THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 43, pp. 37615–37624, October 28, 2011
© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

The RhoG/ELMO1/Dock180 Signaling Module Is Required for Spine Morphogenesis in Hippocampal Neurons*

Received for publication, June 3, 2011, and in revised form, August 26, 2011 Published, JBC Papers in Press, September 7, 2011, DOI 10.1074/jbc.M111.268029

Jeong-Yoon Kim[‡1], **Mi Hee Oh**[‡], **Laura P. Bernard**[§], **Ian G. Macara**[¶], **and Huaye Zhang**[§2]

From the [‡]*Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, Korea, the* [§]*Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, University of Medicine and Dentistry, New Jersey (UMDNJ), Piscataway, New Jersey 08854, and the* [¶]*Center for Cell Signaling, University of Virginia Health System, Charlottesville, Virginia 22908*

Background: Dendritic spines are actin-rich structures that receive most of the excitatory synaptic inputs in the brain.
Results: ELMO1/Dock180 regulates spine morphogenesis through activating Rac, and RhoG functions upstream of this process.
Conclusion: A RhoG/ELMO1/Dock180 signaling module is important for spine morphogenesis in hippocampal neurons.
Significance: Our data reveal a novel role for RhoG/ELMO1/Dock180 and provide insight into the molecular mechanisms of spine morphogenesis.

Dendritic spines are actin-rich structures, the formation and plasticity of which are regulated by the Rho GTPases in response to synaptic input. Although several guanine nucleotide exchange factors (GEFs) have been implicated in spine development and plasticity in hippocampal neurons, it is not known how many different Rho GEFs contribute to spine morphogenesis or how they coordinate the initiation, establishment, and maintenance of spines. In this study, we screened 70 rat Rho GEFs in cultured hippocampal neurons by RNA interference and identified a number of candidates that affected spine morphogenesis. Of these, Dock180, which plays a pivotal role in a variety of cellular processes including cell migration and phagocytosis, was further investigated. We show that depletion of Dock180 inhibits spine morphogenesis, whereas overexpression of Dock180 promotes spine morphogenesis. ELMO1, a protein necessary for *in vivo* functions of Dock180, functions in a complex with Dock180 in spine morphogenesis through activating the Rac GTPase. Moreover, RhoG, which functions upstream of the ELMO1/Dock180 complex, is also important for spine formation. Together, our findings uncover a role for the RhoG/ELMO1/Dock180 signaling module in spine morphogenesis in hippocampal neurons.

Dendritic spines are highly specialized protrusions that form synapses with axons and receive most of the excitatory neurotransmission in the central nervous system (1, 2). Abnormalities in spine morphology and plasticity are closely associated with neurological disorders such as mental retardation, epilepsy, schizophrenia, and Alzheimer disease (3). Therefore, elucidating the molecular mechanisms by which spine morphogenesis and plasticity are regulated is crucial to understanding how information is processed and stored in the brain and the pathogenesis of various neurological diseases.

Dendritic spines are dynamic, actin-rich structures that are modulated in response to synaptic inputs (4–6). Formation and organization of the actin cytoskeleton are mediated by the Rho GTPase family members, including RhoA, Rac1, and Cdc42 (7–11). The Rho GTPases switch between an active GTP-bound form, which is generated by guanine nucleotide exchange factors (GEFs)[3] that catalyze exchange of GDP for GTP, and an inactive GDP-bound form, which is produced by GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity (12).

There are 21 members of the family of Rho GTPases in mammals but nearly 80 Rho GEFs. These GEFs can be divided into two distinct subfamilies: Dbl homology-pleckstrin homology (DH-PH) domain-containing proteins and CDM (Ced-5, Dock180 and Myoblast city) and zizimin homology (CZH) proteins (13, 14). Several Rho GEFs belonging to the Dbl homology-pleckstrin homology domain-containing subfamily, such as Tiam1, Kalirin-7, β-PIX (p21-activated kinase [PAK]-interacting exchange factor), ArhGEF6, GEFT, Lfc/GEF-H1, and intersectin, have been reported to affect spine development and plasticity in hippocampal neurons (15–23). However, it is not known how many Rho GEFs play a role in spine morphogenesis or how the Rho GEFs coordinate the initiation, establishment, and maintenance of spines.

Dock180, a prototype member of CDM and zizimin homology subfamily proteins, is an unconventional Rho GEF in that Dock180 is catalytically active toward Rac1 in cells only when it is in a complex with ELMO (24). Dock180 plays an essential role in a wide variety of biological functions including cell migration, phagocytosis of apoptotic cells, and myoblast fusion (25–29). Furthermore, Dock180 was implicated in the invasive phe-

* This work was supported, in whole or in part, by National Institutes of Health Grants GM070902 (to I. G. M.) and NS065183 (to H. Z.). This work was also supported by a National Alliance for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award (to H. Z.).

S The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. S1–S3.

[1] Recipient of financial support from Chungnam National University during sabbatical leave at the University of Virginia.

[2] To whom correspondence should be addressed: Dept. of Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes La. W., Piscataway, NJ 08854. Tel.: 732-235-3433; Fax: 732-235-4029; E-mail: zhang29@umdnj.edu.

[3] The abbreviations used are: GEF, guanine nucleotide exchange factor; DIV, days *in vitro*; EGFP, enhanced GFP; ELMO, Engulfment and Cell Motility.

notype of glioma cells (30). In neurons, Dock180 is crucial for coupling netrin stimulation to Rac1 activation (31) and is required for axon retraction/pruning following activation of ephrin-B3 reverse signaling (32). Despite the important functions of Dock180 in different types of cells in normal and pathological conditions, a role for Dock180 in spine morphogenesis has yet to be revealed.

In this study, we screened an RNAi library consisting of 70 short hairpin RNAs (shRNAs) targeting rat Rho GEFs and identified a number of candidate proteins that may be involved in spine morphogenesis. We show that Dock180 is required for spine formation and demonstrate that the RhoG/ELMO1/Dock180 signaling module plays a role in spine morphogenesis in cultured hippocampal neurons.

EXPERIMENTAL PROCEDURES

Plasmids—FLAG-Dock180, Dock180-ISP, RhoG, and ELMO constructs were kindly provided by Kodi Ravichandran and have been described previously (24, 25, 33). pCXN2-m2Venus-Dock180 was a gift from Michiyuki Matsuda. For knockdown of Dock180, ELMO1, and RhoG, oligonucleotides targeting different regions of each gene were synthesized, annealed, and inserted into pSUPER. Sequences of the oligonucleotides are as follows: Dock180 shRNA 1, 5′-gatccccCAGCAAACACCAAGAGATAttcaagagaTATCTCTTGGTGTTTGCTGtttttggaaa-3′ (forward), 5′-agcttttccaaaaaCAGCAAACACCAAGAGATAtctcttgaaTATCTCTTGGTGTTTGCTGggg-3′ (reverse); Dock180 shRNA 2, 5′-gatccccGCAGAGGAGACGAGCAATAttcaagagaTATTGCTCGTCTCCTCTGCtttttggaaa-3′ (forward), 5′-agcttttccaaaaaGCAGAGGAGACGAGCAATAtctcttgaaTATTGCTCGTCTCCTCTGCggg-3′ (reverse); Dock180 shRNA 3, 5′-gatccccCCTATAAACTGCCGGGAATttcaagagaATTCCCGGCAGTTTATAGCtttttggaaa-3′ (forward), 5′-agcttttccaaaaaCCTATAAACTGCCGGGAATtctcttgaaATTCCCGGCAGTTTATAGCggg-3′ (reverse); ELMO1 shRNA, 5′-gatccccGGAGACAGGAGATGGCTAAttcaagagaTTAGCCATCTCCTGTCTCCtttttggaaa-3′ (forward), 5′-agcttttccaaaaaGGAGACAGGAGATGGCTAAtctcttgaaTTAGCCATCTCCTGTCTCCggg-3′ (reverse); RhoG shRNA, 5′-gatccccCCACTGTGTTCGACAATTAttcaagagaTAATTGTCGAACACAGTGGtttttggaaa-3′ (forward), 5′-agcttttccaaaaaCCACTGTGTTCGACAATTAtctcttgaaTAATTGTCGAACACAGTGGggg-3′ (reverse). For generation of the Dock180 and RhoG rescue constructs, the following oligonucleotides were synthesized to make silent mutations in the constructs, rendering them resistant to the shRNAs: pCXN2-m2Venus-mtDock180, 5′-GAAGGGGCGATGAACAGTA-3′ (residues 3416–3464); and pEGFP-C3-mtRhoG, 5′-CTACCGTGTTTGATAACTA-3′ (residues 101–119).

