

The Guanine Nucleotide Exchange Factor (GEF) Asef2 Promotes Dendritic Spine Formation via Rac Activation and Spinophilin-dependent Targeting*

Received for publication, August 17, 2014, and in revised form, March 4, 2015. Published, JBC Papers in Press, March 6, 2015, DOI 10.1074/jbc.M114.605543

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Background: The role of Rho family GEFs in dendritic spine formation is currently not well understood.

Results: The Rho family GEF Asef2 promotes spine and synapse formation through activation of Rac and spinophilin-mediated localization to spines.

Conclusion: Asef2 is a critical regulator of spines and synapses.

Significance: Asef2-spinophilin signaling is an important, new mechanism for inducing spine and synapse development.

Dendritic spines are actin-rich protrusions that establish excitatory synaptic contacts with surrounding neurons. Reorganization of the actin cytoskeleton is critical for the development and plasticity of dendritic spines, which is the basis for learning and memory. Rho family GTPases are emerging as important modulators of spines and synapses, predominantly through their ability to regulate actin dynamics. Much less is known, however, about the function of guanine nucleotide exchange factors (GEFs), which activate these GTPases, in spine and synapse development. In this study we show that the Rho family GEF Asef2 is found at synaptic sites, where it promotes dendritic spine and synapse formation. Knockdown of endogenous Asef2 with shRNAs impairs spine and synapse formation, whereas exogenous expression of Asef2 causes an increase in spine and synapse density. This effect of Asef2 on spines and synapses is abrogated by expression of GEF activity-deficient Asef2 mutants or by knockdown of Rac, suggesting that Asef2-Rac signaling mediates spine development. Because Asef2 interacts with the F-actin-binding protein spinophilin, which localizes to spines, we investigated the role of spinophilin in Asef2-promoted spine formation. Spinophilin recruits Asef2 to spines, and knockdown of spinophilin hinders spine and synapse formation in Asef2-expressing neurons. Furthermore, inhibition of N-methyl-D-aspartate receptor (NMDA) activity blocks spinophilin-mediated localization of Asef2 to spines. These results collectively point to spinophilin-Asef2-Rac signaling as a novel mechanism for the development of dendritic spines and synapses.

Neurons form cell-cell junctions called synapses that comprise pre- and postsynaptic terminals that propagate signals from one neuron to another. Most excitatory synapses form on dendritic spines, which are postsynaptic protrusive structures enriched in actin (1–3). Spines display a wide range of morphologies, from immature, filopodia-like protrusions to more mature protrusions composed of a mushroom-shaped spine head and a thin neck (4–6). Spine development and plasticity are essential for normal cognitive function and underlie processes such as learning and memory. Abnormalities in spine formation and morphology are associated with numerous neurological and intellectual disorders, including autism, schizophrenia, epilepsy, Fragile X syndrome, and Alzheimer's disease (7, 8), underscoring the importance of these structures in cognition.

The development and morphological plasticity of dendritic spines is associated with the assembly and disassembly of actin filaments (9–11). Actin organization, in turn, is tightly regulated by the Rho family of small GTPases, including Rac, Cdc42, and Rho (12). Like other small GTPases, the Rho GTPases cycle between an active (GTP-bound) form and an inactive (GDP-bound) form. This cycling is regulated by two families of proteins: GEFs,³ which catalyze the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), which promote GTP hydrolysis (13–15). Although the roles of Rac, Cdc42, and Rho in modulating spine and synapse formation have been characterized (16–20), much less is known about the GEFs and GAPs that regulate them. Recent work, however, suggests that these proteins play a critical role in spine development (21). For example, the Rac GEF Tiam1 has been shown to mediate spine morphogenesis through its association with NMDA-type glutamate receptors, Eph receptors, and the polarity protein PAR-3 (22–24). In addition, the Rho GAP oligophrenin-1 regulates the plasticity and maturation of spines and synapses, and loss of function of this protein is associated with some intellec-

* This work was supported, in whole or in part, by National Institutes of Health Grants GM092914 and MH071674 and by National Center for Research Resources Grant S10RR025524 (to D. J. W.).

¹ Supported by National Institutes of Health Predoctoral Training Grant GM008554.

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³ The abbreviations used are: GEF, guanine nucleotide exchange factor; AP5, DL-2-amino-5-phosphonopentanoic acid; DIV, day *in vitro*; GAP, GTPase activating protein; PSD95, postsynaptic density protein 95; R2F, rat 2 fibroblast; SV2, synaptic vesicle protein 2; TRITC, tetramethylrhodamine isothiocyanate; NT shRNA, non-targeting shRNA.

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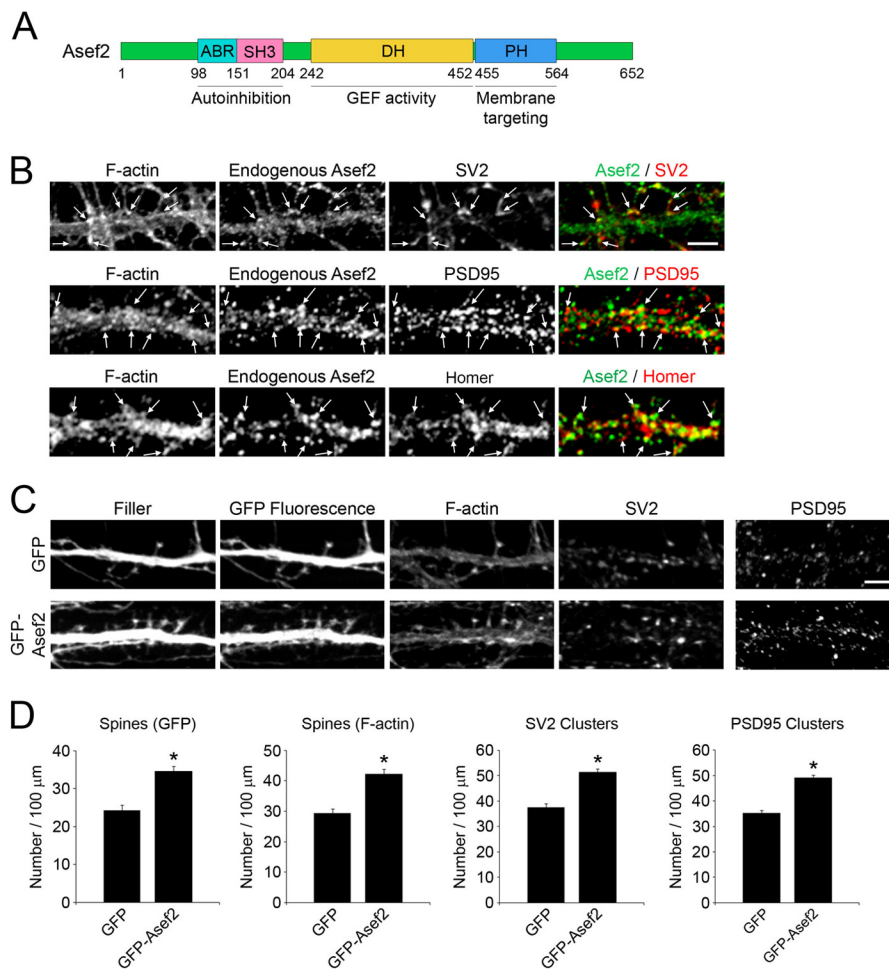


FIGURE 1. Asef2 promotes the formation of dendritic spines and synapses. *A*, schematic of Asef2 showing the conserved domains. The domain numbering is based on Kawasaki *et al.* (29). *ABR*, adenomatous polyposis coli binding region; *SH3*, Src homology 3 domain; *DH*, Dbl homology domain; *PH*, pleckstrin homology domain. *B*, hippocampal neurons were fixed at DIV14 and stained for endogenous Asef2, F-actin with phalloidin, and either SV2 (*upper panels*), PSD95 (*middle panels*), or Homer (*lower panels*). Overlays of the images show that Asef2 puncta are found with the synaptic markers (*right panels, arrows*). *Bar*, 5 μ m. *C*, DIV5 neurons were co-transfected with mCerulean (*Filler*) and either GFP or GFP-Asef2, then fixed and stained for F-actin (phalloidin) and SV2 or PSD95 at DIV11. *Bar*, 5 μ m. *D*, quantification of spine density using either GFP fluorescence or F-actin (phalloidin) staining as well as synapse density (SV2 and PSD95 clusters) in GFP- and GFP-Asef2-expressing neurons. *Error bars* represent S.E. for 40–72 dendrites from five independent experiments (*, $p < 0.001$).

tual disorders (25–27). Therefore, these studies highlight the importance of GEFs and GAPs in regulating spine and synapse function and point to a need to investigate the contribution of other GEFs and GAPs to these processes.

Asef2 (SPATA13, FLJ31208) is a 652-amino acid GEF that activates Rac and Cdc42 (28, 29). Asef2 is composed of several conserved domains that include an adenomatous polyposis coli binding region, a Src homology 3 domain, a Dbl homology domain, and a pleckstrin homology domain (28) (Fig. 1A). The Dbl homology domain promotes GTP exchange, whereas the pleckstrin homology domain contributes to membrane localization (28–31). The adenomatous polyposis coli binding region and Src homology 3 domains work together to modulate the activation state of Asef2 (28). The autoinhibited form of Asef2 prohibits nucleotide exchange via the Dbl homology domain; however, binding of the tumor suppressor adenomatous polyposis coli to the adenomatous polyposis coli binding region-Src homology 3 tandem induces a conformational change in Asef2, thus stimulating the GEF activity of this protein (28, 32). In the mammalian brain, Asef2 is expressed in

various regions, including the cerebral cortex, the amygdala, the olfactory bulb, and the hippocampus (33–36), but its function in the central nervous system is currently not known.

Although most of the Asef2 binding partners remain to be identified, Asef2 has been shown to interact with the actin-binding protein spinophilin (neurabin II) (37). Spinophilin is highly expressed in the brain, and it localizes to dendritic spines in hippocampal neurons through an N-terminal F-actin binding domain (38–41). Spinophilin has been shown to regulate the formation and morphology of dendritic spines and to modulate glutamatergic synaptic transmission (42). Moreover, spinophilin knock-out mice display defects in associative learning (43), further emphasizing the importance of this protein in regulating synaptic function. Spinophilin could mediate these effects on spines and synapses at least in part through its interaction with proteins such as Asef2. This led us to investigate the role of Asef2 in the development of dendritic spines and synapses.

