RasGRF1 is a neuron-specific guanine nucleotide exchange factor for the small GTPases Ras and Rac. It is implicated in the regulation of memory formation and in the development of tolerance to drug abuse, although the mechanisms have been elucidated only in part. Here we report the isolation, by the yeast two-hybrid screen, of the microtubule-destabilizing factor SCLIP (SCG10-like protein) as a novel RasGRF1-interacting protein. This interaction requires the region spanning the Dbl-homology domain of RasGRF1, endowed with catalytic activity on Rac. In search for a possible function we found by biochemical means that SCLIP influences the signaling properties of RasGRF1, greatly reducing its ability to activate the Rac/p38 MAPK pathway, while the Ras/Erk one remains unaffected. Moreover, a potential role is suggested by transfection studies in neuronal PC12 cells in which RasGRF1 induces neurite outgrowth, and coexpression of SCLIP counteracts this effect, causing a dramatic decrease in the percentage of cells bearing neurites, which also appear significantly shortened. This study unveils a physical and functional interaction between RasGRF1 and SCLIP. We suggest that this novel interplay may have possible implications in mechanisms that regulate neuronal morphology and structural plasticity.

SCLIP, a Microtubule-destabilizing Factor, Interacts with RasGRF1 and Inhibits Its Ability to Promote Rac Activation and Neurite Outgrowth*

Simona Baldassa‡1, Nerina Gnesutta‡2, Umberto Fascio§, Emmapaola Sturani‡, and Renata Zippel§

From the ‡Department of Biomolecular Sciences and Biotechnology and the §Interdepartmental Center for Advanced Microscopy, University of Milan, Via Celoria 26, 20133 Milan, Italy

RasGRF1 is a bifunctional guanine nucleotide exchange factor (GEF) that catalyzes the activation of the small GTP-binding proteins Ras and Rac, by facilitating the release of GDP thus favoring the loading of GTP. Its expression is restricted to postnatal neurons of the central nervous system, enriched in postsynaptic densities (1, 2) where it plays a key role in the regulation of some forms of synaptic plasticity.

Indeed, RasGRF1 knock-out mice display defects in memory consolidation associated to different areas of the brain, fail to develop behavioral tolerance to chronic treatment with cannabinoids, and show abnormal synaptic properties to repeated exposure to Δ9-tetrahydrocannabinol (3–7). The involvement of RasGRF1 and its closely related, widely distributed homologue RasGRF2 in intracellular signaling is developmentally regulated, because recent findings demonstrated that the components of signaling cascades from both types of ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA) receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, to mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated protein kinases (ERKs) switch during development and that RasGRFs play a major role in these signaling events only in mature neurons (8, 9). In addition, regarding the signaling from NMDA receptor, recent experiments on single and double knock-out mice revealed that RasGRF1 and -2 respond to diverse subclasses of NMDA receptors and contribute to different forms of synaptic plasticity, because RasGRF1 mediates mainly long term depression by regulating Rac and its effector p38 MAPK, whereas RasGRF2 predominantly signals to Ras/Erk cascade and mediates long term potentiation (10).

RasGRF1 is a 140-kDa modular protein containing multiple domains involved in interactions with specific binding partners and second messenger systems. In addition to its C-terminally located CDC25 homology domain (with exchange activity on Ras), it contains, in N- to C-terminal order, a pleckstrin homology (PH) domain followed by a coiled-coil region and an ill-defined domain, a pleckstrin homology (PH) domain, and a Dbl-homology domain (DblH). Its catalytic activity towards Rac is inhibited by SCLIP (SCG10-like protein), a microtubule-destabilizing factor that interacts with the Ras GRF1 guanine exchange factor (GEF) and inhibits its ability to promote Rac activation and neurite outgrowth. This study unveils a physical and functional interaction between RasGRF1 and SCLIP, suggesting that this novel interplay may have possible implications in mechanisms that regulate neuronal morphology and structural plasticity.
SCLIP Interaction with RasGRF1

To identify novel connections and give more insight into the physiological function of RasGRF1, in this work we performed a yeast two-hybrid screen with the DH PH tandem as bait and isolated SCLIP (SCG10-like protein) as a new RasGRF1 binding partner. SCLIP was originally identified by a screening of expressed sequence tag databases and is a member of the stathmin family of phospho-proteins (15, 16). Its expression was originally found to be restricted to brain structures in rodents (both neonatal and adult brain), and later a much more widespread expression profile was described in human tissues (15–17). The stathmin family also includes the founding member stathmin (18), SCG10 (superior cervical ganglion-10 protein) (19), and RB3 (20). These proteins regulate microtubule dynamics, and this property mostly resides in their ability to sequester free tubulin dimers through their conserved stathmin-like domains (SLDs), thus interfering with microtubule assembly, although other mechanisms have also been proposed (21–23). All stathmin-related proteins except stathmin itself, which is diffusely distributed in the cytosol, possess additional N-terminal palmitoylated regions that anchor them to the Golgi apparatus (24–26). In contrast to the ubiquitous expression of stathmin, all the other proteins are mainly restricted to the nervous system. Moreover, all of them are developmentally regulated in neuronal tissues, because expression of stathmin and SCG10 progressively increases during embryogenesis until birth and then decreases during postnatal development, whereas SCLIP and RB3, expressed embryonically, persist during postnatal development and into adulthood (15, 16, 27). This suggests that, despite their similarity, they might have distinct functional roles. Stathmin and SCG10 are the family members that have been mainly characterized, and growing evidence suggests their involvement in neurite formation both during development and regeneration after injury and, at least for stathmin, in the regulation of neuronal cell motility (28–32). Conversely, up to now little is known regarding the functional roles of SCLIP and RB3.