Antibodies—Primary antibodies used in this study include rabbit polyclonal anti-Dock180 (1:1000, provided by Dr. Matsuda), goat polyclonal anti-Dock180 antibodies (1:100, N19 and C19, Santa Cruz Biotechnology), rabbit polyclonal anti-ELMO1 (1:1000, provided by Dr. Ravichandran) (24), goat polyclonal anti-ELMO1 (1:2000, Abcam), mouse monoclonal anti-β-catenin (1:3000, BD Biosciences), mouse monoclonal anti-α-tubulin (1:5000, Sigma-Aldrich), mouse monoclonal anti-SV2 (1:500, Developmental Studies Hybridoma Bank, University of Iowa), rabbit polyclonal anti-Synapsin 1 (1:500, Millipore), mouse monoclonal anti-PSD-95 (1:200, Santa Cruz Biotechnology), rabbit polyclonal anti-GFP (1:2000, Molecular Probes), mouse monoclonal anti-Cdc42 (1:250, Abcam), rabbit polyclonal anti-RhoA (1:1000, Abcam), and mouse monoclonal anti-Rac1 (1:500, Abcam). Secondary antibodies used in this study include HRP-conjugated goat anti-mouse IgG antibody, HRP-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch), Alexa Fluor 488-conjugated donkey anti-goat IgG, Texas Red-conjugated sheep anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Molecular Probes).

Neuronal Culture and Transfection—Hippocampal neuron cultures were prepared from embryonic day 19 rat embryos as described previously (34) with minor modifications. Briefly, hippocampi were dissected from embryonic day 19 rat embryos, trypsinized, and triturated through a glass Pasteur pipette. Dissociated neurons were plated on glass coverslips coated with 1 mg ml^{-1} poly-L-lysine. After initial attachment, the coverslips were transferred to dishes containing a monolayer of glia cells. Cultures were grown in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and 2 mM GlutaMAX (Invitrogen). Neurons were transfected using the calcium phosphate method as described (34).

Cerebral cortical regions were isolated and dissociated with 0.25% (v/v) trypsin digestion and trituration with a Pasteur pipette. Six-well plates coated with 1% (w/v) poly-L-lysine were seeded with 1×10^6 cells. The cells were maintained in Neurobasal medium supplemented with B27, 0.5 mM glutamine, and glucose.

Construction, Production, and Transduction of Lentiviral Vectors—Lentiviral vectors expressing shRNA for Dock180, Elmo1, or RhoG were constructed by replacing the tetO-H1 promoter region in pLVTHM (Addgene plasmid 12247 (51)) with the H1-shRNA cassette, bearing a BamHI and a ClaI site, from the pSuper-shRNA vectors. Lentiviral vectors expressing Dock180 or Elmo1 were constructed by inserting the Dock180 or Elmo1 cDNA into the NotI or BamHI/SpeI sites of pLV-Venus (35). All vectors were verified by sequencing. Lentivirus production and titering were carried out as described previously (35) using 293T cells. Hippocampal and cerebral cortical neurons cultured for 7 days were infected with the lentiviral vectors. The virus-containing medium was removed 2 h later and replaced with fresh Neurobasal medium. Three days after infection, the cultures were harvested and analyzed by Western blotting.

Small GTPase Activity Assay—Hippocampal neurons infected with the Dock180- or ELMO1-expressing lentiviral vector were harvested with ice-cold lysis buffer. The whole cell lysate was incubated overnight at 4 °C with GST-PBD (p21-binding domain from PAK, for Rac1 and Cdc42) or GST-RBD (Rho-binding domain from Rhotekin, for RhoA) immobilized to GSH beads. Bound small GTPases were separated by 15% SDS-PAGE and detected by immunoblotting with antibodies against Rac1, Cdc42, or RhoA.

Immunoprecipitation and Western Blotting—For co-immunoprecipitation experiments, hippocampal neurons at DIV 10

were lysed by lysis buffer (20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1.0% Nonidet P-40, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, and EDTA-free protease inhibitor mixture (Roche Applied Science)). Samples were centrifuged for 20 min at 15,000 rpm. Cleared lysates were incubated with anti-Dock180 antibody (2 μg) or IgG (2 μg) for 1 h at 4 °C. The immune complexes were then precipitated with protein A/G beads (Santa Cruz Biotechnology). Beads were washed three times with lysis buffer. Bound proteins were eluted with 2× Laemmli sample buffer and subjected to SDS-PAGE and Western blot analysis as described previously (22).

Immunocytochemistry—For endogenous immunostaining of Dock180 and ELMO1, hippocampal neurons were fixed in 1:1 methanol:acetone for 20 min at −20 °C. They were blocked using Roche Applied Science Western blocking reagent (diluted 1:10 in 0.2% Triton X-100 in PBS) at room temperature for 1 h. Neurons were then incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. Secondary antibodies diluted in blocking buffer were incubated with the neurons at room temperature for 1 h. Neurons were then washed with PBS and mounted using VECTASHIELD (Vector Laboratories).

Microscopy and Image Quantification—Hippocampal neurons were fixed in 4% paraformaldehyde with 4% sucrose in phosphate-buffered saline (PBS) at room temperature for 15 min. Fluorescence images were acquired using a Zeiss LSM510 confocal microscope with a 63× oil immersion lens, an Olympus FV1000 confocal microscope with a 60× water immersion lens, or an inverted epifluorescence microscope (Nikon TE-200) with a 60× water immersion lens (Plan Achromatic, NA 1.2) coupled to a CCD camera (Hamamatsu OrcaER), controlled by Openlab 5.0.1 software (Improvision, Boston, MA). Dendritic spines were defined as mushroom- or stubby-shaped protrusions contacted by presynaptic terminals. Filopodia were defined as long, thin protrusions without an enlarged spine head and not contacted by presynaptic terminals. Lamellipodia were defined as broad (width of >3 μm) protrusions. Lamellipodia were excluded in the spine head size quantifications. Spine density was measured by counting the number of spines on 15–20 neurons. Spine head size was measured using ImageJ. All experiments were repeated at least three times. A two-tailed, two-sample, unequal variance Student's t test was used to calculate the p values.

RESULTS

Dock180 Is Required for Dendritic Spine Morphogenesis in Cultured Hippocampal Neurons—The Rho GTPases are central regulators of the actin cytoskeleton, which is highly enriched in the spines. To identify potential regulators of spine morphogenesis, we generated a library of 70 shRNAs in pSUPER against the rat Rho family GEFs. A screen using the shRNA library revealed a number of interesting candidates (supplemental Fig. S1 and supplemental Table S1), including those known to be involved in spine morphogenesis, such as intersectin (16). One of the candidate proteins was Dock180, which has been known to regulate the actin cytoskeleton through Rac1 for phagocytosis, cell migration, and neuronal polarization (26, 27, 36–38). ELMO1, which forms a complex with Dock180 to activate Rac1 (24, 26), was implicated in the regulation of axonal and dendritic development (37). Thus, we decided to investigate the role for Dock180 in spine morphogenesis. First, we examined Dock180 expression levels at different stages of neuronal development and found that the protein is expressed at all developmental stages examined (Fig. 1*a*), consistent with a role in neurite extension as well as synaptogenesis. We then examined the endogenous distribution of Dock180 in cultured hippocampal neurons. Dock180 accumulated in punctate structures that partially co-localize with the excitatory synaptic marker PSD-95 (Fig. 1*b*), further indicating a potential role in synaptogenesis.

To explore a role for Dock180 in the morphogenesis of spines and synapses, we transfected hippocampal neurons with wild type Dock180 and dominant negative Dock180 (Dock180-ISP). Expression of Dock180-ISP significantly reduced spine density, whereas expression of WT Dock180 resulted in increased spine density (Fig. 1, *c* and *d*). In addition, overexpression of Dock180 resulted in the formation of many branched spines and lamellipodia-like protrusions. Quantification showed that 76% of the Dock180-overexpressing neurons form lamellipodia-like protrusions, which are defined as protrusions with a width of >3 μm. There was no significant effect of Dock180 overexpression on spine head size (Fig. 1*e*, lamellipodia-like structures are excluded in the quantification). Finally, the spines induced by Dock180 overexpression are apposed to presynaptic terminals, indicating that these spines are functional (Fig. 1*f*).