In this study, we show that spinophilin recruits Asef2 to synaptic sites. Asef2, in turn, promotes the formation of dendritic

spines and synapses in hippocampal neurons by a Rac-dependent signaling mechanism. These results indicate that spinophilin-Asef2-Rac signaling is important in spine and synapse development.

EXPERIMENTAL PROCEDURES

Reagents—Asef2 polyclonal antibody was made by 21st Century Biochemicals (Marlboro, MA), as previously described (30). Spinophilin polyclonal antibody was obtained from Novus Biologicals (Littleton, CO). SV2 monoclonal antibody was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). PSD95 monoclonal antibody (clone 7E3-1B8) was purchased from EMD Millipore (Billerica, MA). Homer 1b/c polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). FLAG monoclonal antibody (clone M2) and DL-2-amino-5-phosphonopentanoic acid (AP5) were obtained from Sigma. Alexa Fluor® 488 anti-rabbit, Alexa Fluor® 555, 647, and 680 anti-mouse (for Western blotting), Alexa Fluor® 546 phalloidin, and ProLong® Gold antifade reagent were purchased from Life Technologies. Aqua-Poly/Mount was from Polysciences, Inc. (Warrington, PA).

Plasmids—Full-length human Asef2 cDNA tagged with enhanced green fluorescent protein (EGFP, from Clontech, Mountain View, CA) (30) was inserted into a neuronal expression vector (pTαS2) that contains the neuron-specific α 1-tubulin promoter (44). This vector was kindly provided by Freda Miller (University of Toronto, Toronto, Ontario). Asef2 GEF activity-deficient mutants were generated by site-directed mutagenesis using the following primers: Asef2-K382A, forward (5'-CTCACACCAGTGCAGGCGATCTGCAAATAC-3') and reverse (5'-GTATTTGCAGATCGCCTGCACTGGTGTGAG-3'); Asef2-K385A, forward (5'-CAGAAGATCTGCGCATACCCGCTGCAG-3') and reverse (5'-CTGCAGCGGTATGCGCAGATCTTCTG-3'). Asef2 short hairpin RNA (shRNA) constructs were created by inserting 64-mer sense and antisense oligonucleotides into the pSUPER vector as described previously (45). The oligonucleotides contained the following target sequences: Asef2 shRNA #1 (5'-CCAGCAGATGATCGATATA-3') and Asef2 shRNA #2 (5'-GCGACTACAACAATATAAAA-3'). A non-targeting shRNA was constructed by inserting the sequence 5'-CAGTCGCGTTTTCGACTGG-3' into the pSUPER vector. Similarly, Rac shRNAs were generated using the following target sequences: Rac shRNA #1 (5'-GTGGTATCCTGAAGTACGA-3') and Rac shRNA #2 (5'-GCAAACAGACGTGTTCTTA-3'). shRNAs targeting spinophilin were made using the following target sequences: spinophilin shRNA #1 (5'-GGACTATGACCGACGCAAT-3'), spinophilin shRNA #2 (5'-AGGAGAATAAGGAGCGGAT-3'), and spinophilin shRNA #3 (5'-ACAAGGACCTGCAGACCAA-3'). Rac1 cDNA and glutathione S-transferase (GST)-tagged p21 binding domain cDNA were generously provided by Alan Hall (Memorial Sloan-Kettering Cancer Center, New York, NY). mCherry cDNA was a kind gift from Roger Tsien (University of California, San Diego, CA). Spinophilin cDNA, kindly provided by Roger Colbran (Vanderbilt University, Nashville, TN), was inserted into pTαS2 vector containing mCherry. The C-terminal deletion mutant of spinophilin, which was previously described (37), was kindly

provided by Tetsu Akiyama (University of Tokyo, Tokyo, Japan) and cloned into pTαS2-mCherry. The Asef2 C-terminal deletion mutant, which was previously described (37), was prepared by PCR and cloned into pTαS2-GFP. mCerulean cDNA, which was a generous gift from David Piston (Vanderbilt University, Nashville, TN), was inserted into pTαS2 vector.

Cell Culture and Transfection—Hippocampal neurons were isolated from day 19 rat embryos and were cultured at low density using an established protocol (46). At day *in vitro* (DIV) 5, neurons were transfected using a modified calcium phosphate protocol (20). Rat 2 fibroblasts (R2Fs) and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) that was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and penicillin/streptomycin (Life Technologies). HT1080 cells were transfected using Lipofectamine® 2000 (Life Technologies), and R2Fs were transfected using an Amaxa Nucleofector™ kit (Lonza, Cologne, Germany) according to the manufacturer's instructions.

Immunocytochemistry—For SV2 and phalloidin staining, neurons were fixed with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) for 15 min at room temperature. For PSD95 staining, neurons were fixed with paraformaldehyde/sucrose for 3 min at room temperature followed by ice-cold methanol for 10 min. Separate sets of neurons were used to stain for SV2 and PSD95. For endogenous protein staining, neurons were fixed with either paraformaldehyde/sucrose for 3 min and then cold 10% formalin for 10 min, or paraformaldehyde/sucrose for 15 min. After 3 washes in PBS, coverslips were permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed 3 times again. The coverslips were blocked with 20% goat serum in PBS for ~1 h. Primary antibodies were diluted in 5% goat serum in PBS, and coverslips were incubated with the antibodies overnight at 4 °C. After at least 1 h of washing in PBS, the coverslips were incubated with secondary antibodies, which were diluted in 5% goat serum in PBS, for 45 min at room temperature. The coverslips were washed again for 1 h and were then mounted with either ProLong® Gold antifade reagent or Aqua Poly/Mount for visualization.

Microscopy and Image Analysis—In some experiments, fixed neurons were visualized with a Retiga EXi CCD camera (QImaging, Surrey, British Columbia) linked to an Olympus IX71 inverted microscope (Melville, NY) with a PlanApo 60X OTIRFM objective (NA 1.45). MetaMorph software (Molecular Devices, Sunnyvale, CA), which was integrated with a Lambda 10-2 automated controller (Sutter Instruments, Novato, CA), was used to acquire and analyze images. For Alexa Fluor® 488 and enhanced GFP images, an Endow GFP Band-pass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT) was utilized. A TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror) was used to visualize mCherry as well as Alexa Fluor® 546 and 555. For Alexa Fluor® 647 imaging, a Cy5™ cube (excitation HQ620/60, emission HQ700/75, Q660LP dichroic mirror) was utilized.

For some experiments, a Quorum WaveFX-X1 spinning disk confocal system containing a Yokogawa CSU-X1 spinning disk (Yokogawa Electric Corp., Newnan, GA) with Borealis

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upgrade/modifications (Guelph, Canada) was utilized for fixed and live-cell imaging. Images were obtained via an EM-CCD camera (Hamamatsu, Hamamatsu City, Japan) on a Nikon Eclipse Ti microscope (Melville, NY) with MetaMorph software and an Apo TIRF 60 \times objective (NA 1.49). mCerulean, GFP, mCherry, and Alexa Fluor 647 images were acquired by exciting laser lines at 441, 491, 561, and 642 nm, respectively (Semrock, Rochester, NY); the emission filters for these fluorophores were 470/24, 525/50, 593/40, and 700/75, respectively (Semrock, Rochester, NY). Neurons were maintained in 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 30 mM glucose, pH 7.4, at 37 °C using a temperature-controlled chamber (Live Cell Instrument, Seoul, Korea). In some experiments, neurons were treated with 50 μ M AP5 for 30 min to 1 h before imaging.

Dendritic spine and synapse densities were quantified along primary and secondary dendrites. Spines were defined as protrusions that contacted presynaptic terminals (identified by immunostaining for SV2). For analysis of knockdown efficiency in neurons, cells were immunostained for the endogenous protein, and the average fluorescence intensity of staining in the soma was obtained. To measure Asef2 localization to spines, neurons were analyzed as previously described (47). Statistical analyses were performed using SPSS Statistics, version 22 (Armonk, NY). Comparison of two means was performed using t-tests. Comparison of multiple means was performed using one-way analysis of variance followed by post hoc tests (Tukey's test or Games-Howell pairwise comparison test). Data are shown as the means \pm S.E.

Rac Activity Assay—This assay was performed as described previously (48, 49). Briefly, HT1080 cells were transiently cotransfected with FLAG-tagged Rac cDNA and either GFP, GFP-Asef2, GFP-Asef2-K382A, or GFP-Asef2-K385A cDNAs. After 24 h, the cells were lysed and incubated with GST-tagged p21 binding domain, which was bound to glutathione-Sepharose beads (GE Healthcare) for 1 h at 4 °C with end-over-end mixing. The amount of active Rac pulled down by the GST-p21 binding domain beads was assayed by Western blot. For quantification, the amounts of active Rac were normalized to the total Rac amounts.

RESULTS

Endogenous Asef2 Is in Dendritic Spines and Synapses—Previous work has shown that Asef2 is expressed in various regions of the mammalian brain (33–36), including the hippocampus (33, 34). Because the hippocampus is functionally linked to learning and memory (50, 51), which are processes that are dependent on proper dendritic spine formation (52–56), we hypothesized that Asef2 plays a role in the development of spines. To test this hypothesis, we first assessed the localization of endogenous Asef2 in hippocampal neurons. In DIV14 neurons, Asef2 was seen throughout the dendrites, including in small puncta along the dendrites that appeared to be spines (Fig. 1*B*). To demonstrate that these dendritic puncta were spines, we co-stained for three synaptic markers, SV2, postsynaptic density protein 95 (PSD95), and Homer. As shown in Fig. 1*B*, Asef2 puncta were observed with the synaptic markers. Quantification showed that $62.7 \pm 1.7\%$ ($n = 24$ dendrites from

three separate experiments) of the Asef2 puncta along the dendrites co-localized with PSD95 clusters. Thus, these results suggest that Asef2 is present at synaptic sites (*i.e.* dendritic spines).