Here we report a novel interaction between RasGRF1 and SCLIP. In addition, we show a functional effect, because co-expression of SCLIP considerably reduced the ability of RasGRF1 to activate the Rac/p38 MAPK pathway and to promote neurite outgrowth in a neuronal model system. The cross-talk between RasGRF1 and SCLIP provides new insights into the properties of these molecules and opens possibilities for future studies in neurons.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen and Constructs—The yeast two-hybrid screen of an adult mouse brain cDNA library (BALB/c males, ages 9–12 weeks) constructed in the GAL4 activation domain plasmid pACT2 was carried out using the DH PH region of mouse RasGRF1 (aa 249–592) as bait. All vectors, yeast strains, and the library were purchased from Clontech (Mountain View, CA, MATCHMAKER GAL4 Two-Hybrid System 3) and used according to the manufacturer’s instructions. The DH PH tandem was PCR-amplified and subcloned into EcoRI/BamHI sites of pGBK7T7 in-frame with the GAL4 DNA binding domain. Primers were designed to introduce an EcoRI and a BamHI site, respectively, at the 5’- and 3’-ends of the DNA fragment. AH109 yeast strain was sequentially co-transformed with both the bait and the library plasmids, and cells were plated on highly selective SD-Ade-His-Trp-Leu medium (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with essential amino acids in the presence of 5 mM 3-amino-1,2,4-triazole (Sigma) and 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) (Clontech). Library plasmids from positive clones grown within 5 days after plating were used to transform Escherichia coli cells for DNA isolation. After re-testing the phenotype by pairwise matings between yeast cells of AH109 and Y187 strains, single transformed with the bait or the isolated library plasmid, inserts were amplified by PCR, sequenced, and identified by Blast alignments. Deletion analysis was performed according to the experimental procedure described above. Full-length SCLIP and the indicated deletion constructs were subcloned in EcoRI/BamHI sites of pGADT7 in-frame with the GAL4 activation domain by PCR upon introduction of the proper restriction sites. Similarly, the fragments corresponding to the PHCCIQ region (aa 1–235) and the isolated DH (aa 240–440) and PH2 (aa 453–592) domains of RasGRF1 were subcloned into EcoRI/BamHI sites of pGBK7T7 in-frame with GAL4 DNA binding domain. All the primers used in this study were synthesized by MWG-Biotech (Ebersberg, Germany), and their sequences are available upon request. All the constructs were verified by DNA sequencing, and the correct expression of the proteins was tested by Western blotting with antibodies to tags.

Mammalian and Bacterial Expression Plasmids—Constructs encoding SCLIP, full-length or SLD, were generated by amplifying fragments by standard PCR techniques with primers designed to introduce proper restriction sites and then subcloning them in pcDNA3 (Invitrogen) or PCR-SCLIP or in pEBG-mBad (Cell Signaling Technology, Danvers, MA), to express GST-SCLIP. Plasmids encoding GFP and myc-tagged versions of SCG10 and RB3 (respectively, in pEGFP-N3 and pCDNA3myc) were kindly provided by A. Sobel (INSERM, Paris, France) and described in previous studies (25, 33). The plasmid encoding pFLAG CMV-6-Stathmin was kindly provided by A. Colombatti (Istituto Nazionale Tumori, Aviano, Italy) and cited before (34). The plasmids encoding full-length RasGRF1 and the truncated PHC21 mutant (aa 1–630) have also been described previously (35, 36).

For expression of the myc-tagged DH PH tandem of RasGRF1 in mammalian cells the DNA fragment corresponding to aa 238–595 of mouse sequence was amplified by PCR with primers designed to introduce proper restriction sites and then subcloned into pcDNA3 downstream to a previously introduced myc epitope. Bacterial expression plasmids encoding the DH domain (aa 239–480) or the DH PH tandem (aa 239–591) of RasGRF1 fused to maltose-binding protein (MBP) were a generous gift by E. Jacquet (Ecole Polytechnique, Palaiseau Cedex, France) and have been previously described (37). MBP-DH PH fusions of mouse RasGRF2 (aa 235–599) and human SOS1 (aa 191–556) were generated by subcloning PCR-amplified fragments into pMAL-cRI (New England Biolabs, Ipswich, MA).
**Cell Culture and Transfections**— Cultures of hippocampal neurons were obtained from E17 Sprague-Dawley rat embryos (Harlan, Italy). Hippocampi were dissected, incubated with a solution of 0.05% trypsin/0.53 mM EDTA for 10 min at 37 °C, and mechanically dissociated through a fire-polished Pasteur pipette. Cells were plated in culture dishes previously coated with 10 μg/ml poly-l-lysine (Sigma) in Neurobasal medium supplemented with B27 (Invitrogen) and cultured at 37 °C in a humidified atmosphere with 5% CO₂.

HEK293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (EuroClone, Pero, Italy). Transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

PC12 cells were maintained in RPMI 1640 containing 10% heat-inactivated horse serum (EuroClone) and 5% fetal bovine serum supplemented with antibiotics as above. All media were from Invitrogen. For studies on the effects of the expression of different proteins on differentiation, PC12 cells were plated in culture dishes at a density of 6 × 10⁴ cells per cm² and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions with a 1:3 DNA to Lipofectamine ratio, using 1 μg of DNA per 35-mm dish (0.5 μg for each indicated plasmid in double transfections). When necessary empty vector (pcDNA3) was included in the transformation to ensure that each dish received the same amount of DNA. Approximately 20–22 h post-transfection, cells were reseded at a cell density of 1.5 × 10⁴ cells per cm² on glass coverslips previously coated with 0.1 g/liter poly-l-lysine (Sigma) in low serum medium (RPMI 1640 supplemented with 1% heat-inactivated horse serum) with or without the addition of 50 ng/ml human β-NGF (PeproTech, London, UK) for 48 h before being fixed and processed for immunofluorescence.

**In Vitro Pull-down and in Vivo Coimmunoprecipitation Assays**—MBP fusion proteins were expressed in E. coli and bacterial lysates prepared as described (37). HEK293 cells transfected with the indicated plasmids were solubilized in lysis buffer (12 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, supplemented with Complete EDTA-free protease inhibitor mixture (Roche diagnostics, Mannheim, Germany)). Lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C, and supernatants (500 μg of whole cell extract) were incubated with amylose-covered beads (New England Biolabs) precoupled to the specific MBP fusion proteins (10–30 μg for each protein), overnight at 4 °C. Precipitates were washed with lysis buffer, and bound proteins were eluted by addition of Laemmli sample buffer, boiled 5 min, and detected by immunoblotting using specific antibodies. For GST-based pull-down assays, pEBG or pEBG-SCLIP plasmids (encoding, respectively, GST and GST-SCLIP) were transfected in HEK293 cells. 24 h post-transfection lysates were prepared (as described above except that 1 mM dithiothreitol was added and Nonidet P-40 was replaced by 2% Triton X-100), and 1 mg of cell lysate was incubated with glutathione-Sepharose beads (Amersham Biosciences). After rocking for 2 h at 4 °C, the beads were washed with lysis buffer and incubated with 1 mg of total protein extract for each sample from HEK293 cells transfected with RasGRF1 full-length or myc-DH PH, overnight at 4 °C, and then processed as above.