To further confirm a role for Dock180 in dendritic spine morphogenesis, we constructed new shRNAs against different regions of the Dock180 gene. shRNA 2 caused a substantial decrease in endogenous Dock180 expression in Rat2 fibroblasts (supplemental Fig. S2*a*) as well as neurons (Fig. 1*g*) and was used for subsequent experiments. Neurons transfected with shRNA 2 showed a dramatic reduction in spine density (Fig. 1, *h* and *i*), similar to the phenotype seen with our original screen. To verify that the phenotype observed was due specifically to the loss of Dock180, we constructed a Dock180 mutant (mtDock180) that harbors silent mutations, making it resistant to the shRNA construct. We then performed rescue experiments using this mtDock180. As seen in Fig. 1, *h* and *i*, mtDock180 was able to partially rescue the spine formation defect seen in the Dock180-depleted neurons, suggesting that the effect of the RNAi was specific.

ELMO1 Is Essential for Spine Formation—The binding of Dock180 to Rac1 is insufficient for GTP loading, and a Dock180-ELMO1 interaction is necessary to modulate actin cytoskeleton for phagocytosis and cell migration (24, 26). To address whether ELMO1 also plays a role in spine morphogenesis, we first examined the localization of endogenous ELMO1 in hippocampal neurons. As shown in Fig. 2*a*, endogenous ELMO1 localizes to punctate structures that co-localize with the excitatory synaptic marker PSD-95, indicating a possible role for ELMO1 in the formation of spines and their associated synapses.

To see whether ELMO1 is required for spine morphogenesis, we constructed shRNAs against ELMO1 and tested their efficiency of knockdown in Rat2 fibroblasts (supplemental Fig. S2*b*) and neurons (Fig. 2*b*). ELMO1 depletion inhibited spine

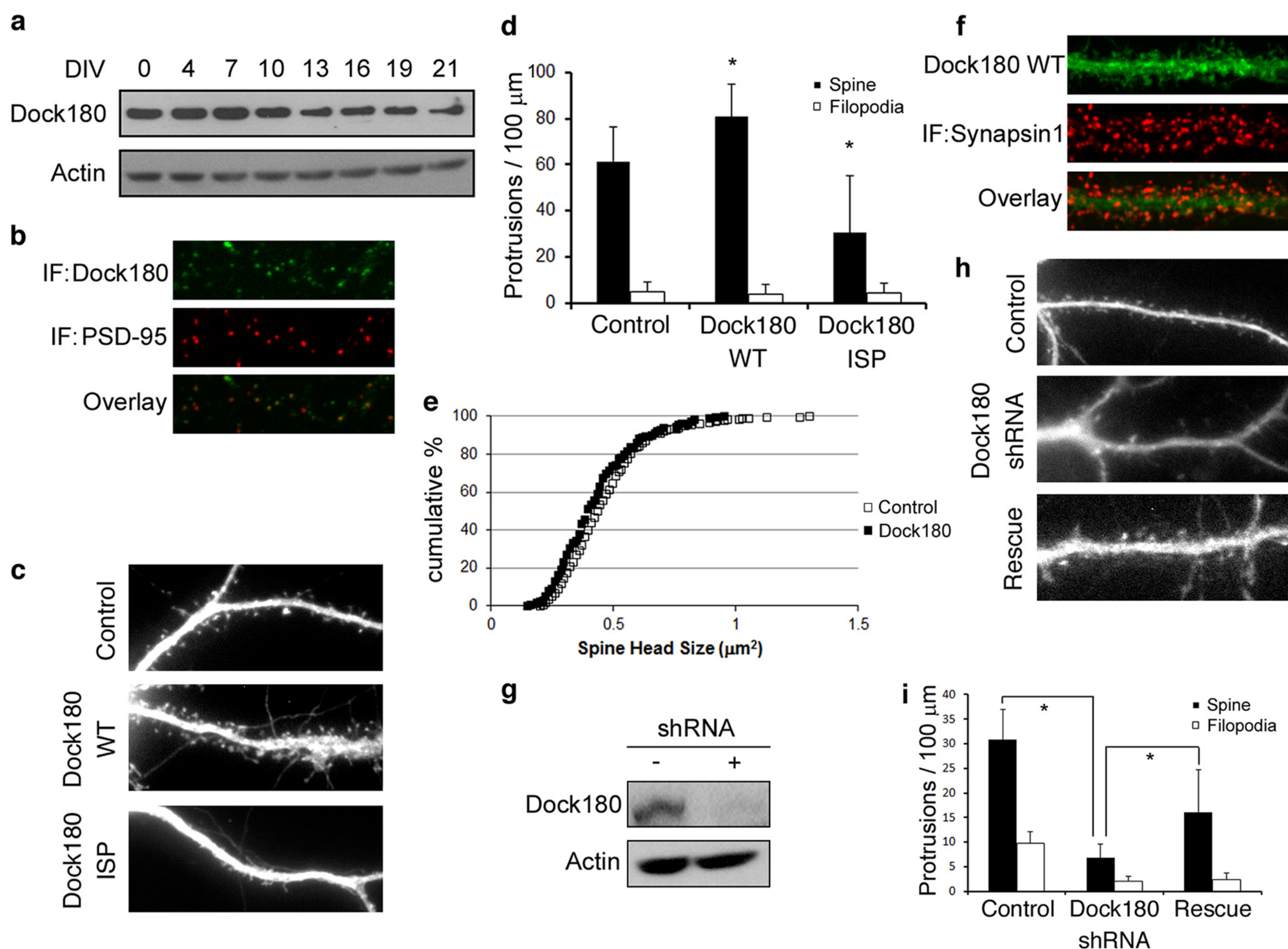

FIGURE 1. **Dock180 is necessary for spine morphogenesis in cultured hippocampal neurons.** *a*, Dock180 expression in hippocampal neurons. Cultured hippocampal neurons were harvested at the indicated days *in vitro*, and the lysates were analyzed by Western blotting. *b*, Dock180 localizes to excitatory synapses. Hippocampal neurons (DIV 27) were co-immunostained for Dock180 and PSD-95. Dock180 accumulates in puncta that partially co-localize with PSD-95. *IF*, immunofluorescence. *c*, effects of overexpression of Dock180 WT and a dominant negative form of Dock180 (*Dock180-ISP*) on spine formation. Venus (super-enhanced YFP) was co-expressed to visualize cell morphology. Overexpression of Dock180 WT causes an increase in the number of spines, but overexpression of Dock180-ISP significantly decreases spine density. Dissociated hippocampal neurons were transfected with each construct at DIV 6 and imaged at DIV 19. *d*, quantification of protrusion density in hippocampal neurons transfected with pSuper (*Control*), Dock180 WT, or Dock180-ISP. The values are means ± S.D. *, $p < 0.001$ by Student's *t* test. For each construct, images of 15–20 neurons from three independent culture preparations were analyzed. *e*, quantification of spine head size in neurons overexpressing Dock180. Lamellipodia-like protrusions (defined by protrusions with a width of >3 μm) were excluded in the quantification. *f*, spines in Dock180-overexpressing neurons are apposed to presynaptic terminals. Hippocampal neurons expressing Dock180 WT were immunostained with a Synapsin 1 antibody at DIV 17. *g*, knockdown efficiency of Dock180 shRNA. Cortical neurons were infected at DIV 7 with lentivirus expressing Dock180 shRNA 2. Seventy-two hours after infection, neurons were lysed and analyzed by Western blotting. *h*, effects of Dock180 knockdown on spine formation. Dissociated hippocampal neurons were transfected with pSuper-luciferase (*Control*), pSuper-Dock180 shRNA, or pSuper-Dock180 shRNA plus a Dock180 rescue construct at DIV 6 and imaged at DIV 17. Venus was co-expressed to visualize the spines. *i*, quantification of protrusion density in neurons transfected with pSuper-luciferase (*Control*), pSuper-Dock180 shRNA, or pSuper-Dock180 shRNA and a Dock180 rescue construct. The decreased spine density caused by Dock180 shRNA was restored by co-expressing a Dock180 rescue construct. The values are means ± S.D. *, $p < 0.001$ by Student's *t* test.

formation to a similar level as the Dock180 shRNA (Fig. 2, *c* and *d*). However, in contrast to Dock180 knockdown, ELMO1 depletion caused the formation of filopodia-like protrusions (Fig. 2, *c* and *d*). This result suggests that ELMO1 does not exert its functions entirely through Dock180.

