregulation (approx. 10 minutes), and long after cellular collapse is triggered.

Altogether, the above evidence indicates that Rho-GTP down-regulation and up-regulation elicited by plexins of the B subfamily may derive from two independently regulated pathways. However, how can they both have a role in semaphorin signalling? One bit of direct evidence that Rho activation may be required for certain functions is the finding that the axonal collapse induced by Sema4D in hippocampal neurons was blocked by Rho and ROCK inhibitors (Swiercz et al., 2002). Nonetheless, we have recently shown that ROCK activity is not required for Sema4D-mediated cellular collapse (Barberis et al., 2004), and this is consistent with results previously reported for growth cone collapse in response to Sema3A and Sema3F (Jin et al., 1997; Kuhn et al., 1999; Arimura et al., 2000; Atwal et al., 2003; Turner et al., 2004). In fact, direct Rho activation might only occur downstream of plexins of the B subfamily, which associate with RhoGEFs, and therefore it is unlikely to play a role in the signalling pathway downstream of other semaphorin receptors. Interestingly, it was recently reported that the downregulation of Rho-GTP levels correlates with PLXNA1-mediated inhibition of endocardial cell migration (Toyofuku et al., 2004). Here we found that p190-RhoGAP is required to mediate plexin signalling in a range of cells, and that it is required for plexin-induced Rho downregulation. Moreover, we show that a mutant form of p190 that specifically lacks GAP activity and cannot downregulate Rho-GTP is unable to mediate plexin signalling. The functional requirement for p190 is observed not only in plexin over-expressing cells, but also in immortalized epithelial and neuronal cells, as well as in primary endothelial cells, which express endogenous semaphorin receptors. Thus, we propose that a transient inhibition of Rho signalling is a common mechanism required for plexin-mediated cell collapse, for the regulation of cell adhesion and cell migration, and in neurite outgrowth induced by semaphorins. However, Rho-GTP upregulation may be selectively elicited by plexins of the B subfamily as an independent signalling pathway, involved in specific functional responses.

Rho-GTP downregulation mediated by p190 may account for F-actin depolymerization and cytoskeletal rearrangements typically observed in semaphorin-treated cells and axonal growth cones. In addition, we showed here that p190 is required for the early inhibition of integrin function mediated by plexins, which is crucially implicated in the control of cell migration and angiogenesis. How can p190-RhoGAP mediate this effect? Integrin-based complexes are very dynamic structures, as

suggested by the fact that both inhibition of adhesion and the inability to break adhesive complexes can hamper cell migration (Webb et al., 2002). Interestingly, low levels of active Rho at the leading edge guarantee a fast turnover of newly formed adhesive sites, to allow changes in the direction of migration induced by environmental guidance cues. However, a major inhibition of Rho signalling at the cell membrane (such as that mediated by p190 activation) can hamper the stability of all adhesive complexes, by weakening their connection with the contractile actin cytoskeleton (Burgstaller et al., 2004). When presented non-directionally, these signals induce retraction of all existing pseudopodia, or result in the appearance of random poorly organized cell protrusions, and eventually lead to impaired migration (Tatsis et al., 1998; Fincham et al., 1999; Worthylake et al., 2003). However, when the same signals are presented locally in a polarized manner, as we showed here in the endothelial cell co-culture assay, they elicit directional cell repulsion, probably explained by the selective retraction of cellular processes closer to the factor inhibiting cell-substrate adhesion (such as the semaphorins).

It remains to be determined whether Rho-GAP activity is also important for semaphorin-mediated axon guidance in vivo. It should be noted that the prevailing concept is that Rho activation hampers axonal outgrowth, while Rho inhibition promotes it. A localized turnover of Rho signalling, however, might be required for the dynamic reshaping of growth cones steering away from repelling signals and towards attractive ones. The demonstration of localized defects in axon pathfinding in p190 knockout mice (Brouns et al., 2000) is consistent with such a hypothesis and merits further investigation. Intriguingly, at least three semaphorins (Sema3A, Sema3E and Sema4D) can induce both axonal collapse and neurite outgrowth in different neuronal cells (Sakai et al., 1999; Schwamborn et al., 2004; Masuda et al., 2004). While axonal collapse and cellular collapse are fast functional responses thought to share certain effector mechanisms, the signalling pathways involved in semaphorin-mediated neurite outgrowth were not known. Intriguingly, Rho inactivation by p190 can induce neurite outgrowth by disassembling the cortical actin network (Brouns et al., 2001), and in this paper we provide the first evidence that the neurite outgrowth induced by semaphorins requires p190-RhoGAP. Based on our results, we thus conclude that p190-Rho-GAP is recruited to plexins upon ligand stimulation and is a pivotal mediator of multiple functional responses mediated by semaphorins.