For immunoprecipitations, lysates from HEK293 cells transfected with the indicated plasmids (1 mg for each sample) were incubated with anti-FLAG M2 monoclonal antibodies (5 μg, Sigma) overnight at 4 °C. Cultures of hippocampal neurons were solubilized in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, supplemented with Complete EDTA-free protease inhibitor mixture. Lysates were clarified by centrifugation at 10,000 rpm for 10 min at 4 °C, and supernatants (800 μg) were subjected to immunoprecipitation with 2 μg of anti-RasGRF1 antibody (sc-224, Santa Cruz Biotechnology, Santa Cruz, CA), 10 μl of anti-SCLIP antibody (a generous gift by A. Sobel, INSERM, Paris, France) or 2 μg of nonimmune rabbit IgGs, overnight at 4 °C. Immune complexes were collected with protein G-agarose or protein A-Sepharose (Sigma). Immunoprecipitates were washed with lysis buffer, resuspended in Laemmli sample buffer, and analyzed by Western blotting.

**Ras and Rac Activation Assays**—Ras-GTP and Rac-GTP levels were detected by performing previously described pull-down assays. Bacterially expressed GST-RBD (Ras-binding domain) of cRaf or GST-PBD (p21 Cdc42/Rac1 binding domain) of PAK3 were immobilized on glutathione-Sepharose beads and used to precipitate, respectively, active myc-tagged Ras or HA-tagged Rac from transfected HEK293 cells. Cells were transfected with RasGRF1 and FLAG-SCLIP (400 ng each per 35-mm dish), alone or in combination, in the presence of myc-Ras (200 ng) or Rac-HA (100 ng). PHC21 was used in place of full-length RasGRF1 in Rac activation assays, and, in this case, the amount of plasmid to be transfected was optimized to 50 ng. Cells were serum starved for 18 h, and lysates were prepared as previously described (38) (except that sodium deoxycholate was omitted to precipitate Rac-GTP). Equal amounts of proteins (150 μg) were subjected to affinity precipitation. After washing precipitates with lysis buffer, bound proteins were eluted with Laemmli sample buffer and detected by immunoblotting with specific antibodies. Aliquots of whole cell lysates were analyzed by Western blotting to normalize for the total amount of transfected Ras and Rac.

**Western Blotting**—Samples were resolved by SDS-PAGE and transferred to nitrocellulose Protran membranes (Schleicher & Shuell GmbH, Dassel, Germany). Membranes were blocked with 5% BSA in Tris-buffered saline for 30 min at 42 °C and probed with different primary antibodies diluted in Tris-buffered saline-5% BSA-Tween 20 (0.1–0.2%): 1:5000 anti-FLAG M2 and anti-Erk2, 1:1000 anti-GST and anti-RasGRF1 (sc-224, Santa Cruz Biotechnology); 1:2000 anti-myc (9E10), 1:1000 anti-GST and anti-RasGRF1 (sc-224, Santa Cruz Biotechnology); 1:2000 anti-phospho Erk1/2, 1:1000 anti-phospho p38 MAPK (Cell Signaling technology, Beverly, MA), 1:2000 anti-N-terminal region of RasGRF1 (PH) (3). Membranes were incubated with the proper
peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and chemiluminescent signals were detected using the ECL detection system (Amersham Biosciences). Band intensities were quantitated by densitometry with the Scion Image program (Scion Corp., Frederick, MD), and signals specific for phosphorylated or GTP-bound forms were equalized to the total amount of the corresponding proteins. The increase in phosphorylation or GTP loading was normalized to the basal level of the control treatments and expressed as -fold increase.

**Immunofluorescence**—Cells were washed with PBS, fixed with a PBS solution containing 4% paraformaldehyde and 60 mm saccharose for 15 min, permeabilized for 4 min with 0.1% Triton X-100, and blocked with 3% BSA in PBS for 1 h at room temperature. Coverslips were then incubated for 1 h at room temperature with primary antibodies (anti-α-tubulin or anti-RasGRF1) diluted 1:500 in a solution of 1% BSA in PBS supplemented with 0.1% Triton X-100. After several washes, cells were incubated with the proper Alexa Fluor-conjugated goat anti-rabbit or anti-mouse secondary antibodies diluted in PBS-1% BSA (1:1000 Alexa Fluor 594, 1:500 Alexa 488, and 1:200 Alexa 350, Molecular Probes, Invitrogen). Where indicated, coverslips were further incubated with 1 μg/ml phalloidin-TRITC (Sigma) for 30 min before being mounted with gel mount (Biomedia, Foster City, CA). Microscopy analysis was carried out with an Olympus BX-60 fluorescence microscope or a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany), equipped with an argon and HeNe laser and a PLAPO 40×/1.25-0.75 oil immersion objective. The figures were assembled using Adobe Photoshop 5.5 or higher.

**Analysis of Neurite Outgrowth in PC12 Cells**—Differentiation was evaluated by two parameters: the percentage of transfected cells bearing neurites and the length of the longest protrusion per cell. Cell edges were outlined by fluorescence of GFP, Alexa-Fluor-conjugated antibodies for uniformly distributed molecules or, when necessary, phalloidin staining.

Neurites were defined as protrusions with lengths that were at least 2-fold longer than cell bodies. Transfected cells bearing neurites were counted, and data were plotted using Microsoft Excel. Three independent experiments with at least 400 cells per condition were evaluated.

Transfected cells were randomly selected using a 40× objective, and the length of the longest projection per cell was quantified using the National Institutes of Health Image analysis program Image J by comparison with measurements from a graduated reference. At least 50 cells per treatment were scored in each experiment, and the experiments were performed in triplicate. Student’s t test was applied for statistical analysis between two treatments.