To verify that the effect of the ELMO1 shRNA was specific, we performed rescue experiments in which we depleted endogenous ELMO1 using the shRNA construct and co-expressed mouse ELMO1 fused with GFP. Exogenously expressed GFP-ELMO1 was able to effectively rescue the defects caused by ELMO1 shRNA, showing that the observed defects were specifically due to the loss of ELMO1 (Fig. 2, *c* and *d*).

C Terminus of ELMO1 Is Sufficient to Promote Spine Formation—Next, we asked whether ELMO1 and Dock180 interact with each other to regulate spine formation through Rac1 in hippocampal neurons. To answer this question, we examined the effect of various mutant and wild type ELMO1 constructs on spine morphogenesis (Fig. 3*a*). Overexpression of wild type ELMO1 caused an increase in spine density, similar to Dock180-overexpressing neurons (Fig. 3, *b* and *c*). ELMO1 overexpression also caused a small fraction of the spines to adopt a highly branched or lamellipodia-like shape, although the effect is not as dramatic as Dock180 overexpression. Quantification demonstrated that 31% of the ELMO-overexpressing neurons show lamellipodia-like protrusions, but there was no

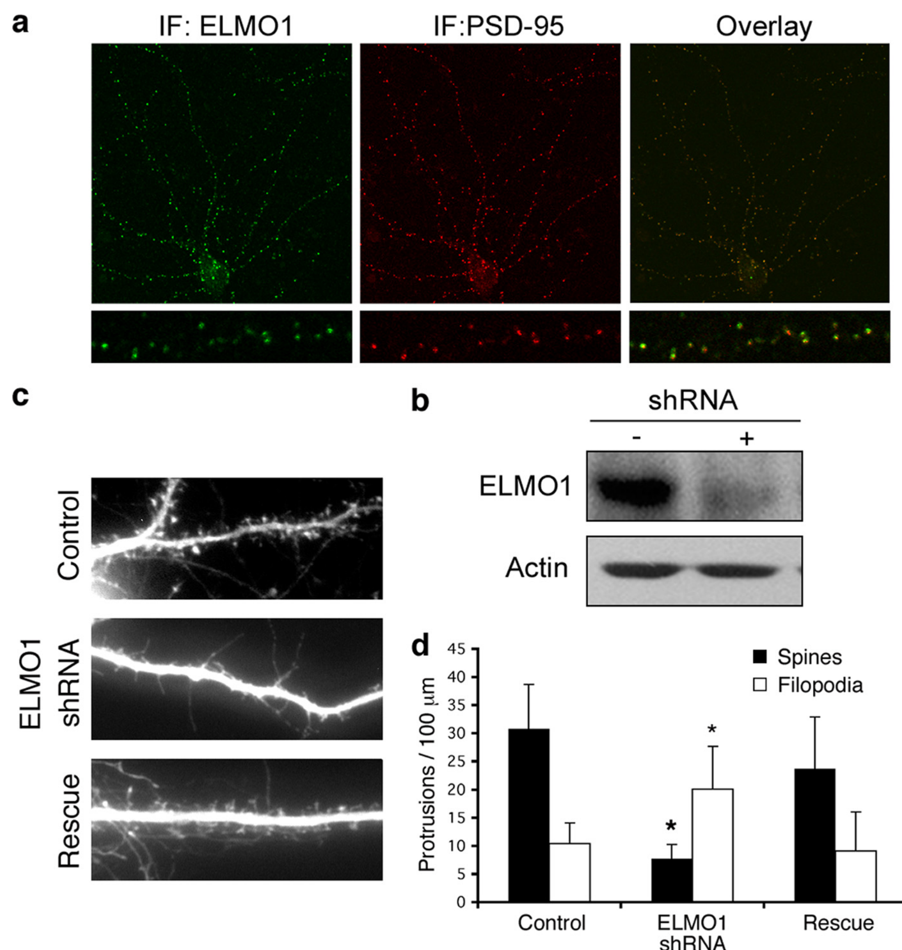

FIGURE 2. **ELMO1 localizes to excitatory synapses and is required for spine formation in hippocampal neurons.** *a*, ELMO1 localizes to excitatory synapses. Hippocampal neurons (DIV 27) were co-immunostained for ELMO1 and PSD-95. ELMO1 accumulates in puncta that co-localize with PSD-95. *IF*, immunofluorescence. *b*, knockdown efficiency of ELMO1 shRNA. Cortical neurons were infected at DIV 7 with lentivirus expressing ELMO1 shRNA. Seventy-two hours after infection, neurons were lysed and analyzed by Western blotting. *c*, effects of ELMO1 knockdown on spine formation. Hippocampal neurons were transfected with pSuper-luciferase (*Control*), pSuper-ELMO1 shRNA, or pSuper-ELMO1 shRNA and a mouse ELMO1 rescue construct at DIV 6 and imaged at DIV 17. *d*, quantification of protrusion density of neurons shown in *b*. The values are means ± S.D. *, $p < 0.001$ by Student's *t* test.

significant effect on the average spine head size when lamellipodia-like structures were excluded (Fig. 3*d*). Expression of an N-terminally deleted ELMO1 mutant (326–727) or an ARM1 mutant resulted in increased spine density, similar to overexpression of wild type ELMO1 (Fig. 3, *b* and *c*). By contrast, a C-terminally truncated ELMO1 mutant (1–280) clearly inhibited dendritic spine formation (Fig. 3, *b* and *c*). We observed a similar phenotype with a longer ELMO1 (1–558) mutant lacking the C-terminal pleckstrin homology domain and proline-rich motif (data not shown). These results suggest that the C terminus of ELMO1 is necessary for activation of Dock180 in hippocampal neurons, which is consistent with previous studies reporting that the C terminus of ELMO1 mediates the interaction with Dock180 and is required for the ELMO1/Dock180 complex to function in phagocytosis and cell migration (39–41).

We then tested whether spines in ELMO1-expressing neurons are able to form synapses. Immunostaining for the synaptic vesicle protein SV2 confirmed that these spines are apposed to presynaptic terminals, showing that ELMO1-induced spines bear synapses (Fig. 3*e*).

ELMO1 and Dock180 Form a Complex in Hippocampal Neurons and Function through the Rac1 GTPase—ELMO1 and Dock180 function as a complex in various cellular processes. To confirm that they form an endogenous complex in hippocampal neurons, we performed co-immunoprecipitation experiments using lysates from cultured hippocampal neurons. ELMO1 was specifically detected in Dock180 immunoprecipitate (Fig. 4*a*), showing that they form a complex in these neurons.

The ELMO1/Dock180 complex functions through Rac1 in various cellular contexts including migration and phagocytosis (33, 42). To see whether this complex functions through Rac1 in hippocampal neurons, we used lentivirus to overexpress ELMO1 and Dock180 in hippocampal neurons and examined the activation of Rac1, RhoA, and Cdc42, using GST pulldown assays. As seen in Fig. 4*b*, we observed a significant increase in the activation of Rac1, but no detectable activation of RhoA and Cdc42 in neurons overexpressing ELMO1 and Dock180. These data suggest that ELMO1 and Dock180 function through the Rac1 GTPase in hippocampal neurons.

To further confirm that Dock180 and ELMO1 function through Rac in regulating spine morphogenesis, we co-expressed a dominant