Expression of Asef2 Promotes Dendritic Spine and Synapse Formation—To continue investigating the role of Asef2 in spine development, we generated a GFP-tagged Asef2 construct and expressed it in hippocampal neurons. We transfected neurons at DIV5, before spines and synapses have formed, and assessed spine and synapse density at DIV11, when these structures are prevalent. Expression of GFP-Asef2 caused an increase in the density of dendritic spines compared with expression of GFP alone, as determined using GFP fluorescence (Fig. 1, *C* and *D*). The neurons were also stained with fluorescently labeled phalloidin, which binds to F-actin and is a commonly used marker for dendritic spines (57–59). Similar to the spine density quantified using GFP fluorescence, the density of spines quantified using phalloidin was increased in GFP-Asef2-expressing neurons (Fig. 1, *C* and *D*). Furthermore, GFP-Asef2 expression caused an increase in the number of synapses, as determined by SV2 and PSD95 staining, compared with GFP expression alone (Fig. 1, *C* and *D*). These results suggest that Asef2 promotes the formation of dendritic spines and synapses in hippocampal neurons.

Knockdown of Endogenous Asef2 Impairs Spine and Synapse Development—We next used a shRNA approach to knock down endogenous Asef2 in neurons and examined the effect on spines and synapses. We generated two shRNAs, which targeted the rat sequence of Asef2, and assessed their ability to reduce endogenous Asef2 expression. Neurons were stained for endogenous Asef2, and the intensity of Asef2 staining in the soma was used to quantify the percentage of Asef2 knockdown (Fig. 2*A*). Both shRNAs significantly reduced endogenous Asef2 expression compared with endogenous Asef2 expression in non-transfected neurons (Fig. 2*B*). Expression of either Asef2 shRNA caused an $\sim 50\%$ decrease in dendritic spine density as compared with expression of empty pSUPER vector or a non-targeting shRNA (NT shRNA) (Fig. 2, *C* and *D*). Similar decreases in synaptic density were also observed in Asef2 shRNA-transfected neurons (Fig. 2, *C* and *D*). To further show that the effects of the Asef2 shRNAs were due to the loss of the endogenous protein, we performed a rescue experiment in which GFP-tagged human Asef2 was co-expressed with Asef2 shRNA #1. Because the Asef2 shRNA targets the rat sequence, it should not affect the expression of GFP-tagged human Asef2 due to four nucleotide mismatches. Expression of human GFP-Asef2 with Asef2 shRNA led to an increase in the amount of total Asef2 and caused a significant increase in the number of spines and synapses compared with Asef2 shRNA expression (Fig. 2, *C–E*). Collectively, these results suggest that endogenous Asef2 is an important regulator of dendritic spine and synapse formation in developing neurons.

The Effect of Asef2 on Spines and Synapses Is Dependent on Its GEF Activity—Because GEF activity is important for Asef2 function (29, 30, 48, 60), we hypothesized that Asef2 promotes spine and synapse formation through activation of GTPases. To initially test this hypothesis, we mutated two residues, lysine 382 or lysine 385, to alanine (K382A and K385A, respectively). These residues are highly conserved among members of the Dbp

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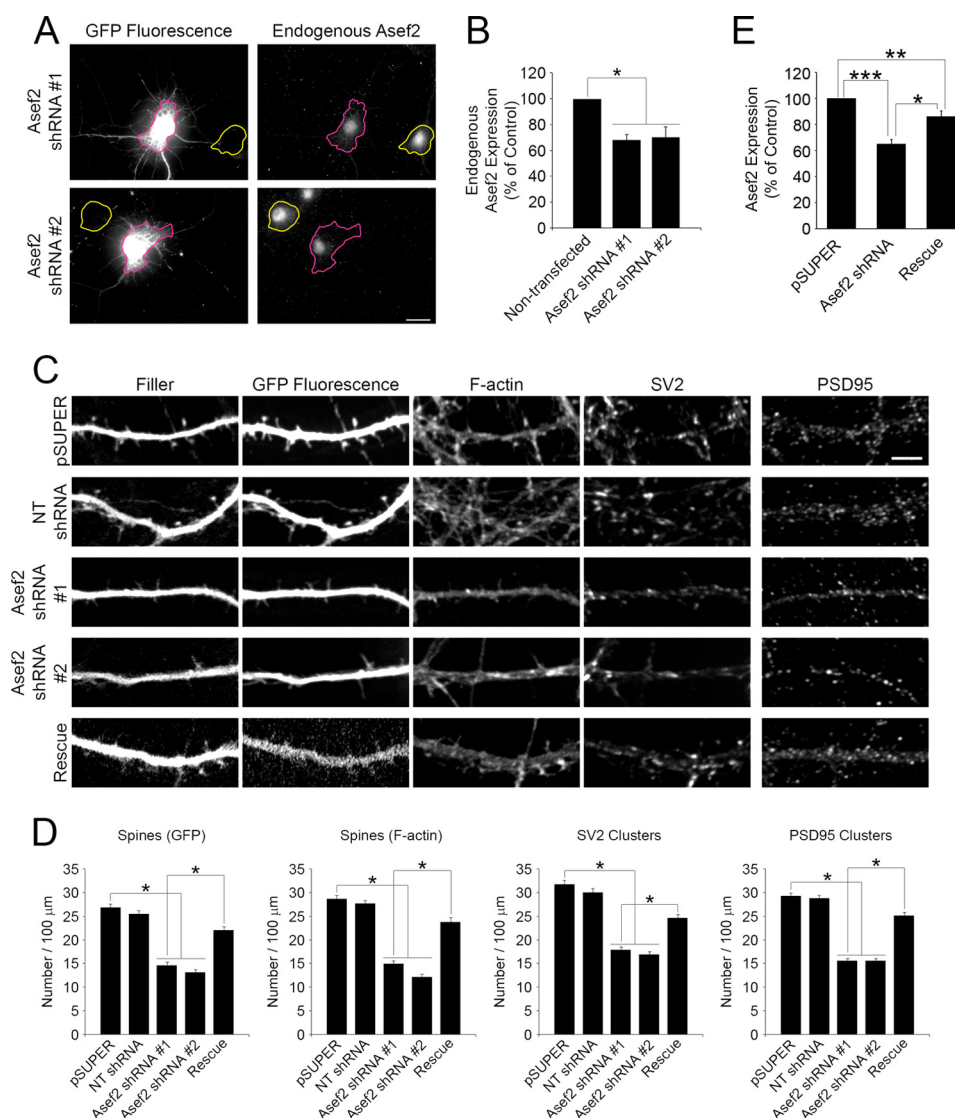


FIGURE 2. Knockdown of endogenous Asef2 hinders spine and synapse development. *A*, neurons were co-transfected at DIV5 with GFP and shRNAs targeting Asef2, then fixed at DIV11 and immunostained for endogenous Asef2. Transfected cell somas are outlined in magenta, and non-transfected cell somas are outlined in yellow. Bar, 20 μm. *B*, the amount of endogenous Asef2 was quantified by measuring the fluorescence intensity of Asef2 in the somas of non-transfected and shRNA-expressing neurons. Error bars represent S.E. for 45 cells from three separate experiments (*, $p < 0.02$). *C*, neurons were co-transfected with mCerulean (Filler), GFP, or GFP-Asef2 (Rescue) and either empty pSUPER vector, non-targeting shRNA (NT shRNA), or Asef2 shRNAs at DIV5, then fixed and stained for F-actin (phalloidin) and SV2 or PSD95 at DIV11. Bar, 5 μm. *D*, quantification of spine and synapse density for control (empty pSUPER vector or NT shRNA) and Asef2 shRNA-expressing neurons. Error bars represent S.E. for at least 45 dendrites from at least three separate experiments (*, $p < 0.001$). *E*, neurons were co-transfected with mCerulean, GFP, or GFP Asef2 (Rescue) and either pSUPER vector or Asef2 shRNA #2 at DIV5, fixed, and stained for Asef2 at DIV11. The total amount of Asef2 was quantified by measuring the fluorescence intensity of Asef2 in the somas of transfected cells. Error bars represent S.E. for 45 neurons from three separate experiments (*, $p < 0.04$; **, $p = 0.004$; ***, $p < 0.001$).

GEF family, which includes Asef2 (Fig. 3A); previous studies showed that lysine-to-alanine mutation of these residues in Dbl family GEFs, including Tiam1 and collybistin, greatly diminished their GEF activity (61–63). We tested these mutants for their GEF activity toward Rac using a Rac activation assay. For this assay, the GST-tagged binding domain from the Rac effector PAK (GST-p21 binding domain) was used to detect the active form of Rac from cell lysates of GFP-, GFP-Asef2-, GFP-Asef2-K382A-, and GFP-Asef2-K385A-expressing cells. Expression of GFP-Asef2 resulted in an increase in active Rac compared with expression of GFP (Fig. 3, B and C). However, expression of either Asef2-K382A or Asef2-K385A abolished the Asef2-promoted effect on active Rac (Fig. 3, B and C), indicating that these residues are crucial for the ability of Asef2 to activate Rac. We

then transfected neurons with either GFP, GFP-Asef2, or the GEF activity-deficient mutants and quantified spine and synapse density. Expression of GFP-Asef2 caused a significant increase in spine and synapse density compared with GFP expression (Fig. 3, D and E). In contrast, expression of GFP-Asef2-K382A or GFP-Asef2-K385A did not lead to an increase in the number of spines and synapses (Fig. 3, D and E), suggesting that the GEF activity of Asef2 is critical for its ability to promote spine and synapse formation.