**RESULTS**

Identification of SCLIP as a RasGRF1-interacting Protein—To identify novel binding partners of RasGRF1 we performed a two-hybrid screen. Because its expression is mainly restricted to mature neurons of the central nervous system (2) we screened a cDNA library from adult mouse brain (ages 9–12 weeks) and used the DH PH tandem of mouse RasGRF1 (aa 2336–592) as bait. A screen of ~3.8 × 10⁶ co-transformants led to the isolation of 55 positive clones with the ability to grow on histidine- and adenine-deficient medium and to develop the blue color in the presence of X-α-Gal. 42 clones were further analyzed and, among these, four independent cDNA inserts encoded for SCLIP (SCG10-like protein) (Fig. 1). Each library clone encoded the full open reading frame of SCLIP (encoding 180 amino acids) with an additional short sequence before the start codon (corresponding to part of the 5′-untranslated region) fused to the GAL4-activation domain, and deletion of this 5′-extra cDNA region did not affect the interaction. Moreover, the interaction was specific, because SCLIP failed to interact with multiple control proteins, including the isolated GAL4 DNA binding domain, the N-terminal region (spanning the PHCCIQ motifs) of RasGRF1 (aa 1–235) or laminin, a protein unrelated to RasGRF1 (see also Fig. 3A). The interaction was further confirmed by yeast mating (data not shown).

RasGRF1 Interacts with SCLIP Both in Vitro and in Vivo—We first proceeded to further verify this interaction in vitro by MBP pull-down assays. The isolated DH PH tandem of RasGRF1 (aa 239–591) was expressed in E. coli as fusion with the affinity tag MBP and immobilized on amylose-covered beads. Extracts from FLAG-SCLIP-transfected HEK293 cells were then incubated with MBP-DH PH fusion protein or MBP as a control. Bound proteins were eluted by the addition of SDS sample buffer and then analyzed by Western blotting using antibodies directed to tags. Fig. 2A (left panel) shows that MBP-DH PH, but not MBP, pulled down FLAG-SCLIP. Similar results were obtained by using GFP-tagged SCLIP (data not shown).

Despite several attempts using different N- or C-terminally located commonly used tags, we failed to express SCLIP protein in a soluble form in E. coli. This may be due to the presence of an additional N-terminal membrane-targeting extension with respect to stathmin that may compromise its solubility in bacterial cells. Therefore, we cloned SCLIP in fusion with GST in a mammalian expression vector and expressed the recombinant protein in transfected cells. GST-SCLIP or GST were immobilized on glutathione-Sepharose beads and used as baits to affinity-precipitate myc-DH PH or full-length RasGRF1, individually expressed in HEK293 cells. Fig. 2A (middle and right panels)
panels) shows that both myc-DH PH and RasGRF1 could be enriched in the fraction pulled down by GST-SCLIP compared with GST.

We next proceeded to determine whether this association occurs also in vivo by performing co-immunoprecipitation assays from transfected HEK293 cells. As depicted in Fig. 2B, both myc-DH PH and full-length RasGRF1-HA were found to co-immunoprecipitate with FLAG-SCLIP using anti-FLAG antibodies. Similar results were obtained by co-expressing GST-SCLIP and RasGRF1 and then pulling down the complex by glutathione-Sepharose beads (data not shown).

Lastly, we examined whether this interaction might be physiologically relevant by performing immunoprecipitation assays from cultures of rat hippocampal neurons. Neuronal cells express both SCLIP and RasGRFs (1 and 2) (Fig. 2C). Interestingly, antibodies to SCLIP visualize a pattern of multiple bands that might represent distinct phosphorylation states of the protein, as already reported for other stathmin family members (39, 40). This profile does not seem to undergo readily discernible changes during culture development, while the expression of RasGRFs clearly increases at the various time points tested (from 7 to 17 days in vitro (DIV)), in line with previous reports (8, 41) (Fig. 2C). Consequently, analysis was performed at 17 DIV, when all these proteins are fully expressed. Lysates were subjected to immunoprecipitation with anti-RasGRF1 or anti-SCLIP antibodies, followed by Western blotting. Incubation with nonimmune rabbit IgGs was used as a control. Immunoprecipitation with anti-SCLIP antibodies allowed to pull down, in addition to a pattern of multiple bands corresponding to SCLIP, also two bands corresponding to both RasGRFs, 1 and 2, suggesting that the interaction does occur in its physiological context (Fig. 2C, right panel). Vice versa, in anti-RasGRF1 precipitates we could recover a clearly identifia ble band corresponding to just one of the multiple signals detected for SCLIP (Fig. 2C, left panel). This band was absent in the control sample treated with nonimmune IgGs and might suggest the involvement of a specific form of SCLIP in this association. Taken together these results provide compelling evidence that RasGRF1 interacts with SCLIP both in vitro as well as in vivo.

Interaction Domain Requirements for RasGRF1 and SCLIP Association—To map the specific regions responsible for the interaction, we co-transformed a series of deletion mutants fused to the DNA binding or activation domains of GAL4 in yeast cells together with full-length SCLIP or the DH PH fragment of RasGRF1. For each pair, we analyzed the ability to interact by monitoring cell growth on highly selective medium. All fusion proteins were correctly expressed in yeast cells as tested by Western blots with antibodies to tags (data not shown).

Results shown in Fig. 3A demonstrated that the minimal interaction motif of RasGRF1 could be narrowed down to the region spanning the DH domain, whereas the PH domain was dispensable. These data were confirmed by in vitro pull-down assays, showing that the isolated DH domain is sufficient to pull down FLAG-SCLIP (Fig. 3B, upper panel).

Conversely, deletion analysis of SCLIP failed to define a smaller region involved in the interaction, because neither the isolated SLD (aa 39–180), nor two different extensions of the N-terminal region (aa 1–38 and 1–80) could rescue the growth defect of yeast cells in highly selective medium (Fig. 3A). In addition, a FLAG-tagged version of SLD expressed in HEK293 cells was unable to bind the DH PH of RasGRF1 fused to MBP in in vitro pull-down assays (Fig. 3B, lower panel). Collectively, these results point to the region spanning the DH domain as the main binding motif on RasGRF1, whereas multiple regions and/or a complex protein folding may be required for SCLIP.