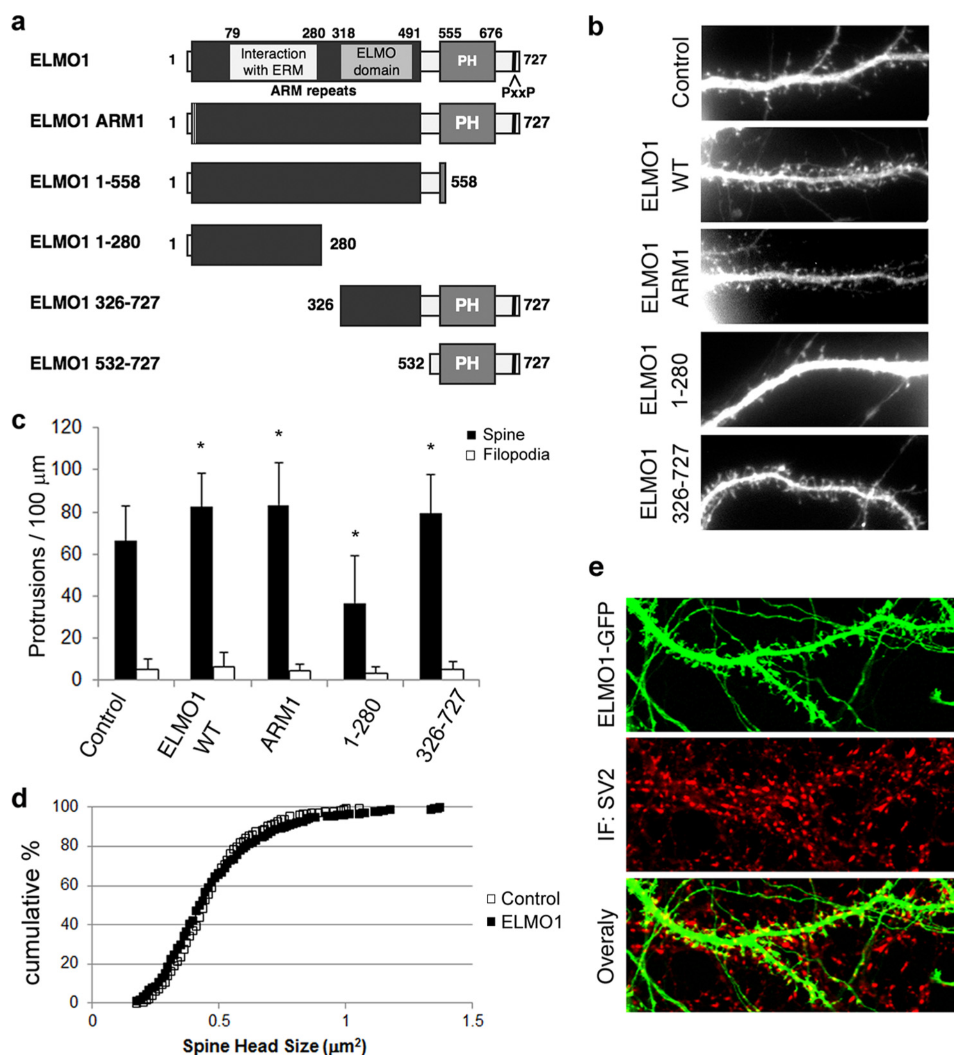

FIGURE 3. **C terminus of ELMO1 is enough to promote spine morphogenesis in hippocampal neurons.** *a*, schematic diagram of ELMO1 mutants used in this study. *PH*, pleckstrin homology; *ERM*, ezrin, radixin and moesin; *ARM*, armadillo. *b*, effects of overexpression of different ELMO1-GFP forms on spine formation. Hippocampal neurons were transfected with different ELMO1 constructs at DIV 6 and imaged at DIV 19. *c*, quantification of protrusion density in neurons overexpressing different ELMO1 mutants. The values are means $\pm$ S.D. *, $p < 0.001$ by Student's *t* test. For each construct, images of 15–20 neurons from three independent culture preparations were analyzed. *d*, quantification of spine head size in neurons overexpressing ELMO1. Lamellipodia-like protrusions (defined by protrusions with a width of >3 μm) were excluded in the quantification. *e*, spines in ELMO1-overexpressing neurons are apposed to presynaptic terminals. Hippocampal neurons transfected with wild type mouse ELMO1-GFP at DIV 6 were immunostained with an anti-SV2 antibody at DIV 19. *IF*, immunofluorescence.

negative Rac, RacN17, with either ELMO or Dock180. RacN17 reversed the phenotype of ELMO1 and Dock180 overexpression (Fig. 4, *c–f*), further showing that the ELMO/Dock180 complex functions through Rac in spine morphogenesis.

ELMO1/Dock180/Rac Functions Downstream of RhoG in Spine Morphogenesis—RhoG regulates neurite outgrowth in PC12 cells through direct interaction with ELMO (37, 43, 44) and functions upstream of ELMO/Dock180/Rac during engulfment and cell migration (25, 45). However, it was also reported that RhoG is dispensable for cell spreading and migration and could induce membrane ruffling via both Rac-dependent and Rac-independent pathways (46). Furthermore, RhoG promotes neural progenitor cell proliferation and regulates anoikis not through interaction with ELMO, but through a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism (47, 48). To investigate whether RhoG is part of the ELMO/Dock180 module that functions in spine morphogenesis, we first examined the expression levels of RhoG and ELMO1 at different developmental stages. Both RhoG and ELMO1 are expressed at low levels during neurite extension, but their levels significantly increased during the synaptogenesis period (Fig. 5*a*), consistent with a function in synaptogenesis. To investigate whether they form an endogenous complex in hippocampal neurons, we performed co-immunoprecipitation experiments using lysates from cultured hippocampal neurons. ELMO1 was specifically detected in RhoG immunoprecipitate (Fig. 5*b*), showing that they form a complex in these neurons. To explore the role of endogenous RhoG in this process, we designed shRNA constructs to deplete RhoG in hippocampal neurons. As seen in Fig. 5*c*, the shRNA construct was able to significantly reduce RhoG expression in neurons. Neurons transfected with RhoG shRNA showed dramatically reduced spine density, similar to Dock180-depleted neurons (Fig. 5, *d* and *e*). This defect was partially rescued by co-expressing a mutant RhoG that is resistant to the shRNA construct (Fig. 5, *d* and *e*, and supplemental Fig. S2*c*), indicating that the effect was specific.