Knockdown of Endogenous Rac Impedes Asef2-dependent Spine and Synapse Formation—Because the GEF activity of Asef2 is crucial for its effect on spines and synapses and previous work has shown that Rac regulates spine formation (16, 17, 64), we hypothesized that Asef2-mediated spine and synapse

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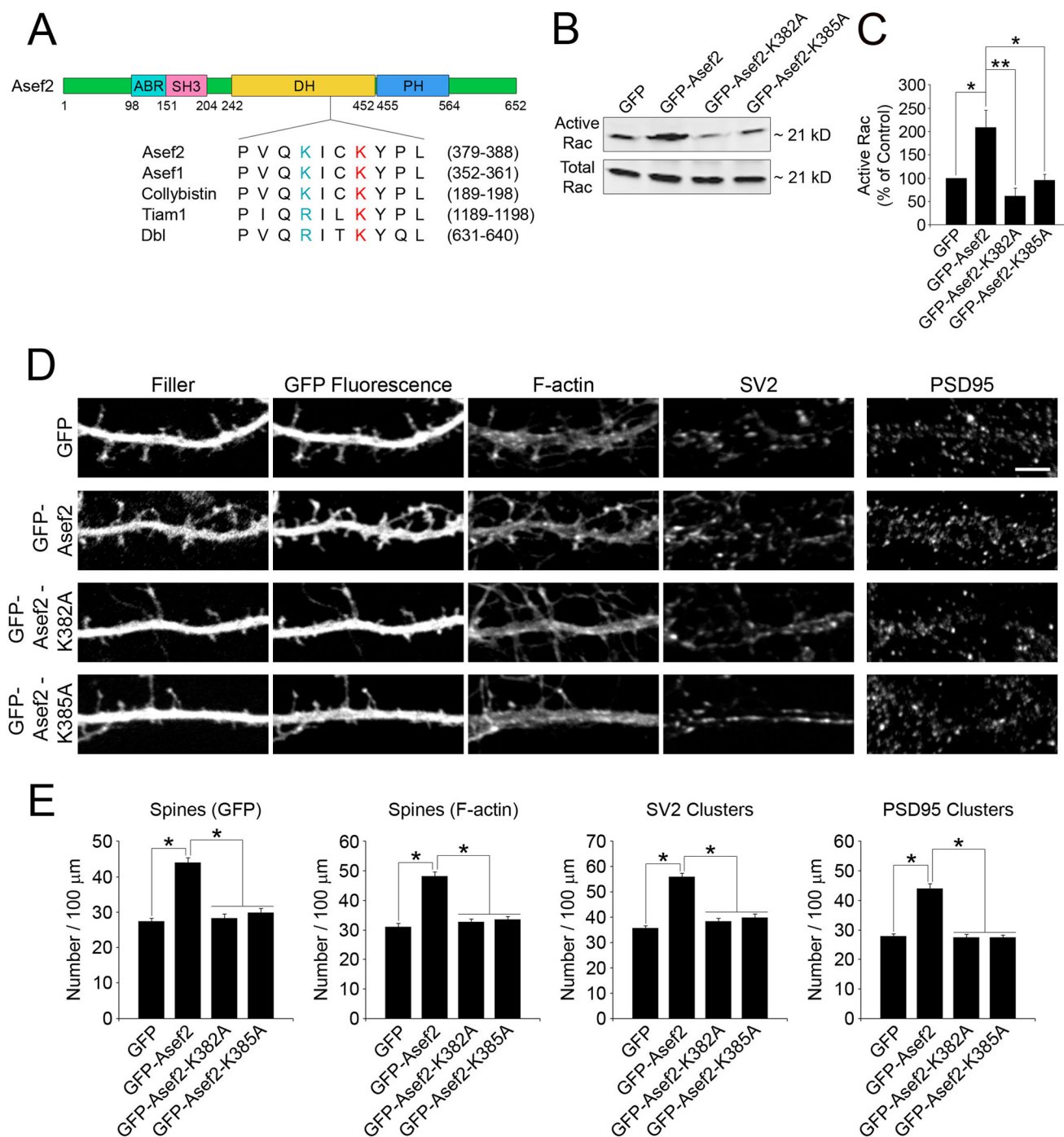


FIGURE 3. Asef2 GEF activity mediates spine and synapse formation. *A*, schematic comparing a portion of the Dbl homology domain amino acid sequences of several Dbl family GEFs. Asef2-K382 (and corresponding residues in other GEFs) is shown in blue, and Asef2-K385 (and corresponding residues in other GEFs) is shown in red. ABR, adenomatous polyposis coli binding region; SH3, Src homology 3 domain; DH, Dbl homology domain; PH, pleckstrin homology domain. *B*, HT1080 cells were co-transfected with FLAG-Rac and either GFP, GFP-Asef2, GFP-Asef2-K382A, or GFP-Asef2-K385A. Three days later the active form of Rac was pulled down from lysates from these cells. The amounts of total Rac in the lysates is shown as a control. *C*, quantification of the amount of active Rac from four independent experiments. Error bars represent S.E. (*, $p < 0.02$; **, $p < 0.002$). *D*, neurons were transfected with the indicated constructs and stained for F-actin (phalloidin) and SV2 or PSD95. Bar, 5 μm . *E*, quantification of spine and synapse density in GFP-, GFP-Asef2-, GFP-Asef2-K382A-, and GFP-Asef2-K385A-expressing neurons. Error bars represent S.E. for 45–59 dendrites from three independent experiments (*, $p < 0.001$).

formation is Rac-dependent. To test this hypothesis, we knocked down endogenous Rac using shRNAs. Two shRNAs, which targeted the rat sequence of Rac, reduced the expression of the endogenous protein by ~80% compared with empty pSUPER vector or to NT shRNA (Fig. 4, *A* and *B*). We then co-transfected these shRNAs with either GFP or GFP-Asef2 in

neurons and assessed spine and synapse density. Knockdown of Rac by transfection with Rac shRNAs in GFP-expressing neurons caused a decrease in the density of spines and synapses compared with neurons transfected with either empty pSUPER vector or NT shRNA (Fig. 4, *C* and *D*), supporting the importance of Rac signaling for the formation of spines. Transfection