Non-exclusive RasGRF1/SCLIP Association: Extension to Other Dbl-related GEFs and Stathmin-like Family Members—We next asked whether the interaction of RasGRF1 with SCLIP was exclusive or could be shared with other stathmin-like

**FIGURE 2. Co-precipitation of RasGRF1 and SCLIP in mammalian cells.** A, in vitro pull-down assays. HEK293 cells were transfected with plasmids encoding FLAG-SCLIP (left panel) myc-DH PH (middle) or RasGRF1 full-length (right) and lysates incubated with the indicated fusion proteins. Precipitates and total cell lysates (20 μg, input) were separated by SDS-PAGE and immunoblotted (IB) with specific antibodies. MBP fusion proteins were visualized by staining with Ponceau S, whereas GST fusions were visualized by immunoblot with anti-GST antibodies. B, co-immunoprecipitation of SCLIP and RasGRF1 full-length or myc-DH PH from transfected HEK293 cells. Lysates were subjected to immunoprecipitation with anti-FLAG antibodies (IP). Precipitates and total cell extracts (inputs) were analyzed by Western blotting with specific antibodies. C, co-immunoprecipitation of endogenous RasGRF1 and SCLIP from rat hippocampal cultures. Upper panel: expression profile of RasGRF1 and SCLIP at different days in vitro (DIV). 40 μg of whole cell lysates was analyzed by Western blotting using antibodies to RasGRF1, SCLIP, or actin to normalize for loading. Lower panel: extracts from cultures at 17 DIV were incubated with anti-RasGRF1 or anti-SCLIP antibodies. Nonimmune rabbit IgGs were used as a control. Immunoprecipitates were then analyzed by Western blotting with the indicated antibodies. The lower band recognized by anti-RasGRF1 antibodies represents RasGRF2 (48). Immunoblots shown are representative of multiple independent experiments.
proteins and/or GEFs of the Dbl family. Therefore, we performed in vitro pull-down assays using the MBP-DH PH fragment to precipitate tagged versions of stathmin-like proteins from transfected HEK293 cells.

The stathmin family includes the founding member stathmin, SCG10, SCLIP, and RB3. As shown in Fig. 4A, the MBP-DH PH fragment of RasGRF1 precipitated, in addition to SCLIP (lanes 4–6, left panel), also SCG10 and RB3 (lanes, respectively, 1–3 and 4–6, right panel), but not stathmin (lanes 1–3, left panel) (similar results were obtained using myc-tagged versions of SCG10 and RB3).

Lastly, we used the DH PH fragments of other two Dbl-related GEFs, the highly homologue mouse RasGRF2 and human SOS1, fused to MBP as baits in pull-down assays. Fig. 4B shows that both protein fragments were able to pull down FLAG-SCLIP, thus spreading this feature also among other GEFs for the Rho family GTPases.

Effect of RasGRF1 and SCLIP Co-expression on the Microtubule Network—To gain insight into this interaction, we first analyzed possible effects of co-expressing RasGRF1 on the microtubule-destabilizing activity of SCLIP. We transiently transfected HeLa cells with GFP-SCLIP alone or in combination with full-length RasGRF1 and, 24 h post-transfection, we looked at the microtubule network by immunofluorescence using anti-α-tubulin antibodies. Transfected proteins were visualized by GFP fluorescence or immunostaining with antibodies directed to RasGRF1. As shown in Fig. 5a, staining for α-tubulin revealed, in untransfected cells in interphase, a highly organized network of microtubules (MTs) and treatment with nocodazole resulted in a dramatic collapse of this structure (Fig. 5e). An organized MT network, without readily detectable defects, could also be observed upon expression of RasGRF1 (Fig. 5d). By contrast, GFP-SCLIP-expressing cells showed a largely perturbed MT array that lost, at least in part, its network organization (Fig. 5b). The effect on MTs varied among the cells depending on the expression level of GFP-SCLIP, as reported elsewhere for stathmin (33). Therefore, cells expressing low levels of GFP-SCLIP were not considered in our analysis.

More importantly, when we expressed GFP-SCLIP together with RasGRF1 we were unable to reveal any improvement in the organization of the tubulin cytoskeleton, that appeared similar to that observed in cells expressing GFP-SCLIP alone (Fig. 5c, compare cells expressing GFP-SCLIP + RasGRF1, indicated by arrowheads, with that transfected with GFP-SCLIP alone, indicated by asterisk). Similar results were also obtained using the myc-tagged DH PH fragment (data not shown). It should be noted that expression of RasGRF1 resulted in a slight reduction of the average cell area (~10%) both in single as well as double transfected cells (data not shown), but at the moment the reason is not clear.
Because the dynamics of microtubules can influence the organization of the actin cytoskeleton (42, 43) we also evaluated possible effects on F-actin visualized by phalloidin staining, but we did not reveal any significant effect of ectopically expressed proteins (data not shown). Therefore, in our experimental condition, RasGRF1 seems unable to overcome the effect of SCLIP in destabilizing the microtubule network, although we cannot exclude the existence of more subtle and/or locally restricted effects on the regulation of microtubule dynamics.

**SCLIP Affects the Ability of RasGRF1 to Activate the Rac/p38 MAPK Pathway but Not the Ras/Erk One**—We then examined the possibility that SCLIP might alter the ability of RasGRF1 to signal to its target GTPases, Ras and Rac, and to their downstream effectors, Erk1/2 and p38 MAPKs, respectively. For this purpose, we used HEK293 cells to achieve high transfection efficiencies to perform biochemical assays. Cells were transfected with RasGRF1 and FLAG-SCLIP, alone or combined, together with a tagged form of the specific GTPase. A truncated mutant of RasGRF1 lacking the C-terminal half (PHC21, spanning aa 1–630, up to the second PH domain) was used in Rac activation assays, because it was a better activator of Rac as compared with the full-length protein, as reported by others (44). This mutant retains the ability to bind SCLIP as assessed by in vivo co-precipitation assays (not shown). GTPase activity was evaluated in vivo by using a region of their effector molecules (RBD of cRaf for Ras and PDB of PAK3 for Rac) to specifically capture the GTP-bound active form from a cell lysate; affinity-precipitated myc-Ras and Rac-HA were then visualized by immunoblotting with antibodies to tags. MAPK activation was evaluated by immunoblotting of whole cell extracts with antibodies that specifically recognize the phosphorylated forms of Erk1/2 or p38.