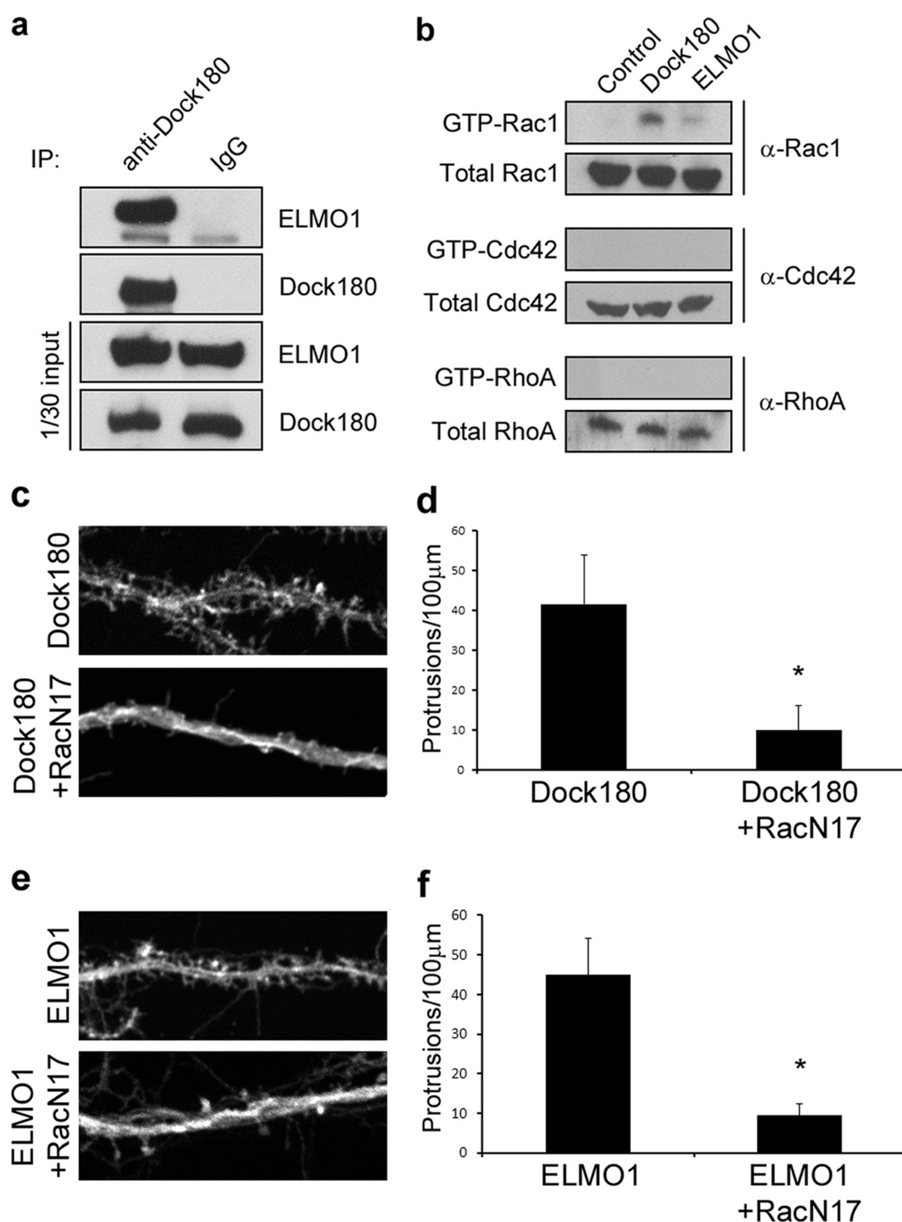

FIGURE 4. **ELMO and Dock180 form a complex in hippocampal neurons and function through activating Rac1.** *a*, co-immunoprecipitation of ELMO1 and Dock180 from hippocampal lysates. Hippocampal neurons (DIV 10) were lysed, and endogenous Dock180 was immunoprecipitated with a Dock180 antibody. The immunoprecipitated complex was analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. *IP*, immunoprecipitation. *b*, cell lysates from hippocampal neurons infected with the Dock180- or Elmo1-overexpressing lentivirus were incubated with GST-PBD or GST-RBD, and bound small GTPases were detected with antibodies against Rac1, Cdc42, and RhoA. *c*, dominant negative Rac (RacN17) reverses the Dock180 overexpression phenotype. Hippocampal neurons were transfected with Dock180 and either an empty vector or RacN17 and imaged at DIV 17. *d*, quantification of protrusion density of neurons in *c*; *, $p < 0.001$ by Student's *t* test. *e*, dominant negative Rac (RacN17) reverses the ELMO1 overexpression phenotype. Hippocampal neurons were transfected with ELMO1 and either an empty vector or RacN17 and imaged at DIV 17. *f*, quantification of protrusion density of neurons in *e*; *, $p < 0.001$ by Student's *t* test.

To further analyze the effects of RhoG in spine morphogenesis, we expressed WT RhoG, constitutively active RhoG-Q61L, and dominant negative RhoG-T17N in hippocampal neurons. The ectopic expression of RhoG-Q61L increased spine density (Fig. 6, *a* and *b*) as well as spine head size (Fig. 6*c*). There was also an increase in abnormally shaped spines. By contrast, overexpression of RhoG-T17N inhibited normal spine formation (Fig. 6, *a* and *b*). The spines induced by RhoG Q61L overexpression are apposed to presynaptic terminals (Fig. 6*d*), indicating that these spines are functional. We conclude, therefore, that RhoG functions to promote spine morphogenesis.

RhoG has been shown to have both Rac-dependent and Rac-independent functions (46–48). To determine whether RhoG functions through ELMO1/Dock180/Rac in spine morphogenesis, we depleted ELMO1 in the context of the constitutively active RhoG-Q61L. As seen in Fig. 7, *a* and *b*, we were able to reverse the RhoG-Q61L phenotype by depleting ELMO1. In addition, overexpression of ELMO1 was able to rescue the defects caused by RhoG depletion (Fig. 7, *c* and *d*). Finally, RacN17 was able to reverse the phenotype induced by RhoG Q61L (Fig. 7, *e* and *f*). Taken together, these results argue that RhoG functions upstream of ELMO1/Dock180/Rac in spine morphogenesis.

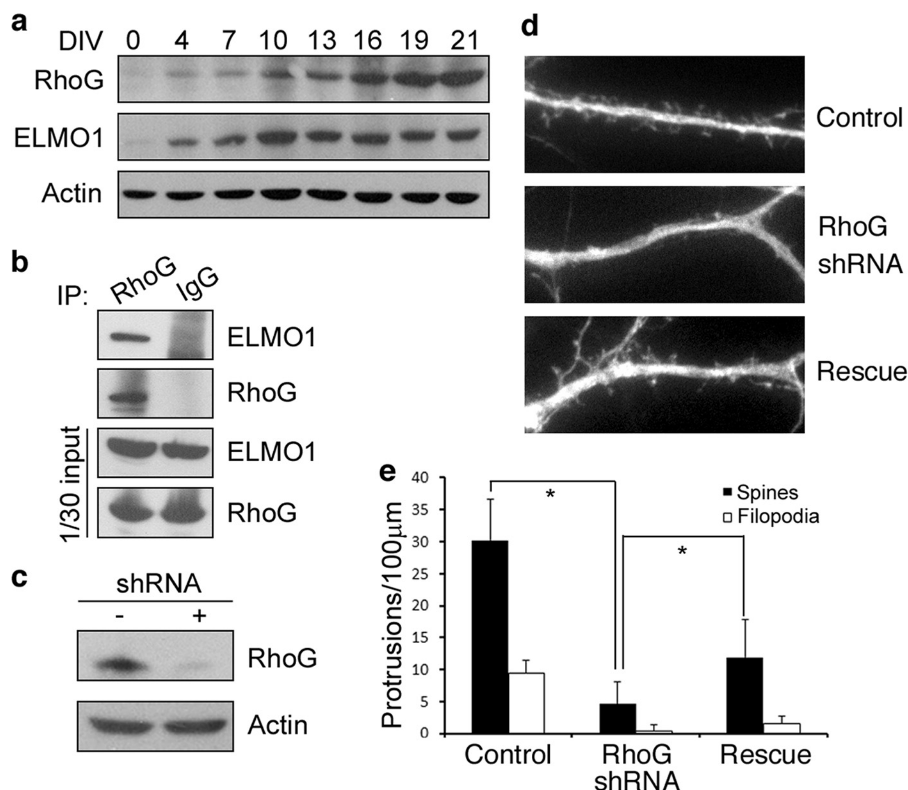

FIGURE 5. **RhoG is required for spine formation in cultured hippocampal neurons.** *a*, expression of RhoG and ELMO1 in hippocampal neurons. Cultured hippocampal neurons were harvested at the indicated days *in vitro*, and the lysates were analyzed by Western blotting. *b*, co-immunoprecipitation of RhoG and ELMO1 from hippocampal lysates. Hippocampal neurons (DIV 10) were lysed, and endogenous RhoG was immunoprecipitated with a RhoG antibody. The immunoprecipitated complex was analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. *IP*, immunoprecipitation. *c*, knockdown efficiency of RhoG shRNA. Cortical neurons were infected at DIV 7 with lentivirus expressing RhoG shRNA. Seventy-two hours after infection, neurons were lysed and analyzed by Western blotting. *d*, effects of RhoG knockdown on spine formation. Hippocampal neurons were transfected with either pSuper (*Control*) or pSuper-RhoG shRNA at DIV 6 and imaged at DIV 17. Venus was co-expressed to visualize spine morphology. Knockdown of RhoG significantly reduced the number of spines. *e*, quantification of protrusion density of neurons in *b*; *, $p < 0.001$ by Student's *t* test.

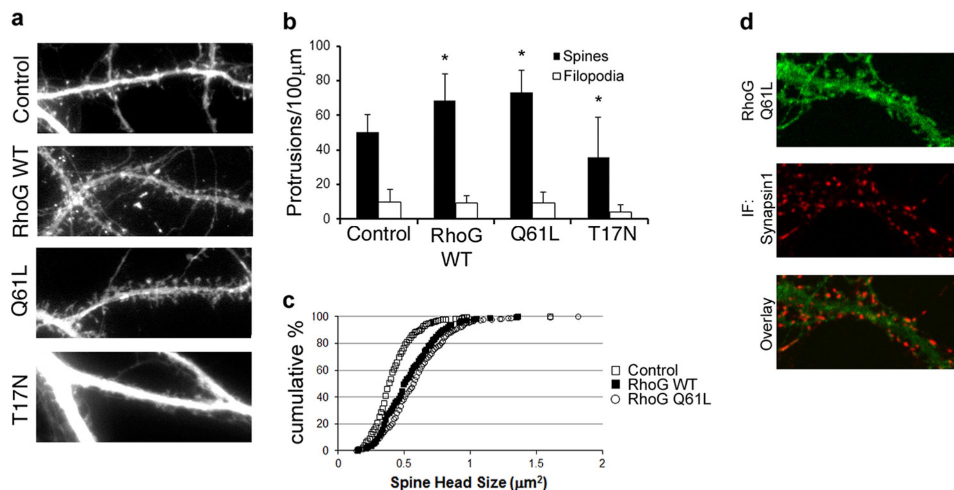

FIGURE 6. **Activation of RhoG causes increased spine density and enlargement of the spine head.** *a*, effects of overexpression of EGFP wild type RhoG, a dominant negative EGFP-RhoG T17N, and a constitutively active EGFP-RhoG Q61L on spine formation. Hippocampal neurons were transfected with pSuper, EGFP-RhoG Q61L, or EGFP-RhoG T17N at DIV 6 and imaged at DIV 19. *b*, quantification of protrusion density in neurons expressing EGFP-RhoG Q61L or EGFP-RhoG T17N. The values are means ± S.D. *, $p < 0.001$ by Student's *t* test. For each construct, images of 15–20 neurons from three independent culture preparations were analyzed. *c*, quantification of spine head size in neurons overexpressing RhoG Q61L. *d*, spines in RhoG Q61L-overexpressing neurons are apposed to presynaptic terminals. Hippocampal neurons expressing RhoG Q61L were immunostained with a Synapsin 1 antibody at DIV 17. *IF*, immunofluorescence.