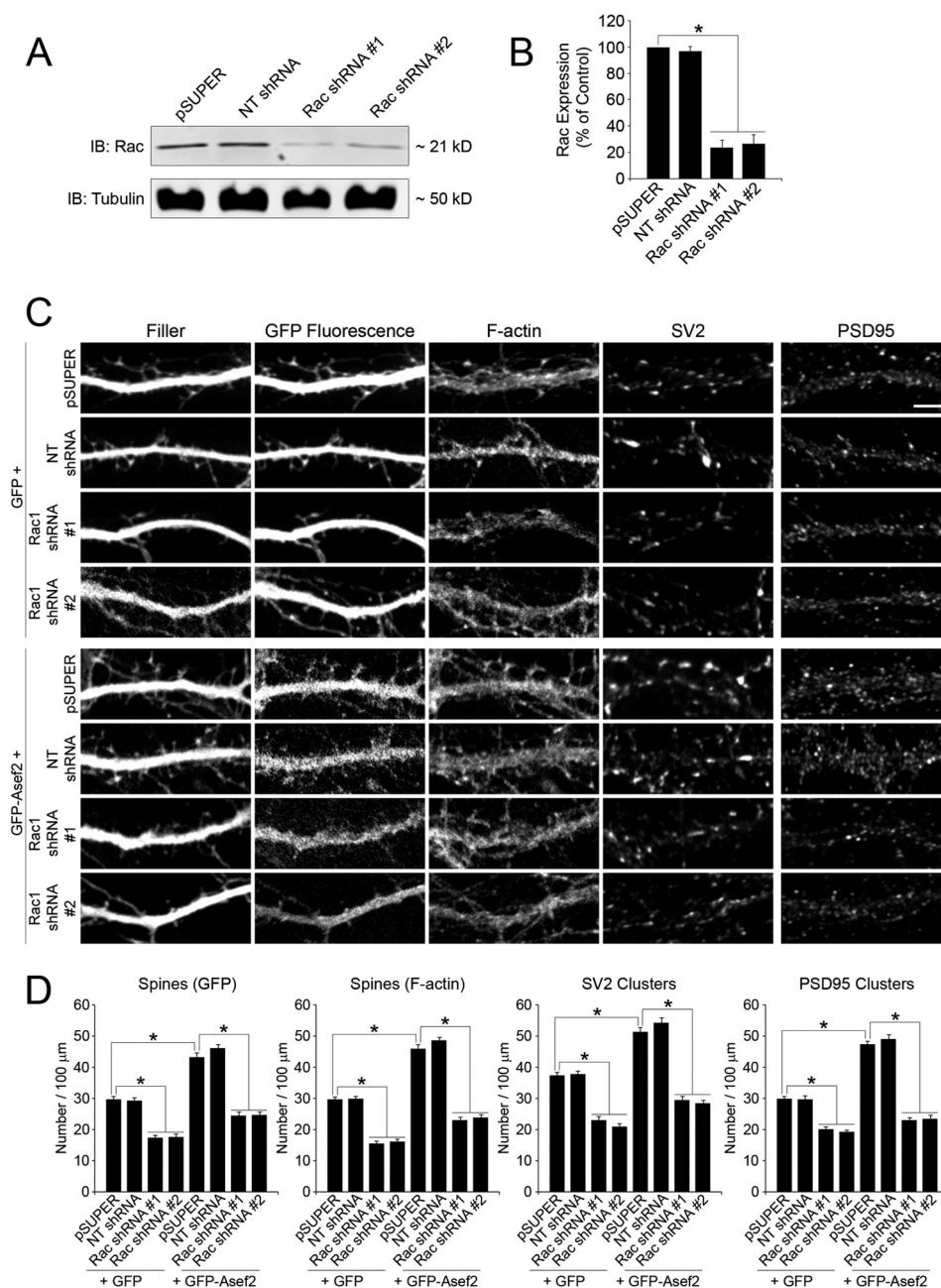


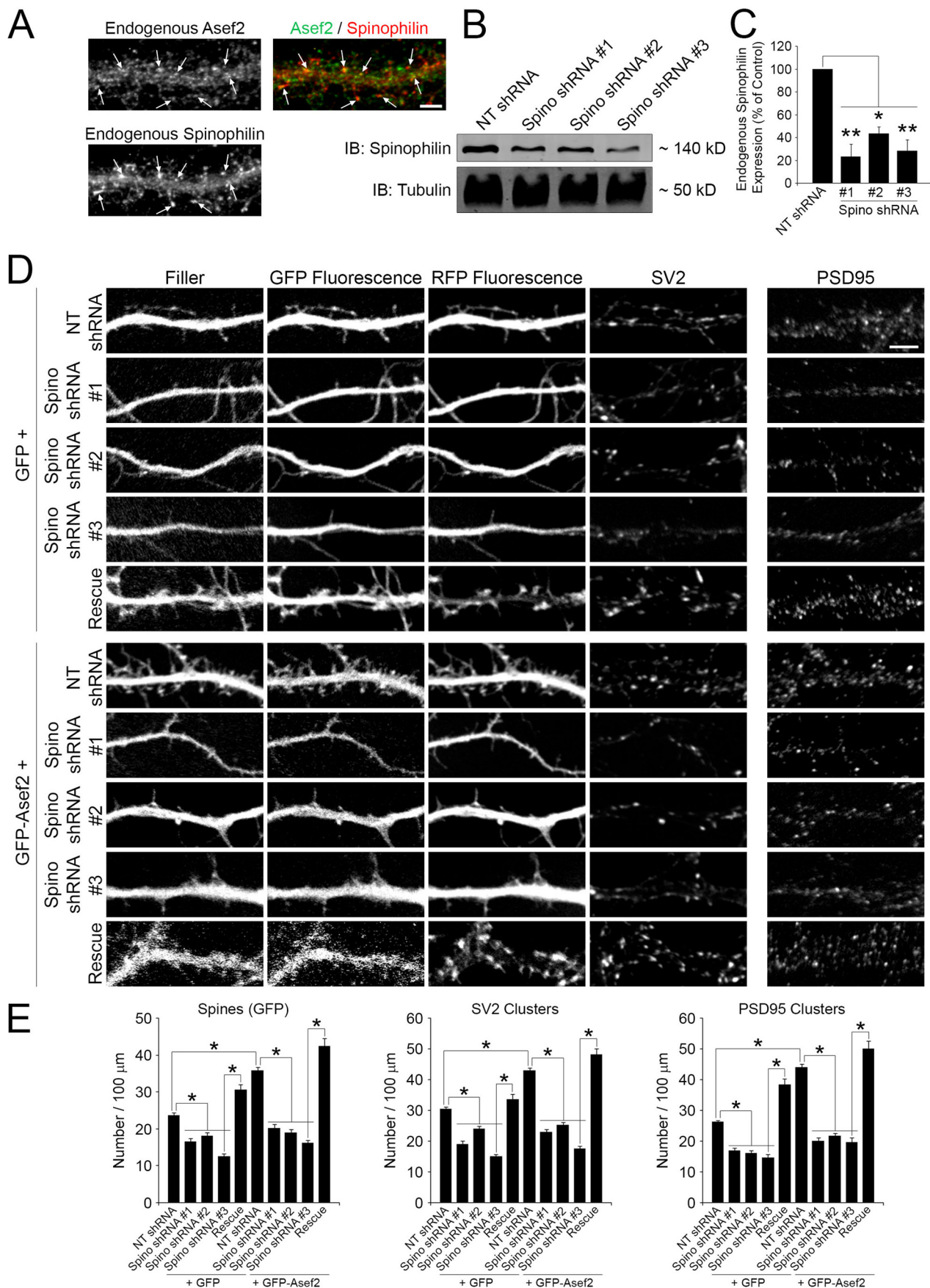
FIGURE 4. Knockdown of Rac impedes Asef2-mediated spine and synapse formation. *A*, cell lysates from R2Fs, which were transfected with either empty pSUPER vector, NT shRNA, or Rac shRNAs, were immunoblotted (IB) for Rac as well as tubulin for a loading control. *B*, quantification of endogenous Rac from three independent experiments. Error bars represent S.E. (*, $p < 0.001$). *C*, neurons were co-transfected with mCerulean (Filler), GFP, or GFP-Asef2 and either empty pSUPER vector, NT shRNA, or Rac shRNAs at DIV5. The cells were then fixed at DIV11 and stained for F-actin (phalloidin) and SV2 or PSD95. Bar, 5 μ m. *D*, quantification of spine and synapse density in GFP- and GFP-Asef2 neurons transfected with either control (empty pSUPER vector or NT shRNA) or Rac shRNAs. Error bars represent S.E. for 45 dendrites from three separate experiments (*, $p < 0.001$).

of Rac shRNAs in Asef2-expressing neurons resulted in a significant decrease in spine and synapse density (Fig. 4, *C* and *D*), which abolished the Asef2-promoted effect on these structures. These results suggest that Asef2 signaling through Rac mediates the formation of dendritic spines and synapses.

Spinophilin Regulates Asef2-promoted Spine and Synapse Formation—Spinophilin, which binds to F-actin, is a known Asef2-interacting protein that has previously been shown to regulate dendritic spine formation and synaptic function (37, 38, 42, 65, 66). Therefore, we next investigated the role of spinophilin in Asef2-mediated spine and synapse formation.

Immunostaining of DIV14 neurons revealed that endogenous Asef2 and spinophilin co-localized at distinct sites along the dendrite (Fig. 5*A*). Quantification showed that $63.6 \pm 2.6\%$ ($n = 24$ dendrites from three separate experiments) of Asef2 puncta co-localized with spinophilin. To determine whether spinophilin affects the function of Asef2 in spines and synapses, we generated three shRNAs to knock down endogenous expression of the protein. Transfection of the spinophilin shRNAs into R2Fs decreased endogenous levels of the protein by 60–80% (Fig. 5, *B* and *C*). Furthermore, the shRNAs decreased expression of endogenous spinophilin by $\sim 50\%$ when transfected into neu-

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rons (data not shown). The spinophilin shRNAs caused a significant decrease in spine and synapse density as compared with NT shRNA in GFP-expressing neurons (Fig. 5, *D* and *E*), indicating that endogenous spinophilin is a key regulator of spine and synapse formation. Moreover, the spinophilin shRNAs completely abrogated the Asef2-promoted effect on spines and synapses (Fig. 5, *D* and *E*), suggesting that spinophilin is an important contributor to Asef2 signaling in the regulation of spine and synapse formation. Because shRNA #3 targeted the 3'-untranslated region of spinophilin, we could perform rescue experiments with rat spinophilin. Expression of mCherry-tagged spinophilin with spinophilin shRNA #3 caused a significant increase in spine and synapse density compared with spinophilin shRNA #3 expression (Fig. 5, *D* and *E*), further showing that the effect of spinophilin shRNAs on spines and synapses is due to loss of the endogenous protein.

Spinophilin Recruits Asef2 to Dendritic Spines—Because spinophilin localizes prominently to dendritic spines and is a binding partner for Asef2 (37, 38, 41), we hypothesized that spinophilin targets Asef2 to spines. Intriguingly, co-expression of mCherry-spinophilin and GFP-Asef2 caused a stark change in the localization of Asef2 (Fig. 6*A*). Although GFP-Asef2 alone was seen throughout dendrites, GFP-Asef2, when co-expressed with mCherry-spinophilin, was observed to localize predominantly to dendritic spines with much less accumulation in the dendrites (Fig. 6*A*). Indeed, the localization of Asef2 mirrored that of mCherry-spinophilin with both proteins co-localizing in dendritic spines (Fig. 6*A*). Quantification of the dendritic spine to shaft ratio of the fluorescence intensities of GFP and GFP-Asef2 showed an ~2.5-fold increase when mCherry-spinophilin was co-expressed with GFP-Asef2 (Fig. 6*B*), suggesting that spinophilin localizes Asef2 to dendritic spines.

To further investigate the function of spinophilin in targeting Asef2 to spines, we generated an mCherry-tagged spinophilin mutant in which the C terminus was deleted (mCherry-spinophilin Δ Cterm) and expressed it in neurons with GFP-Asef2. As we previously observed, expression of full-length, mCherry-tagged spinophilin caused a significant increase in Asef2 localization to spines compared with mCherry expression (Fig. 6, *C* and *D*). In contrast, expression of mCherry-spinophilin Δ Cterm completely abrogated this increase in Asef2 spine localization (Fig. 6, *C* and *D*). This effect was not due to a decrease in spinophilin localization to spines because the N-terminal F-actin binding domain targets spinophilin to spines (39). Next, we prepared a GFP-tagged Asef2 C-terminal deletion construct (GFP-Asef2 Δ Cterm). This region of Asef2 has been previously shown to mediate its interaction with spinophilin (37). Expression of GFP-Asef2 Δ Cterm with mCherry-spinophilin caused a $57.6 \pm 9.7\%$ ($n = 90$ spines from three separate experiments) reduction in Asef2 localization to spines

compared with that observed with expression of GFP-tagged full-length Asef2 with mCherry-spinophilin. These results collectively indicate that the interaction of spinophilin with Asef2 targets Asef2 to spines where it regulates spine and synapse development.

NMDA Receptor Signaling Is Important for Spinophilin-dependent Asef2 Localization to Dendritic Spines—NMDA receptors mediate the activity of numerous dendritic spine-associated proteins, and they are integral for the biochemical processes that underlie learning and memory (67, 68). To assess whether NMDA receptors regulate spinophilin-mediated Asef2 localization, we used the NMDA receptor antagonist AP5, which acts as a competitive inhibitor (69). Neurons expressing GFP-Asef2 and mCherry-spinophilin were treated with 50 μ M AP5 or a vehicle control for 30 min to 1 h before imaging. As previously seen, expression of mCherry-spinophilin promoted the localization of Asef2 to spines (Fig. 7, *A* and *B*). Treatment of neurons with AP5, however, caused a significant reduction in the localization of Asef2 to spines (Fig. 7, *A* and *B*), as determined by calculating the ratio of the GFP-Asef2 fluorescence intensity in dendritic spines to shafts (Fig. 7*B*). Thus, these results suggest that NMDA receptor signaling is an upstream modulator of spinophilin-mediated Asef2 localization to spines.