We wish to thank M. Shwartz and K. Burrige for generously providing expression constructs for Rhotekin-RBD-GST, and Rho-Q63L-GST and p190-R₁₂₈₃A-GFP, respectively. We thank G. Scita, H. Kikutani, A. Kolodkin, S. Giordano, G. Gilestro and L. Trusolino for comments and advice, and M. De Petrini, L. Palmas and R. Albano for help. This work was supported by funds from the Italian Association for Cancer Research-AIRC (to L.T. and P.M.C.) and from the EMBO Young Investigator Programme (to L.T.).

References

- Arimura, N., Inagaki, N., Chihara, K., Menager, C., Nakamura, N., Amano, M., Iwamatsu, A., Goshima, Y. and Kaibuchi, K. (2000). Phosphorylation of collapsin response mediator protein-2 by Rho-kinase: Evidence for two separate signaling pathways for growth cone collapse. *J. Biol. Chem.* **273**, 23973-23980.
- Atwal, J. K., Singh, K. K., Tessier-Lavigne, M., Miller, F. D. and Kaplan, D. R. (2003). Semaphorin 3F antagonizes neurotrophin-induced phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling: a mechanism for growth cone collapse. *J. Neurosci.* **23**, 7602-7609.
- Aurandt, J., Vikis, H. G., Gutkind, J. S., Ahn, N. and Guan, K. L. (2002). The semaphorin receptor plexin-B1 signals through a direct interaction with the Rho-specific nucleotide exchange factor, LARG. *Proc. Natl. Acad. Sci. USA* **99**, 12085-12090.
- Barberis, D., Artigiani, S., Casazza, A., Corso, S., Giordano, S., Love, C. A., Jones, E. Y., Comoglio, P. M. and Tamagnone, L. (2004). Plexin signaling hampers integrin-based adhesion, leading to Rho-kinase independent cell rounding, and inhibiting lamellipodia extension and cell motility. *FASEB J.* **18**, 592-594.
- Behar, O., Golden, J. A., Mashimo, H., Schoen, F. J. and Fishman, M. C. (1996). Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature* **383**, 525-528.
- Bernards, A. and Settleman, J. (2004). GAP control: regulating the regulators of small GTPases. *Trends Cell Biol.* **14**, 377-385.
- Bielenberg, D. R., Hida, Y., Shimizu, A., Kaipainen, A., Kreuter, M., Kim, C. C. and Klagsbrun, M. (2004). Semaphorin 3F, a chemorepellant for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. *J. Clin. Invest.* **114**, 1260-1271.
- Bito, H., Furuyashiki, T., Ishihara, H., Shibasaki, Y., Ohashi, K., Mizuno, K., Maekawa, M., Ishizaki, T. and Narumiya, S. (2000). A critical role for a Rho-associated kinase, p16ROCK, in determining axon outgrowth in mammalian CNS neurons. *Neuron* **26**, 431-441.
- Brouns, M. R., Matheson, S. F., Hu, K. Q., Delalle, I., Caviness, V. S., Silver, J., Bronson, R. T. and Settleman, J. (2000). The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development. *Development* **127**, 4891-4903.
- Brouns, M. R., Matheson, S. F. and 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.
- Brown, C. B., Feiner, L., Lu, M. M., Li, J., Ma, X., Webber, A. L., Jia, L., Raper, J. A. and Epstein, J. A. (2001). PlexinA2 and semaphorin signaling during cardiac neural crest development. *Development* **128**, 3071-3080.
- Brummelkamp, T. R., Bernards, R. and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553.
- Burbelo, P. D., Finegold, A. A., Kozak, C. A., Yamada, Y. and Takami, H. (1998). Cloning, genomic organization and chromosomal assignment of the mouse p190-B gene. *Biochim. Biophys. Acta* **1443**, 203-210.
- Burgstaller, G. and Gimona, M. (2004). Actin cytoskeleton remodelling via local inhibition of contractility at discrete microdomains. *J. Cell Sci.* **117**, 223-231.
- Bussolino, F., Di Renzo, M. F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffey, A. and Comoglio, P. M. (1992). Hepatocyte growth factor is a potent angiogenic factor that stimulates endothelial cell motility and growth. *J. Cell Biol.* **119**, 629-641.
- Conrotto, P., Corso, S., Gamberini, S., Comoglio, P. M. and Giordano, S. (2004). Interplay between scatter factor receptors and B plexins controls invasive growth. *Oncogene* **23**, 5131-5137.
- De Palma, M. and Naldini, L. (2002). Transduction of a gene expression cassette using advanced generation lentiviral vectors. *Methods Enzymol.* **346**, 514-529.
- Driessens, M. H., Hu, H., Nobes, C. D., Self, A., Jordens, I., Goodman, C. S. and Hall, A. (2001). Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho. *Curr. Biol.* **11**, 339-344.
- Eickholt, B. J., Mackenzie, S. L., Graham, A., Walsh, F. S. and Doherty, P. (1999). Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development* **126**, 2181-2189.
- Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.
- Feiner, L., Webber, A. L., Brown, C. B., Lu, M. M., Jia, L., Feinstein, P., Mombaerts, P., Epstein, J. A. and Raper, J. A. (2001). Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. *Development* **128**, 3061-3070.
- Fincham, V. J., Chudleigh, A. and Frame, M. C. (1999). Regulation of p190 Rho-GAP by v-Src is linked to cytoskeletal disruption during transformation. *J. Cell Sci.* **112**, 947-956.
- Fujioka, S., Masuda, K., Toguchi, M., Ohoka, Y., Sakai, T., Furuyama, T. and Inagaki, S. (2003). Neurotrophic effect of Semaphorin 4D in PC12 cells. *Biochem. Biophys. Res. Commun.* **301**, 304-310.

- Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L. and Comoglio, P. M. (2002). The semaphorin 4D receptor controls invasive growth by coupling with Met. *Nat. Cell Biol.* **4**, 720-724.
- Hu, H., Marton, T. F. and Goodman, C. S. (2001). Plexin B mediates axon guidance in *Drosophila* by simultaneously inhibiting active Rac and enhancing RhoA signaling. *Neuron* **32**, 39-51.
- Jin, Z. and Strittmatter, S. M. (1997). Rac1 mediates collapsin-1-induced growth cone collapse. *J. Neurosci.* **17**, 6256-6263.
- Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T. and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126**, 4895-4902.
- Kinbara, K., Goldfinger, L. E., Hansen, M., Chou, F. L. and Ginsberg, M. H. (2003). Ras GTPases: integrins' friends or foes? *Nat. Rev. Mol. Cell Biol.* **4**, 767-776.
- Kolodkin, A. L., Matthes, D. J. and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* **75**, 1389-1399.
- Kuhn, T. B., Brown, M. D., Wilcox, C. L., Raper, J. A. and Bamberg, J. R. (1999). Myelin and collapsin-1 induce motor neuron growth cone collapse through different pathways: inhibition of collapse by opposing mutants of rac1. *J. Neurosci.* **19**, 1965-1975.
- Luo, Y., Raible, D. and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**, 217-227.
- Maestrini, E., Tamagnone, L., Longati, P., Cremona, O., Gulisano, M., Bione, S., Tamanini, F., Neel, B. G., Toniolo, D. and Comoglio, P. M. (1996). A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor. *Proc. Natl. Acad. Sci. USA* **93**, 674-678.
- Masuda, K., Furuyama, T., Takahara, M., Fujioka, S., Kurinami, H. and Inagaki, S. (2004). Sema4D stimulates axonal outgrowth of embryonic DRG sensory neurones. *Genes Cells* **9**, 821-829.
- Moreno-Flores, M. T., Martin-Aparicio, E., Martin-Bermejo, M. J., Agudo, M., McMahon, S., Avila, J., Diaz-Nido, J. and Wandosell, F. (2003). Semaphorin 3C preserves survival and induces neurogenesis of cerebellar granule neurons in culture. *J. Neurochem.* **87**, 879-890.
- Noren, N. K., Arthur, W. T. and Burridge, K. (2003). Cadherin engagement inhibits RhoA via p190RhoGAP. *J. Biol. Chem.* **278**, 13615-13618.
- Oinuma, I., Katoh, H., Harada, A. and Negishi, M. (2003). Direct interaction of Rnd1 with Plexin-B1 regulates PDZ-RhoGEF-mediated Rho activation by Plexin-B1 and induces cell contraction in COS-7 cells. *J. Biol. Chem.* **278**, 25671-25677.
- Oinuma, I., Ishikawa, Y., Katoh, H. and Negishi, M. (2004). The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. *Science* **305**, 862-865.
- Pasterkamp, R. J. and Kolodkin, A. L. (2003). Semaphorin junction: making tracks toward neural connectivity. *Curr. Opin. Neurobiol.* **13**, 79-89.
- Pasterkamp, R. J., Peschon, J. J., Spriggs, M. K. and Kolodkin, A. L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. *Nature* **424**, 398-405.
- Perrot, V., Vazquez-Prado, J. and Gutkind, J. S. (2002). Plexin B regulates rho through the guanine nucleotide exchange factors leukemia-associated Rho GEF (LARG) and PDZ-RhoGEF. *J. Biol. Chem.* **277**, 43115-43120.
- Polleux, F., Morrow, T. and Ghosh, A. (2000). Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* **404**, 567-573.
- Raftopoulos, M. and Hall, A. (2004). Cell migration: Rho GTPases lead the way. *Dev. Biol.* **265**, 23-32.
- Ren, X. D., Kiosses, W. B. and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18**, 578-585.
- Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D. and Schwartz, M. A. (2000). Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J. Cell Sci.* **113**, 3673-3678.
- Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709.
- Rohm, B., Rahim, B., Kleiber, B., Hovatta, I. and Puschel, A. W. (2000). The semaphorin 3A receptor may directly regulate the activity of small GTPases. *FEBS Lett.* **486**, 68-72.