We then examined the effect on the Ras/Erk pathway. Expression of RasGRF1 resulted in a marked increase in GTP-loaded Ras, whereas FLAG-SCLIP alone did not produce any significant effect (Fig. 6A). Similarly, Ras was fully activated also when RasGRF1 was expressed together with FLAG-SCLIP. Consistent with this, Erk1/2 became phosphorylated in the presence of RasGRF1, and this activation was fully achieved also upon co-expression of SCLIP (Fig. 6B). These data indicate that SCLIP does not interfere with RasGRF1-mediated Ras signaling.

We then focused on Rac. Expression of PHC21 led to an appreciable increase of GTP-bound Rac (Fig. 6C). Interestingly, in this case, in contrast to Ras, this stimulation could not be observed when PHC21 was expressed in the presence of SCLIP. A similar trend was also observed using full-length RasGRF1 (data not shown). Therefore, SCLIP impairs the ability of RasGRF1 to stimulate the activity of Rac. To further analyze this effect we also evaluated the phosphorylation level of the Rac target p38 MAPK. As shown in Fig. 6D, RasGRF1 promoted a robust increase in the level of phospho-p38, and, in parallel with the effect on Rac, this activation was reduced in the presence of SCLIP.

Altogether these data indicate that SCLIP interferes with the ability of RasGRF1 to signal to the Rac/p38 MAPK pathway, whereas it does not affect its signaling to the Ras/Erk cascade. These findings seem consistent also with the fact that the interaction occurs in the region spanning the DH domain of RasGRF1, mainly endowed with the exchange activity on Rac.

**Expression of SCLIP Antagonizes RasGRF1-induced Neurite Outgrowth in PC12 Cells**—We then wanted to investigate potential biological effects of this interplay. In view of the fact that RasGRF1 and SCLIP are mainly expressed in neuronal tissues we further investigated the physiological consequences of their co-expression in a neuronal model system.

Rho family GTPases and several Dbl family GEFs play a crucial role in diverse aspects of neuronal development and morphogenesis (45). Among these, RasGRF1 has recently been shown to functionally interact with the NGF receptor TrkA in promoting neurite formation in PC12 cells (12). Additionally, SCG10, the best characterized among the neuron-specific stathmin-like proteins, plays a role in regulating neurite outgrowth, and SCLIP has recently been shown to interact with clusterin, a chaperone-like protein also involved in this process (29, 30, 46). All these clues prompted us to test the effect of expressing RasGRF1 and SCLIP, alone or in combination, on neurite outgrowth in PC12 cells. This effect was quantified by using neurite length as marker (Fig. 6E). As expected, expression of RasGRF1 alone or together with SCLIP, did not produce any appreciable effect on neurite outgrowth. However, co-expression of RasGRF1 and SCLIP resulted in a pronounced diminution of the neurite lengths.
neuritogenesis. We used the PC12 cell line that represents a well characterized cellular model for studying neuronal differentiation.

We were unable to detect the expression of endogenous Ras-GRF1 in these cells, even upon treatment with NGF for a 5-day period (Fig. 7A, left panel). Conversely, endogenous SCLIP was present at low levels in untreated cells and only slightly increased (~1.5-fold) following exposure to NGF, in accordance with previous data on SCLIP mRNA (15).

To promote differentiation the following protocol was adopted in all the experiments described below: cells were transfected with the indicated expression constructs and, 20–22 h post-transfection, re-plated at lower density on poly-lysine-covered coverslips, in low serum conditions, with or without the addition of NGF. 48 h after replating, cells were fixed, processed by immunofluorescence, and assessed for neurite formation.

To quantify the observed effects on neurite outgrowth we scored the percentage of transfected cells that bore neurites and measured the length of the longest process per transfected cell. Cells were scored as positive for bearing neurites when they showed protrusions of at least two cell bodies in length.

First we analyzed the effect of expressed proteins on neurite outgrowth in the absence of NGF (Fig. 7B). Expression of GFP alone did not produce significant morphological changes (Fig. 7B, panel a). Similarly, overexpression of GFP-SCLIP was unable to promote the formation of neurites (Fig. 7B, panel b and inset, showing a higher magnification). By contrast, in the same condition expression of RasGRF1 caused extensive neurite outgrowth (Fig. 7B, panel c). Indeed, ~26% of transfected

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**FIGURE 6. Effect of SCLIP on the activation of the Ras/Erk and Rac/p38 pathways by RasGRF1.** Ras GTP loading (A) and Erk phosphorylation (B) induced by RasGRF1 were unaffected by SCLIP, whereas activation of Rac (C) was impaired and phosphorylation of p38 (D) was significantly reduced. Active GTP-bound Ras or Rac (A–C) were precipitated as described under “Experimental Procedures” from HEK293 cells transfected with myc-Ras or Rac-HA together with empty vector, FLAG-SCLIP, or RasGRF1 (or VSV-tagged PHC21), as shown. Precipitates were then separated by SDS-PAGE and probed with the indicated antibodies. Whole cell lysates (15 µg, representing 10% of total cell lysate used in activation assays) were analyzed by Western blotting for the total amount of tagged Ras or Rac to normalize for loading. To analyze MAPK phosphorylation equal amounts of protein (20 µg) were analyzed by Western blotting using anti-phospho Erk1/2 (B) or anti-phospho p38 (D). Blots were also probed with anti-Erk2 antibodies to normalize for protein loading. Representative immunoblots of several independent experiments are shown. Band intensities of the active forms (GTP-bound or phosphorylated) were equalized to total levels of the proteins and plotted in graphs as -fold increase over control. Results shown are the mean of n independent experiments (A, n = 6; B and D, n = 4; C, n = 7). Error bars indicate S.E.
cells were scored positive for neurite sprouting (bar graph in Fig. 7B, left panel): the majority of differentiation-positive cells exhibited one long and thin process that eventually branched in along cell processes and frequently accumulated in the terminal growth cone (see Fig. 7B, lower panel c, showing a picture taken with a lower exposure time and Fig. 7C, middle panel). GFP-