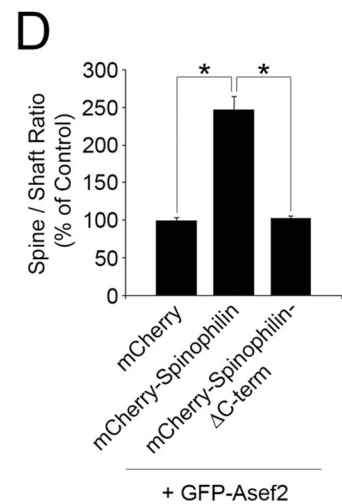
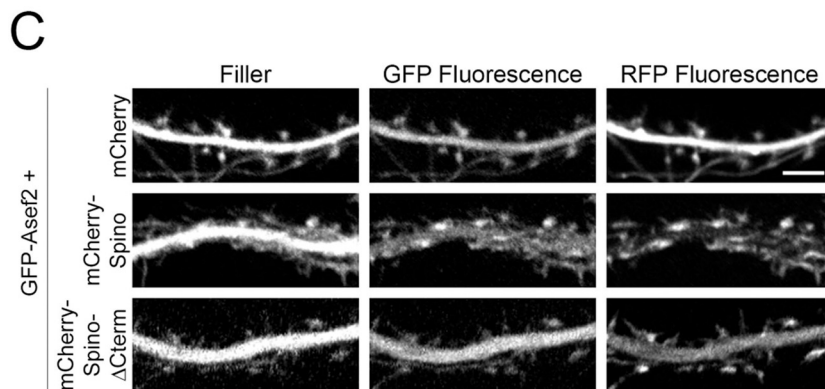
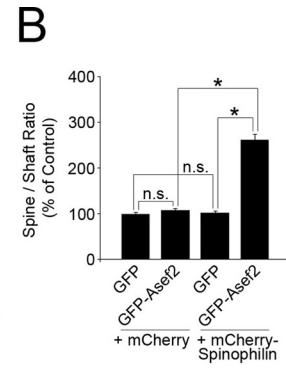
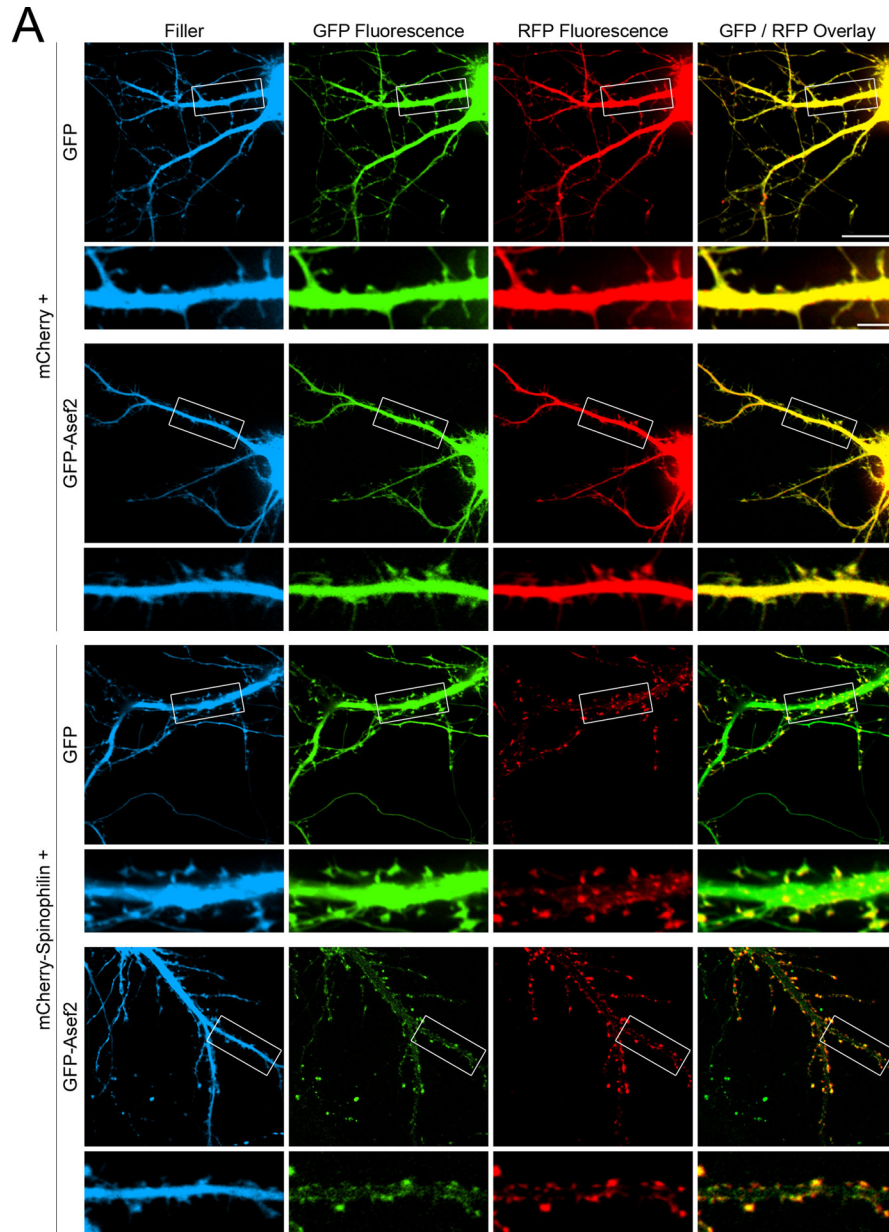
DISCUSSION

The function of Rho family GEFs in spine development represents an increasingly exciting area of study given that not much is currently known about the contribution of these proteins to spine and synapse formation. Our results indicate a previously unknown role for the Rho family GEF Asef2 in the formation of dendritic spines and synapses in hippocampal neurons. Proper development of spines is dependent on the endogenous expression of Asef2, because knockdown of Asef2 causes a decrease in spine and synapse density. Knockdown of other Rho family GEFs, including Tiam1 and kalirin-7, have also been shown to affect spine and synapse function (22–24, 70, 71), pointing to the critical contribution of Rho family GEFs to these processes.

Asef2 signals through Rac to regulate spine formation, as demonstrated by knockdown of Rac in Asef2-expressing neurons. Interestingly, exogenous expression of Asef2 causes a significant increase in spine density, which also occurs when constitutively active Rac is expressed in neurons (16, 17, 64). Although our results indicate that Rac is crucial for the Asef2-promoted increase in spine and synapse density, we cannot exclude the possible contribution of other Rho family GTPases. Asef2 is reported to activate both Rac and Cdc42, and Cdc42 has previously been shown to promote spine and synapse development (20, 28, 29). Therefore, Cdc42 could play a role in mediating the Asef2-promoted increase in the number of spines and synapses. Future studies are needed to determine whether

FIGURE 5. Spinophilin knockdown abolishes Asef2-promoted spine and synapse formation. *A*, neurons were fixed at DIV14 and immunostained for endogenous Asef2 and spinophilin. An overlay of the images shows co-localization of the proteins along the dendrite (arrows). Bar, 5 μ m. *B*, cell lysates from R2Fs transfected with NT shRNA or spinophilin shRNAs were immunoblotted (IB) for spinophilin as well as tubulin for a loading control. *C*, quantification of endogenous spinophilin from three separate experiments. Error bars represent S.E. (*, $p = 0.005$; **, $p = 0.001$). *D*, neurons were co-transfected with mCherry (Filler), GFP or GFP-Asef2, mCherry or mCherry-spinophilin (Rescue), and either NT shRNA or spinophilin shRNAs at DIV5–6. Cells were fixed at DIV11–12 and stained for SV2 or PSD95. Bar, 5 μ m. *E*, quantification of spine and synapse density in GFP- and GFP-Asef2 neurons transfected with either NT shRNA or spinophilin shRNAs. Error bars represent S.E. for 30–90 dendrites from at least three separate experiments (*, $p < 0.001$).

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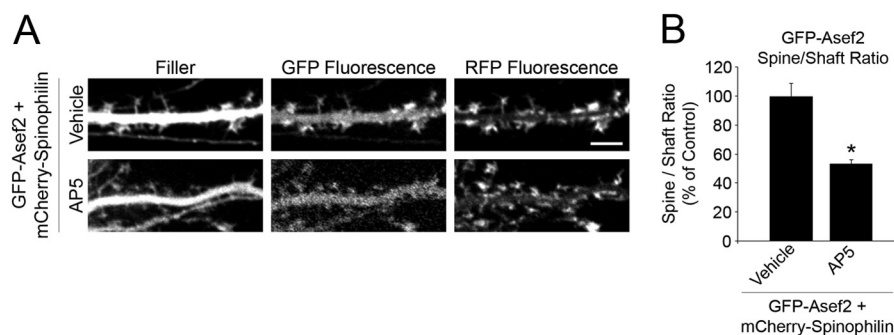


FIGURE 7. **NMDA receptor signaling is critical for spinophilin-mediated Asef2 localization to spines.** *A*, neurons were co-transfected with mCherry (Filler), GFP-Asef2, and mCherry-spinophilin at DIV5–6. DIV11 neurons were incubated with either water (vehicle) or 50 μ M AP5 for 30 min to 1 h and then visualized with live-cell confocal microscopy. Bar, 5 μ m. RFP, red fluorescent protein. *B*, the ratios of the GFP-Asef2 fluorescence intensities in dendritic spines to neighboring shafts was quantified and normalized to the vehicle-treated control. Error bars represent S.E. for 70 spines from three separate experiments (*, $p < 0.001$).

Cdc42 also contributes to the effects of Asef2 on spines and synapses.

To better understand the mechanism of Asef2-dependent spine formation, we investigated the role of the scaffolding protein spinophilin. Our work demonstrates that spinophilin localizes Asef2 to spines, which has not been previously shown. Furthermore, this observation highlights the increasing importance of spinophilin-GEF signaling as a key component of the development of spines. Spinophilin has been shown to interact with several Rho family GEFs in addition to Asef2: kalirin-7, Tiam1, and Lfc (72–75). Interestingly, spinophilin expression alters the localization of endogenous Tiam1 in non-neuronal cells (73, 74), whereas spinophilin expression results in relocation of Lfc to the cell periphery in neuroblast N2a cells (75). Taken together, these data suggest that spinophilin is a key regulator of Rho family GEF localization.