DISCUSSION

Our data reveal a signaling module, consisting of RhoG, ELMO1, and Dock180, which functions in spine morphogenesis. The ELMO1/Dock180 complex has been shown to regulate a number of Rac-dependent processes including cell migration and phagocytosis (24, 26). In addition, RhoG has been shown to function in both a Rac-dependent and a Rac-independent manner (46). Here we identify a novel function for ELMO1/

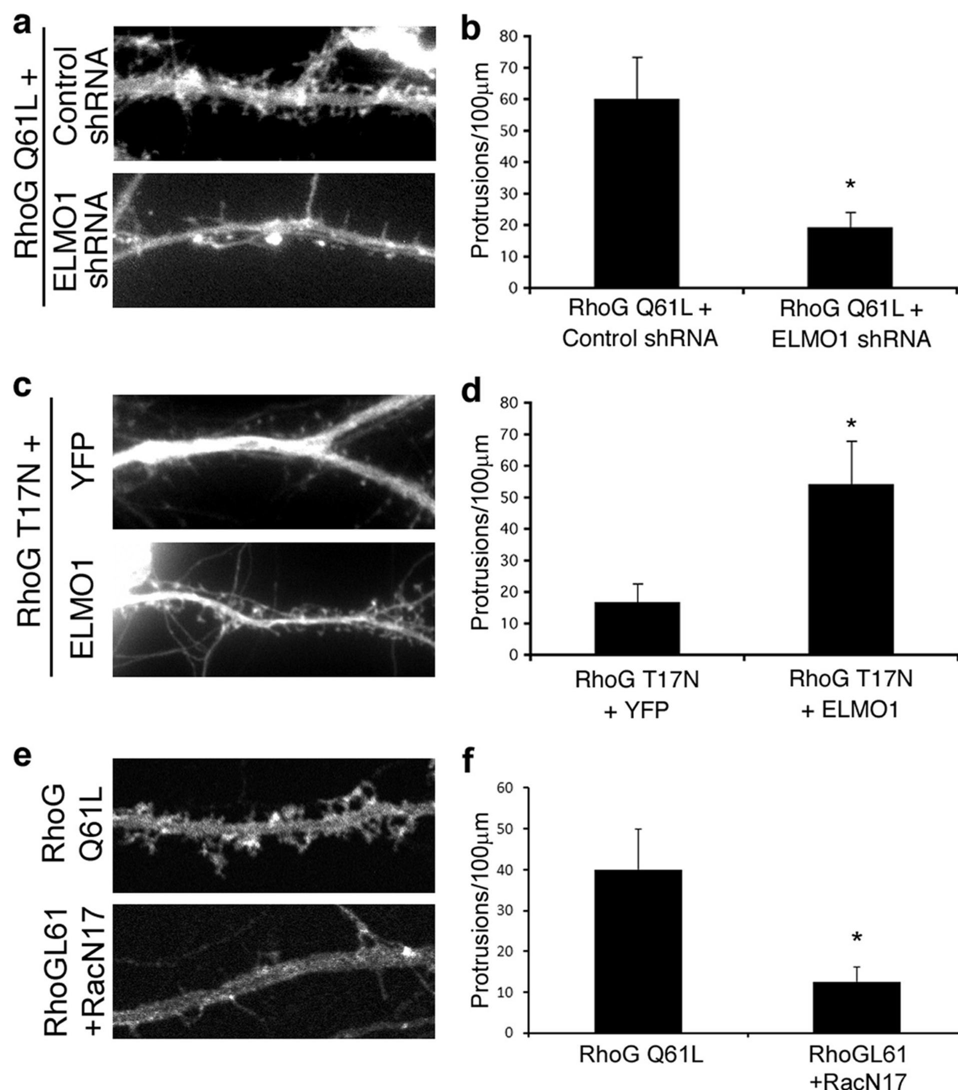

FIGURE 7. **RhoG functions through the ELMO1/Dock180/Rac pathway.** *a*, ELMO1 depletion reverses the RhoG Q61L overexpression phenotype. Hippocampal neurons were transfected with RhoG Q61L and either a control luciferase shRNA or ELMO1 shRNA at DIV 6 and imaged at DIV 17. *b*, quantification of protrusion density of neurons in *a*; *, $p < 0.001$ by Student's *t* test. *c*, ELMO1 overexpression rescues the RhoG T17N phenotype. Hippocampal neurons were transfected with RhoG T17N and either Venus (super-enhanced YFP) or ELMO1. Neurons were transfected at DIV 6 and imaged at DIV 17. *d*, quantification of protrusion density of neurons in *c*; *, $p < 0.001$ by Student's *t* test. *e*, dominant negative Rac (RacN17) reverses the RhoG Q61L overexpression phenotype. Hippocampal neurons were transfected with RhoG Q61L and either an empty vector or RacN17 and imaged at DIV 17. *f*, quantification of protrusion density of neurons in *e*; *, $p < 0.001$ by Student's *t* test.

Dock180 in regulating dendritic spine morphogenesis and show that RhoG functions upstream of this process. Our domain analysis shows that the C terminus of ELMO1, which mediates the interaction with Dock180, is sufficient to promote spine morphogenesis. This indicates that ELMO1 functions through regulating Dock180 in this process. Furthermore, the N terminus of ELMO1, which mediates the interaction with active RhoG, caused a reduction in spine density. This suggests that the N terminus of ELMO1 functions as a dominant negative by binding RhoG but preventing the downstream activation of Dock180 and Rac. Taken together, these data suggest that RhoG, ELMO1, and Dock180 function as a signaling module to regulate spine morphogenesis, with RhoG being upstream of the ELMO1/Dock180 complex.

Interestingly, we observed reproducible differences in phenotypes in RhoG- or ELMO1-depleted *versus* Dock180-depleted neurons. For example, ELMO1 depletion caused a reduction in spine number along with the formation of filopodia-like protrusions; however, we did not observe filopodia-like protrusions in RhoG- or Dock180-depleted neurons. In addition, Dock180 knockdown caused a reduction in spine density as well as in dendrite branching, whereas RhoG or ELMO1 depletion did not have any effect on dendrite branching (supplemental Fig. S3). Thus, it is likely that the separate components of this signaling module are each involved in additional pathways. This is consistent with our finding that Dock180 and RhoG/ELMO1 exhibit differential developmental expression profiles (Figs. 1*c* and 4*a*).

Our results show that activating RhoG caused an enlargement of the spine head (Fig. 6*c*), whereas overexpression of ELMO1 and Dock180 caused the formation of lamellipodia-like protrusions, without changing the size of the remaining spines (Figs. 1*e* and 3*d*). It is likely that RhoG Q61L caused downstream activation of endogenous ELMO1 and Dock180, which are correctly localized

to dendritic spines. Thus, activation of RhoG would cause local activation of Rac in the dendritic spines, leading to spine head enlargement. By contrast, overexpression of ELMO1 and Dock180 would cause a fraction of the proteins to mislocalize outside the spines, leading to more global activation of Rac and the formation of lamellipodia-like structures.

The morphogenesis and plasticity of dendritic spines are critical for cognitive functions such as learning and memory. It is generally believed that activity-dependent spine structural plasticity plays an important role in memory processes; however, the underlying molecular mechanisms still remain unclear. Thus, it would be of great interest to elucidate the upstream regulators of the RhoG/ELMO1/Dock180 module. Possible candidates include neurotransmitter receptors and other cell surface receptors. Further investigation into the upstream pathways will shed more light on the function of this module in spine morphogenesis and potentially activity-dependent structural plasticity of spines.

Our finding of a role for the RhoG/ELMO1/Dock180 complex in dendritic spine morphogenesis adds to the ever growing network of signaling molecules that function in this process. Several other Rho family GEFs are also known to be involved in spine morphogenesis. These include Kalirin-7, β-PIX (p21-activated kinase [PAK]-interacting exchange factor), Tiam1, intersectin, and GEFT (15, 16, 19, 21–23, 49). Further studies are needed to examine the role of these Rho GEFs in different brain regions and types of neurons, as well as different stages of neuronal development. Within the same neuron, these Rho GEFs are likely involved in the spatial and temporal regulations of Rho GTPase activity. Consistent with this, highly localized Rho GTPase activation has been recently observed in hippocampal neurons (23, 50), which is associated with spine structural plasticity. How the convergent signals from different Rho GEFs spatially and temporally regulate Rho GTPase activity awaits further study.