- Rottner, K., Hall, A. and Small, J. V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* **9**, 640-648.
- Sakai, T., Furuyama, T., Ohoka, Y., Miyazaki, N., Fujioka, S., Sugimoto, H., Amasaki, M., Hattori, S., Matsuya, T. and Inagaki, S. (1999). Mouse semaphorin H induces PC12 cell neurite outgrowth activating Ras-mitogen-activated protein kinase signaling pathway via Ca(2+) influx. *J. Biol. Chem.* **274**, 29666-29671.
- Schwamborn, J. C., Fiore, R., Bagnard, D., Kappler, J., Kaltschmidt, C. and Puschel, A. W. (2004). Semaphorin 3A stimulates neurite extension and regulates gene expression in PC12 cells. *J. Biol. Chem.* **279**, 30923-30926.
- Schwartz, M. A. and Shattil, S. J. (2000). Signaling networks linking integrins and rho family GTPases. *Trends Biochem. Sci.* **25**, 388-391.
- Serini, G., Valdembrì, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M. et al. (2003). Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* **424**, 391-397.
- Swiercz, J. M., Kuner, R., Behrens, J. and Offermanns, S. (2002). Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. *Neuron* **35**, 51-63.
- Swiercz, J. M., Kuner, R. and Offermanns, S. (2004). Plexin-B1/RhoGEF-mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. *J. Cell Biol.* **165**, 869-880.
- Takahashi, T. and Strittmatter, S. M. (2001). PlexinA1 autoinhibition by the plexin sema domain. *Neuron* **29**, 429-439.
- Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H. and Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* **99**, 59-69.
- Tamagnone, L. and Comoglio, P. M. (2004). To move or not to move? Semaphorin signalling in cell migration. *EMBO Rep.* **5**, 356-361.
- Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M. et al. (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* **99**, 71-80.
- Tatsis, N., Lannigan, D. A. and Macara, I. G. (1998). The function of the p190 Rho GTPase-activating protein is controlled by its N-terminal GTP binding domain. *J. Biol. Chem.* **273**, 34631-34638.
- Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Suto, F., Kamei, J., Aoki, K., Yabuki, M., Hori, M., Fujisawa, H. et al. (2004). Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. *Genes Dev.* **18**, 435-447.
- Troller, U., Raghunath, A. and Larsson, C. (2004). A possible role for p190RhoGAP in PKCepsilon-induced morphological effects. *Cell Signal.* **16**, 245-252.
- Turner, L. J., Nicholls, S. and Hall, A. (2004). The activity of the plexin-A1 receptor is regulated by Rac. *J. Biol. Chem.* **279**, 33199-33205.
- Van Aelst, L. and Symons, M. (2002). Role of Rho family GTPases in epithelial morphogenesis. *Genes Dev.* **16**, 1032-1054.
- Vincent, S. and Settleman, J. (1999). Inhibition of RhoGAP activity is sufficient for the induction of Rho-mediated actin reorganization. *Eur. J. Cell Biol.* **78**, 539-548.
- Webb, D. J., Parsons, J. T. and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells - over and over and over again. *Nat. Cell Biol.* **4**, E97-E100.
- Wennerberg, K., Forget, M. A., Ellerbroek, S. M., Arthur, W. T., Burridge, K., Settleman, J., Der, C. J. and Hansen, S. H. (2003). Rnd proteins function as RhoA antagonists by activating p190 RhoGAP. *Curr. Biol.* **13**, 1106-1115.
- Winberg, M. L., Tamagnone, L., Bai, J., Comoglio, P. M., Montell, D. and Goodman, C. S. (2001). The transmembrane protein Off-track associates with Plexins and functions downstream of Semaphorin signaling during axon guidance. *Neuron* **32**, 53-62.
- Worthyake, R. A. and Burridge, K. (2003). RhoA and ROCK promote migration by limiting membrane protrusions. *J. Biol. Chem.* **278**, 13578-13584.
- Zanata, S. M., Hovatta, I., Rohm, B. and Puschel, A. W. (2002). Antagonistic effects of Rnd1 and RhoD GTPases regulate receptor activity in Semaphorin 3A-induced cytoskeletal collapse. *J. Neurosci.* **22**, 471-477.