Our results showed that endogenous Asef2 distributed throughout dendrites, including the dendrite shaft and spines, under basal conditions. Other GEFs, such as Lfc, have similarly been found to distribute to dendritic shafts under basal conditions (75). In the case of Lfc, neuronal stimulation caused it to translocate from dendrites to spines (75). This change in localization is thought to occur via targeting by spinophilin (75). Therefore, spinophilin-mediated targeting of Asef2 to spines could occur predominantly in response to synaptic signaling. Indeed, inhibition of NMDA receptor activity via the antagonist AP5 caused a significant loss of spinophilin-dependent Asef2 localization to spines, suggesting that NMDA receptors signaling is an important upstream regulator of Asef2 localization. Further elucidation of the upstream signals that regulate spinophilin-promoted Asef2 localization to spines is an interesting avenue for future study.

In our study knockdown of spinophilin caused a decrease in dendritic spine density. This phenotype differs from that observed by Feng *et al.* (42), who reported that spinophilin

knock-out resulted in an increase in spine density in young mice. A possible explanation for this discrepancy is that the knock-out spine density measurements were performed on neurons from the caudatoputamen. Spinophilin could have a different function in these neurons *versus* the hippocampal neurons used in our study. Another explanation could be that spinophilin mediates these effects on spines by associating with different binding partners. In the study by Feng *et al.* (42), the interaction of spinophilin with protein phosphatase-1 appears to be important in the modulation of spine dynamics, whereas in our study the Asef2-spinophilin interaction is critical for the effects that we observe on spine development.

The C terminus of spinophilin acts as a centralized scaffold for several GEFs, including Asef2 (37). This region of spinophilin contains a PDZ domain and a coiled-coil domain, which both facilitate protein-protein interactions (76). Based on yeast two-hybrid screening, the C terminus of kalirin-7 appears to associate with the PDZ domain of spinophilin (72). Lfc, on the other hand, interacts with the coiled-coil domain of spinophilin, whereas Tiam1 is thought to associate with a region spanning the PDZ and coiled-coil domains (73, 75). The specific region within the spinophilin C terminus that interacts with Asef2 is currently unknown. However, Asef2 most likely does not associate with spinophilin via coiled-coil interactions, because Asef2 does not have a coiled-coil domain (37). Nevertheless, the C-terminal interaction between Asef2 and spinophilin is integral for targeting Asef2 to spines because deletion of this region disrupts Asef2 localization to spines. Continued work is necessary to determine the specific regions that are essential for the Asef2-spinophilin interaction and whether this interaction affects the binding of spinophilin to other GEFs.

In summary, our results reveal a new function for Asef2 in promoting the formation of dendritic spines and synapses in hippocampal neurons. Asef2-mediated spine and synapse development occurs via the scaffold protein spinophilin, which

FIGURE 6. **Spinophilin targets Asef2 to dendritic spines.** *A*, neurons were co-transfected with mCherry (Filler), GFP, or GFP-Asef2 and either mCherry or mCherry-spinophilin at DIV5. The neurons were visualized at DIV11 using live-cell confocal microscopy. Bar, 20 μ m. Higher magnification images of the boxed regions are shown below. Bar, 5 μ m. *B*, the spine-to-shaft ratios of the fluorescence intensities of GFP and GFP-Asef2 were quantified and normalized to the control (GFP + mCherry). Error bars represent S.E. from 150 spines from 5 independent experiments (*, $p < 0.001$). n.s. denotes no statistically significant difference. *C*, DIV5–6 neurons were co-transfected with mCherry (Filler), GFP-Asef2, and either mCherry, mCherry-spinophilin (mCherry-Spino), or mCherry-spinophilin- Δ C terminus (mCherry-Spino- Δ Cterm). The neurons were visualized at DIV11 using live-cell confocal microscopy. Bar, 5 μ m. *D*, the spine-to-shaft ratios of the GFP-Asef2 fluorescence intensities were quantified and normalized to the control (mCherry + GFP-Asef2). Error bars represent S.E. from 90 spines from three independent experiments (*, $p < 0.001$).

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targets Asef2 to spines. Asef2 activates Rac locally to facilitate the formation of new spines. Spinophilin also maintains Asef2 in spines, which suggests that Asef2-mediated Rac signaling is involved in spine stability. The proper formation and maintenance of spines is crucial for efficient synaptic signaling. Interestingly, a nucleotide deletion in Asef2 has been linked to autism, and a single-nucleotide polymorphism in Asef2 has been linked to the co-occurrence of alcohol dependence and depression (77, 78); these disorders are associated with spine defects (7, 79, 80). Collectively, our data point to Asef2 as a key signaling protein in regulating the development of dendritic spines and synapses, which is critical for maintaining normal cognitive and behavioral function.

Acknowledgment—We are grateful to Lan Hu for technical assistance in the preparation of neuronal cultures and cDNA constructs.

REFERENCES

1. Gray, E. G. (1959) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* **93**, 420–433
2. Nimchinsky, E. A., Sabatini, B. L., and Svoboda, K. (2002) Structure and function of dendritic spines. *Annu. Rev. Physiol.* **64**, 313–353
3. Matus, A., Ackermann, M., Pehling, G., Byers, H. R., and Fujiwara, K. (1982) High actin concentrations in brain dendritic spines and postsynaptic densities. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7590–7594
4. Papa, M., Bundman, M. C., Greenberger, V., and Segal, M. (1995) Morphological analysis of dendritic spine development in primary cultures of hippocampal neurons. *J. Neurosci.* **15**, 1–11
5. Dailey, M. E., and Smith, S. J. (1996) The dynamics of dendritic structure in developing hippocampal slices. *J. Neurosci.* **16**, 2983–2994
6. Ziv, N. E., and Smith, S. J. (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* **17**, 91–102
7. Fiala, J. C., Spacek, J., and Harris, K. M. (2002) Dendritic spine pathology: cause or consequence of neurological disorders? *Brain Res. Brain Res. Rev.* **39**, 29–54
8. Penzes, P., Cahill, M. E., Jones, K. A., VanLeeuwen, J. E., and Woolfrey, K. M. (2011) Dendritic spine pathology in neuropsychiatric disorders. *Nat. Neurosci.* **14**, 285–293
9. Fischer, M., Kaech, S., Knutti, D., and Matus, A. (1998) Rapid actin-based plasticity in dendritic spines. *Neuron* **20**, 847–854
10. Korobova, F., and Svitkina, T. (2010) Molecular architecture of synaptic actin cytoskeleton in hippocampal neurons reveals a mechanism of dendritic spine morphogenesis. *Mol. Biol. Cell* **21**, 165–176
11. Matus, A. (2000) Actin-based plasticity in dendritic spines. *Science* **290**, 754–758
12. Jaffe, A. B., and Hall, A. (2005) Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol.* **21**, 247–269
13. West, M., Kung, H. F., and Kamata, T. (1990) A novel membrane factor stimulates guanine nucleotide exchange reaction of ras proteins. *FEBS Lett.* **259**, 245–248
14. Trahey, M., and McCormick, F. (1987) A cytoplasmic protein stimulates normal N-ras p21 GTPase but does not affect oncogenic mutants. *Science* **238**, 542–545
15. Cherfils, J., and Zeghouf, M. (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiol. Rev.* **93**, 269–309
16. Luo, L., Hensch, T. K., Ackerman, L., Barbel, S., Jan, L. Y., and Jan, Y. N. (1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379**, 837–840
17. Tashiro, A., Minden, A., and Yuste, R. (2000) Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of Rac and Rho. *Cereb. Cortex* **10**, 927–938
18. Scott, E. K., Reuter, J. E., and Luo, L. (2003) Small GTPase Cdc42 is required for multiple aspects of dendritic morphogenesis. *J. Neurosci.* **23**, 3118–3123
19. Irie, F., and Yamaguchi, Y. (2002) EphB receptors regulate dendritic spine development via intersectin, Cdc42, and N-WASP. *Nat. Neurosci.* **5**, 1117–1118
20. Wegner, A. M., Nebhan, C. A., Hu, L., Majumdar, D., Meier, K. M., Weaver, A. M., and Webb, D. J. (2008) N-wasp and the arp2/3 complex are critical regulators of actin in the development of dendritic spines and synapses. *J. Biol. Chem.* **283**, 15912–15920
21. Toliás, K. F., Duman, J. G., and Um, K. (2011) Control of synapse development and plasticity by Rho GTPase regulatory proteins. *Prog. Neurobiol.* **94**, 133–148
22. Toliás, K. F., Bikoff, J. B., Burette, A., Paradis, S., Harrar, D., Tavazoie, S., Weinberg, R. J., and Greenberg, M. E. (2005) The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines. *Neuron* **45**, 525–538
23. Toliás, K. F., Bikoff, J. B., Kane, C. G., Toliás, C. S., Hu, L., and Greenberg, M. E. (2007) The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7265–7270
24. Zhang, H., and Macara, I. G. (2006) The polarity protein PAR-3 and TIAM1 cooperate in dendritic spine morphogenesis. *Nat. Cell Biol.* **8**, 227–237
25. Govek, E. E., Newey, S. E., Akerman, C. J., Cross, J. R., Van der Veken, L., and Van Aelst, L. (2004) The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis. *Nat. Neurosci.* **7**, 364–372
26. Nadif Kasri, N., Nakano-Kobayashi, A., Malinow, R., Li, B., and Van Aelst, L. (2009) The Rho-linked mental retardation protein oligophrenin-1 controls synapse maturation and plasticity by stabilizing AMPA receptors. *Genes Dev.* **23**, 1289–1302
27. Billuart, P., Bienvenu, T., Ronce, N., des Portes, V., Vinet, M. C., Zemni, R., Roest Crollius, H., Carrié, A., Fauchereau, F., Cherry, M., Briault, S., Hamel, B., Fryns, J. P., Beldjord, C., Kahn, A., Moraine, C., and Chelly, J. (1998) Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature* **392**, 923–926
28. Hamann, M. J., Lubking, C. M., Luchini, D. N., and Billadeau, D. D. (2007) Asef2 functions as a Cdc42 exchange factor and is stimulated by the release of an autoinhibitory module from a concealed C-terminal activation element. *Mol. Cell. Biol.* **27**, 1380–1393
29. Kawasaki, Y., Sagara, M., Shibata, Y., Shirouzu, M., Yokoyama, S., and Akiyama, T. (2007) Identification and characterization of Asef2, a guanine-nucleotide exchange factor specific for Rac1 and Cdc42. *Oncogene* **26**, 7620–7627
30. Bristow, J. M., Sellers, M. H., Majumdar, D., Anderson, B., Hu, L., and Webb, D. J. (2009) The Rho-family GEF Asef2 activates Rac to modulate adhesion and actin dynamics and thereby regulate cell migration. *J. Cell Sci.* **122**, 4535–4546
31. Muroya, K., Kawasaki, Y., Hayashi, T., Ohwada, S., and Akiyama, T. (2007) PH domain-mediated membrane targeting of Asef. *Biochem. Biophys. Res. Commun.* **355**, 85–88
32. Murayama, K., Shirouzu, M., Kawasaki, Y., Kato-Murayama, M., Hanawa-Suetsugu, K., Sakamoto, A., Katsura, Y., Suenaga, A., Toyama, M., Terada, T., Taiji, M., Akiyama, T., and Yokoyama, S. (2007) Crystal structure of the rac activator, Asef, reveals its autoinhibitory mechanism. *J. Biol. Chem.* **282**, 4238–4242
33. Walther, D. M., and Mann, M. (2011) Accurate quantification of more than 4000 mouse tissue proteins reveals minimal proteome changes during aging. *Mol. Cell Proteomics* **10**.1074/mcp.M110.004523
34. Cajigas, I. J., Tushev, G., Will, T. J., tom Dieck, S., Fuerst, N., and Schuman, E. M. (2012) The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron* **74**, 453–466
35. Yoshizawa, M., Sone, M., Matsuo, N., Nagase, T., Ohara, O., Nabeshima, Y., and Hoshino, M. (2003) Dynamic and coordinated expression profile of dbl-family guanine nucleotide exchange factors in the developing mouse brain. *Gene Expr. Patterns* **3**, 375–381
36. Becker, J. A., Befort, K., Blad, C., Fillion, D., Ghate, A., Dembele, D., Thibault, C., Koch, M., Muller, J., Lardenois, A., Poch, O., and Kieffer, B. L. (2008) Transcriptome analysis identifies genes with enriched ex-