Acknowledgments—We thank Drs. Kodi Ravichandran, Martin Schwartz, and Michiyuki Matsuda for reagents.

REFERENCES

1. Hering, H., and Sheng, M. (2001) *Nat. Rev. Neurosci.* **2,** 880–888
2. Sheng, M., and Hoogenraad, C. C. (2007) *Annu. Rev. Biochem.* **76,** 823–847
3. Fiala, J. C., Spacek, J., and Harris, K. M. (2002) *Brain Res. Brain Res. Rev.* **39,** 29–54
4. Bonhoeffer, T., and Yuste, R. (2002) *Neuron* **35,** 1019–1027
5. Cingolani, L. A., and Goda, Y. (2008) *Nat. Rev. Neurosci.* **9,** 344–356
6. Matus, A. (2000) *Science* **290,** 754–758
7. Govek, E. E., Newey, S. E., and Van Aelst, L. (2005) *Genes Dev.* **19,** 1–49
8. Nakayama, A. Y., and Luo, L. (2000) *Hippocampus* **10,** 582–586
9. Raftopoulou, M., and Hall, A. (2004) *Dev. Biol.* **265,** 23–32
10. Tada, T., and Sheng, M. (2006) *Curr. Opin. Neurobiol.* **16,** 95–101
11. Tashiro, A., Minden, A., and Yuste, R. (2000) *Cereb. Cortex* **10,** 927–938
12. Schmidt, A., and Hall, A. (2002) *Genes Dev.* **16,** 1587–1609
13. Meller, N., Irani-Tehrani, M., Kiosses, W. B., Del Pozo, M. A., and Schwartz, M. A. (2002) *Nat. Cell Biol.* **4,** 639–647
14. Rossman, K. L., Der, C. J., and Sondek, J. (2005) *Nat. Rev. Mol. Cell Biol.* **6,** 167–180
15. Bryan, B., Kumar, V., Stafford, L. J., Cai, Y., Wu, G., and Liu, M. (2004) *J. Biol. Chem.* **279,** 45824–45832
16. Irie, F., and Yamaguchi, Y. (2002) *Nat. Neurosci.* **5,** 1117–1118
17. Kang, M. G., Guo, Y., and Huganir, R. L. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 3549–3554
18. Nodé-Langlois, R., Muller, D., and Boda, B. (2006) *J. Cell Sci.* **119,** 4986–4993
19. Penzes, P., Johnson, R. C., Sattler, R., Zhang, X., Huganir, R. L., Kambampati, V., Mains, R. E., and Eipper, B. A. (2001) *Neuron* **29,** 229–242
20. Ryan, X. P., Alldritt, J., Svenningsson, P., Allen, P. B., Wu, G. Y., Nairn, A. C., and Greengard, P. (2005) *Neuron* **47,** 85–100
21. Tolias, K. F., Bikoff, J. B., Burette, A., Paradis, S., Harrar, D., Tavazoie, S., Weinberg, R. J., and Greenberg, M. E. (2005) *Neuron* **45,** 525–538
22. Zhang, H., and Macara, I. G. (2006) *Nat. Cell Biol.* **8,** 227–237
23. Zhang, H., Webb, D. J., Asmussen, H., Niu, S., and Horwitz, A. F. (2005) *J. Neurosci.* **25,** 3379–3388
24. Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) *Nat. Cell Biol.* **4,** 574–582
25. deBakker, C. D., Haney, L. B., Kinchen, J. M., Grimsley, C., Lu, M., Klingele, D., Hsu, P. K., Chou, B. K., Cheng, L. C., Blangy, A., Sondek, J., Hengartner, M. O., Wu, Y. C., and Ravichandran, K. S. (2004) *Curr. Biol.* **14,** 2208–2216
26. Gumienny, T. L., Brugnera, E., Tosello-Trampont, A. C., Kinchen, J. M., Haney, L. B., Nishiwaki, K., Walk, S. F., Nemergut, M. E., Macara, I. G., Francis, R., Schedl, T., Qin, Y., Van Aelst, L., Hengartner, M. O., and Ravichandran, K. S. (2001) *Cell* **107,** 27–41
27. Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., Shibuya, M., Kurata, T., and Matsuda, M. (1996) *Mol. Cell Biol.* **16,** 1770–1776
28. Laurin, M., Fradet, N., Blangy, A., Hall, A., Vuori, K., and Côté, J. F. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 15446–15451
29. Nolan, K. M., Barrett, K., Lu, Y., Hu, K. Q., Vincent, S., and Settleman, J. (1998) *Genes Dev.* **12,** 3337–3342
30. Jarzynka, M. J., Hu, B., Hui, K. M., Bar-Joseph, I., Gu, W., Hirose, T., Haney, L. B., Ravichandran, K. S., Nishikawa, R., and Cheng, S. Y. (2007) *Cancer Res.* **67,** 7203–7211
31. Li, X., Gao, X., Liu, G., Xiong, W., Wu, J., and Rao, Y. (2008) *Nat. Neurosci.* **11,** 28–35
32. Xu, N. J., and Henkemeyer, M. (2009) *Nat. Neurosci.* **12,** 268–276
33. Grimsley, C. M., Kinchen, J. M., Tosello-Trampont, A. C., Brugnera, E., Haney, L. B., Lu, M., Chen, Q., Klingele, D., Hengartner, M. O., and Ravichandran, K. S. (2004) *J. Biol. Chem.* **279,** 6087–6097
34. Zhang, H., Webb, D. J., Asmussen, H., and Horwitz, A. F. (2003) *J. Cell Biol.* **161,** 131–142
35. McCaffrey, L. M., and Macara, I. G. (2009) *Genes Dev.* **23,** 1450–1460
36. Albert, M. L., Kim, J. I., and Birge, R. B. (2000) *Nat. Cell Biol.* **2,** 899–905
37. Katoh, H., and Negishi, M. (2003) *Nature* **424,** 461–464
38. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) *Genes Dev.* **12,** 3331–3336
39. Komander, D., Patel, M., Laurin, M., Fradet, N., Pelletier, A., Barford, D., and Côté, J. F. (2008) *Mol. Biol. Cell* **19,** 4837–4851
40. Lu, M., Kinchen, J. M., Rossman, K. L., Grimsley, C., deBakker, C., Brugnera, E., Tosello-Trampont, A. C., Haney, L. B., Klingele, D., Sondek, J., Hengartner, M. O., and Ravichandran, K. S. (2004) *Nat. Struct. Mol. Biol.* **11,** 756–762
41. Lu, M., Kinchen, J. M., Rossman, K. L., Grimsley, C., Hall, M., Sondek, J., Hengartner, M. O., Yajnik, V., and Ravichandran, K. S. (2005) *Curr. Biol.* **15,** 371–377
42. Ravichandran, K. S., and Lorenz, U. (2007) *Nat. Rev. Immunol.* **7,** 964–974
43. Estrach, S., Schmidt, S., Diriong, S., Penna, A., Blangy, A., Fort, P., and Debant, A. (2002) *Curr. Biol.* **12,** 307–312
44. Katoh, H., Yasui, H., Yamaguchi, Y., Aoki, J., Fujita, H., Mori, K., and Negishi, M. (2000) *Mol. Cell Biol.* **20,** 7378–7387
45. Katoh, H., Hiramoto, K., and Negishi, M. (2006) *J. Cell Sci.* **119,** 56–65
46. Meller, J., Vidali, L., and Schwartz, M. A. (2008) *J. Cell Sci.* **121,** 1981–1989
47. Fujimoto, S., Negishi, M., and Katoh, H. (2009) *Mol. Biol. Cell* **20,** 4941–4950
48. Yamaki, N., Negishi, M., and Katoh, H. (2007) *Exp. Cell Res.* **313,** 2821–2832
49. Ma, X. M., Kiraly, D. D., Gaier, E. D., Wang, Y., Kim, E. J., Levine, E. S., Eipper, B. A., and Mains, R. E. (2008) *J. Neurosci.* **28,** 12368–12382
50. Murakoshi, H., Wang, H., and Yasuda, R. (2011) *Nature* **472,** 100–104
51. Wiznerowicz, M., and Trono, D. (2003) *J. Virol.* **77,** 8957–8961