FIGURE 7. SCLIP greatly reduces RasGRF1-induced neurite outgrowth in PC12 cells. A, Western blot analysis of endogenous (left panel) and transfected (right) proteins in PC12 cells. Left panel: cells were treated with 50 ng/ml NGF and lysed at the indicated times. α-Tubulin is a marker of stable microtubules, particularly abundant in mature neuronal cells. An extract of HEK293 cells transfected with RasGRF1 and FLAG-SCLIP was loaded as a control. B, representative confocal images of PC12 cells transfected with the indicated plasmids and cultured in the absence of NGF for 3 days before being processed by immunofluorescence. Expressed proteins were revealed by GFP fluorescence (green) or by staining with anti-RasGRF1 antibodies (red, panel c and upper panel d). Where indicated, cell edges were outlined by staining for F-actin with phalloidin-TRITC. Arrowheads in panel d underline the length of the process. Images in the inset in panel b (representing a higher magnification) and lower panel c were acquired by a fluorescence microscope. Bar graphs: quantification of neurite outgrowth. Transfected cells were scored for the presence of neurites whose lengths were at least 2-fold longer than their cell bodies (left). The measures of the lengths of the longest process per transfected cell were sorted into three categories (indicated below the graph), and the percentage of cells in each group was plotted. Results shown are the mean of three independent experiments. In each experiment at least 200 (left) or 50 (right) cells per condition were counted. Error bars represent S.E.; *, p < 0.03; ***, p < 0.0005. C, partial co-localization of RasGRF1 and SCLIP in the perinuclear region. Confocal images of PC12 cells double transfected with the indicated plasmids and merged fluorescence of the same images (yellow signal results from superimposition of green and red fluorescence). The blue staining represents 4′,6-diamidino-2-phenylindole (DAPI). The arrowhead indicates the tip of the neurite. Scale bars, 50 μm; inset in B, panel b, 10 μm.

SCLIP Interaction with RasGRF1

the distal part. A smaller fraction of cells showed multiple neurites (generally two or three) originating from the cell body. In some cells a marked enlargement of the tip of the neurite (growth cone) was also observed. The lengths of the longest process per cell were distributed, for the majority of the cells, close to the value of 100 μm, with some cells extending processes even longer than 200 μm. Additionally, a large fraction of cells that expressed RasGRF1 appeared enlarged compared with untransfected or GFP-transfected control ones (compare panels c with a in Fig. 7B).

Then we evaluated the effect of co-expressing GFP-SCLIP together with RasGRF1. Remarkably, SCLIP severely impaired the observed effect of RasGRF1 on neurite formation. Double transfected cells exhibited two major differences compared with cells expressing RasGRF1 alone: a significant reduction in the percentage of neurite bearing cells from 26% to 10% (~60% decrease) (bar graph in Fig. 7B, left panel), coupled to a dramatic reduction of the length of their neurites. Indeed, when compared with cells transfected with RasGRF1 alone, neurites of double transfected cells appeared unbranched and appreciably shorter (compare panels c and d in Fig. 7B). Consistently, a higher fraction of cells extended very short projections (<50-μm length), and no process longer than 100 μm could be found (bar graph in Fig. 7B, right panel). Only occasionally neurites were longer than 70 μm. Similar results were also obtained using GFP-RasGRF1 and a FLAG-tagged version of SCLIP (not shown).

Staining for RasGRF1, both in single as well as in double transfected cells, revealed a diffuse distribution throughout the cytosol with a more intense fluorescence signal accumulated in a ring-shaped structure around the nucleus. Bright puncta were uniformly distributed along cell processes and frequently accumulated in the terminal growth cone (see Fig. 7B, lower panel c, showing a picture taken with a lower exposure time and Fig. 7C, middle panel). GFP-
SCLIP mainly accumulated in a cap-shaped structure around the nucleus, presumably the Golgi apparatus, as previously reported (26), and in the few double transfected cells that extended neurites, it could also be detected in the tip of the projections and, as barely detectable puncta, along the neurites (Fig. 7C, left panel and Fig. 7B, panel b and inset). The two proteins co-localized, at least in part, in the region around the nucleus (Fig. 7C, right panel), and, in neurite bearing cells, their staining did not seem to extensively overlap in neurites, except for some isolated puncta along the length of the processes and in the tip. To summarize, these experiments show that in PC12 cells expression of RasGRF1 induces the formation of neurites and the concomitant expression of SCLIP critically counteracts this effect.

**SCLIP Does Not Affect Neurite Outgrowth Induced by NGF, but It Impairs the Additive Effect Exerted by RasGRF1**—We next asked whether the effect of SCLIP on RasGRF1-induced neurite outgrowth could result indirectly from a deregulation of a more general mechanism that is relevant to neurite sprouting, primarily its interference with microtubule assembly, rather than to its relationship with RasGRF1. To address this point we assessed the effect of expressing GFP-SCLIP on neuronal differentiation induced by treatment with NGF.

Following treatment of PC12 cells with NGF (50 ng/ml) for 48 h, ~10–15% of cells exhibited neurites (Fig. 8A, left bar graph). Expression of GFP did not alter this phenotype (Fig. 8A, left bar graph; also compare panel a with lower panel b showing an untransfected (“ut”) differentiated cell). More importantly, in contrast with the effect on RasGRF1-induced neuritogenesis, expression of GFP-SCLIP did not affect the ability of cells to differentiate upon treatment with NGF. Indeed, it neither decreased the percentage of cells that formed neurites, nor did it affect the length of neuronal processes (Fig. 8A, panels a and b, and bar graphs).

Thus, SCLIP seems to counteract the ability of RasGRF1 to promote neurite outgrowth and does not act as a negative reg-
SCLIP Interaction with RasGRF1

RasGRF1 is a neuron-specific Ras/Rac guanine nucleotide exchange factor that plays a key role in important neurological processes such as memory formation (3, 5). The signaling network in which it works has been elucidated only in part. Some relevant protein-protein interactions have been reported, but the picture is still incomplete and deserves further investigation. Here we report the identification by the yeast two-hybrid screen of a novel interaction between RasGRF1 and SCLIP, a microtubule-destabilizing protein belonging to the stathmin family, and demonstrate that these two proteins physically and functionally interact in vivo.

To explore possible effects we first considered the possibility that RasGRF1 might influence the ability of SCLIP to destabilize microtubules, but immunofluorescence analysis carried out on transfected HeLa cells did not reveal any discernible effect. We also investigated whether SCLIP might share with stathmin the property of regulating cell migration, but we were unable to reveal any significant effect neither by SCLIP nor by RasGRF1, alone or combined.4

On the other hand, we could show a clear effect of SCLIP on the signaling mediated by RasGRF1. In transfection studies, RasGRF1 activated both Ras and Rac and also caused a marked increase in the phosphorylation level of their major downstream MAPK targets, respectively, Erk1/2 and p38. Although Ras and Erk activation was not affected, concomitant expression of SCLIP severely impaired the GTP loading of Rac and considerably reduced the phosphorylation of p38.

Therefore, SCLIP seems to uncouple RasGRF1 specifically from the Rac/p38 pathway. In view of the fact that the interaction takes place in the region spanning the DH domain of RasGRF1, mainly responsible for its exchange activity on Rac, we hypothesize that SCLIP may be masking the DH domain, or, alternatively, displacing RasGRF1 from its protein scaffold machinery, thus preventing it to activate Rac. The findings we present also further highlight the relevance of RasGRF1 in the signaling mechanisms to p38 MAPK, recently supported by others (10, 14).

Concomitant to these biochemical effects, we could also show the influence of SCLIP on a cellular process triggered by RasGRF1. Indeed, in PC12 cells RasGRF1 efficiently promoted the formation of neurites and exposure to NGF increased this effect. Expression of SCLIP alone failed to promote neuritogenesis and did not interfere with neurite outgrowth induced by NGF. Nevertheless, when expressed together with RasGRF1 it markedly affected its ability to promote neurite formation. This effect was observed both in unstimulated cells as well as upon exposure to NGF. Therefore, SCLIP displays selectivity in inhibiting neuritogenesis promoted by RasGRF1.

While this report was in preparation a paper by Yang and Mattingly was published reporting a requirement of co-expression of RasGRF1 with H-Ras to promote neurite outgrowth in PC12 cells (44). In addition, Robinson et al. (12) reported only a small increase in the percentage of cells with neurites upon transfection with RasGRF1. These discrepancies may reflect differences in culture conditions, particularly in the differentiation protocol (e.g. presence or absence of serum, cell density) or variability among PC12 cell lines used in the studies. For example, Yang and Mattingly did not detect expression of endogenous H-Ras, the selective target of RasGRF1, in their cells and hypothesized that this might be the reason for the requirement of its ectopic expression. Conversely, we did reveal endogenous H-Ras in our cells (data not shown), and its expression may be enough to elicit the observed effect.

It is well documented that Ras and Rac play a pivotal role in promoting neurite outgrowth. Our biochemical data may suggest that SCLIP, by down-regulating the activation of Rac by RasGRF1, may compromise the ability of cells to spread neurites. In line with our findings it has recently been shown that a novel interaction between c-Jun N-terminal kinase 1 (JNK1), another target of Rac, and members of the stathmin family, including SCLIP, seems to be involved in the regulation of the length of neurites in cortical neurons (47).

Yang and Mattingly recently reported the requirement of the Ras/Erk pathway to induce neurite extension by RasGRF1 in PC12 cells, independently of Rac. This was shown by using truncation mutants of RasGRF1, lacking one of its GEF activities, expressed in combination with H-Ras (44). Conversely, as stated above, we transfected full-length RasGRF1 without H-Ras under low serum conditions. It seems plausible that in our system a more fine balance between signals promoting neuritogenesis may exist and that perturbing a given contribution may compromise their coordination and the completion of the neurite extension program.

4 A. Colombatti, personal communication.
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Another possibility is that SCLIP might be recruited by RasGRF1 and locally perturb the dynamics of microtubules. Indeed, in the tip of neurites a highly dynamic pool of microtubules maintains structural dynamism that contributes to growth cone progression and altering this balance may compromise neurite outgrowth. This appears reasonable also, because in an effort to explore other possible RasGRF1 binding partners we recently demonstrated, by biochemical means, that RasGRF1 specifically binds microtubules (48).

A plethora of information is available regarding the involvement of Dbl-GEFs in diverse aspects of neuronal development, including neurite outgrowth and branching, axon pathfinding, spine formation, and remodeling (45, 49–52). On the other side, stathmin family proteins are regulators of microtubule dynamics, and in neurons the coordinated balance between stabilizing and destabilizing factors guarantees a certain structural flexibility of the cytoskeleton that underlies neuronal development, regeneration after damage, and plasticity. A growing body of evidence suggests their involvement in these events and, remarkably, some stathmin-like proteins are also abnormally expressed in Alzheimer’s disease (28, 30, 31, 53–57).

However, up to now, the role of SCLIP is still largely elusive, only in part inferred by analogy with other stathmin-related proteins. Here we provide new insights into the properties of SCLIP by showing a previously uncharacterized interplay with RasGRF1 and a functional effect as a negative regulator of its signaling.

Our data in PC12 cells provide a starting point and open new perspectives for further developments in the study of possible effects of these proteins in neurons. RasGRF1 is expressed only in mature, postnatal neurons, whereas SCLIP is additionally expressed in embryonic brain. Thus, the involvement of this interaction at least in early steps of neuritogenesis seems unlikely. Nevertheless, we suggest that it may have important physiological implications in structural plasticity that support remodeling of pre-existing connections in response to specific stimuli. Mice lacking RasGRF1 exhibit defects in the consolidation of long term memory in the amygdala and in the development of behavioral tolerance to chronic exposure to cannabinoids (3, 6, 7). Both experience-driven learning and chronic exposure to drugs of abuse produce persistent structural and functional reorganization in dendrites and synapses. This novel interplay may be a particularly attractive candidate for future studies on this topic and also on the function of the stathmin-related family in neurons both in normal and, potentially, in disease states.

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