- pression in the mouse central extended amygdala. *Neuroscience* **156**, 950–965
37. Sagara, M., Kawasaki, Y., Iemura, S. I., Natsume, T., Takai, Y., and Akiyama, T. (2009) Asef2 and Neurabin2 cooperatively regulate actin cytoskeletal organization and are involved in HGF-induced cell migration. *Oncogene* **28**, 1357–1365
 38. Allen, P. B., Ouimet, C. C., and Greengard, P. (1997) Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9956–9961
 39. Grossman, S. D., Hsieh-Wilson, L. C., Allen, P. B., Nairn, A. C., and Greengard, P. (2002) The actin-binding domain of spinophilin is necessary and sufficient for targeting to dendritic spines. *Neuromolecular Med.* **2**, 61–69
 40. Barnes, A. P., Smith, F. D., 3rd, VanDongen, H. M., VanDongen, A. M., and Milgram, S. L. (2004) The identification of a second actin-binding region in spinophilin/neurabin II. *Brain Res. Mol. Brain Res.* **124**, 105–113
 41. Satoh, A., Nakanishi, H., Obaishi, H., Wada, M., Takahashi, K., Satoh, K., Hirao, K., Nishioka, H., Hata, Y., Mizoguchi, A., and Takai, Y. (1998) Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell-cell adhesion sites. *J. Biol. Chem.* **273**, 3470–3475
 42. Feng, J., Yan, Z., Ferreira, A., Tomizawa, K., Liauw, J. A., Zhuo, M., Allen, P. B., Ouimet, C. C., and Greengard, P. (2000) Spinophilin regulates the formation and function of dendritic spines. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9287–9292
 43. Stafstrom-Davis, C. A., Ouimet, C. C., Feng, J., Allen, P. B., Greengard, P., and Houpt, T. A. (2001) Impaired conditioned taste aversion learning in spinophilin knockout mice. *Learn Mem.* **8**, 272–278
 44. Gloster, A., El-Bizri, H., Bamji, S. X., Rogers, D., and Miller, F. D. (1999) Early induction of α 1 α -tubulin transcription in neurons of the developing nervous system. *J. Comp. Neurol.* **405**, 45–60
 45. Zhang, H., and Macara, I. G. (2008) The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. *Dev. Cell* **14**, 216–226
 46. Goslin, K., Asmussen, H., and Banker, G. (1998) Rat Hippocampal Neurons in Low Density Culture. In *Culturing Nerve Cells*, pp. 339–370, MIT Press, Cambridge, MA
 47. Lin, W. H., Nebhan, C. A., Anderson, B. R., and Webb, D. J. (2010) Vasodilator-stimulated phosphoprotein (VASP) induces actin assembly in dendritic spines to promote their development and potentiate synaptic strength. *J. Biol. Chem.* **285**, 36010–36020
 48. Jean, L., Majumdar, D., Shi, M., Hinkle, L. E., Diggins, N. L., Ao, M., Brousard, J. A., Evans, J. C., Choma, D. P., and Webb, D. J. (2013) Activation of Rac by Asef2 promotes myosin II-dependent contractility to inhibit cell migration on type I collagen. *J. Cell Sci.* **126**, 5585–5597
 49. Knaus, U. G., Bamberg, A., and Bokoch, G. M. (2007) Rac and Rap GTPase activation assays. *Methods Mol. Biol.* **412**, 59–67
 50. Scoville, W. B., and Milner, B. (1957) Loss of recent memory after bilateral hippocampal lesions. *J. Neurol. Neurosurg. Psychiatry* **20**, 11–21
 51. Bird, C. M., and Burgess, N. (2008) The hippocampus and memory: insights from spatial processing. *Nat. Rev. Neurosci.* **9**, 182–194
 52. Engert, F., and Bonhoeffer, T. (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature* **399**, 66–70
 53. Maletic-Savatic, M., Malinow, R., and Svoboda, K. (1999) Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* **283**, 1923–1927
 54. Leuner, B., Falduo, J., and Shors, T. J. (2003) Associative memory formation increases the observation of dendritic spines in the hippocampus. *J. Neurosci.* **23**, 659–665
 55. Nägerl, U. V., Eberhorn, N., Cambridge, S. B., and Bonhoeffer, T. (2004) Bidirectional activity-dependent morphological plasticity in hippocampal neurons. *Neuron* **44**, 759–767
 56. De Roo, M., Klauser, P., and Muller, D. (2008) LTP promotes a selective long-term stabilization and clustering of dendritic spines. *PLoS Biol.* **6**, e219
 57. Drenckhahn, D., Frotscher, M., and Kaiser, H. W. (1984) Concentration of F-actin in synaptic formations of the hippocampus as visualized by staining with fluorescent phalloidin. *Brain Res.* **300**, 381–384
 58. Wulf, E., Deboben, A., Bautz, F. A., Faulstich, H., and Wieland, T. (1979) Fluorescent phalloidin, a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4498–4502
 59. Allison, D. W., Gelfand, V. I., Spector, I., and Craig, A. M. (1998) Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. *J. Neurosci.* **18**, 2423–2436
 60. Kawasaki, Y., Tsuji, S., Sagara, M., Echizen, K., Shibata, Y., and Akiyama, T. (2009) Adenomatous polyposis coli and Asef function downstream of hepatocyte growth factor and phosphatidylinositol 3-kinase. *J. Biol. Chem.* **284**, 22436–22443
 61. Worthylake, D. K., Rossman, K. L., and Sondek, J. (2000) Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. *Nature* **408**, 682–688
 62. Reddy-Alla, S., Schmitt, B., Birkenfeld, J., Eulenburg, V., Dutertre, S., Böhringer, C., Götz, M., Betz, H., and Papadopoulos, T. (2010) PH-domain-driven targeting of collybistin but not Cdc42 activation is required for synaptic gephyrin clustering. *Eur. J. Neurosci.* **31**, 1173–1184
 63. Zhu, K., Debreceni, B., Li, R., and Zheng, Y. (2000) Identification of Rho GTPase-dependent sites in the Dbl homology domain of oncogenic Dbl that are required for transformation. *J. Biol. Chem.* **275**, 25993–26001
 64. Nakayama, A. Y., and Luo, L. (2000) Intracellular signaling pathways that regulate dendritic spine morphogenesis. *Hippocampus* **10**, 582–586
 65. Yan, Z., Hsieh-Wilson, L., Feng, J., Tomizawa, K., Allen, P. B., Fienberg, A. A., Nairn, A. C., and Greengard, P. (1999) Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. *Nat. Neurosci.* **2**, 13–17
 66. Kelker, M. S., Dancheck, B., Ju, T., Kessler, R. P., Hudak, J., Nairn, A. C., and Peti, W. (2007) Structural basis for spinophilin-neurabin receptor interaction. *Biochemistry* **46**, 2333–2344
 67. Lüscher, C., and Malenka, R. C. (2012) NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). *Cold Spring Harb Perspect Biol* **4**, a005710
 68. Shimizu, E., Tang, Y. P., Rampon, C., and Tsien, J. Z. (2000) NMDA receptor-dependent synaptic reinforcement as a crucial process for memory consolidation. *Science* **290**, 1170–1174
 69. Davies, J., Francis, A. A., Jones, A. W., and Watkins, J. C. (1981) 2-Amino-5-phosphonovalerate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci. Lett.* **21**, 77–81
 70. Ma, X. M., Huang, J., Wang, Y., Eipper, B. A., and Mains, R. E. (2003) Kalirin, a multifunctional Rho guanine nucleotide exchange factor, is necessary for maintenance of hippocampal pyramidal neuron dendrites and dendritic spines. *J. Neurosci.* **23**, 10593–10603
 71. Xie, Z., Srivastava, D. P., Photowala, H., Kai, L., Cahill, M. E., Woolfrey, K. M., Shum, C. Y., Surmeier, D. J., and Penzes, P. (2007) Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. *Neuron* **56**, 640–656
 72. Penzes, P., Johnson, R. C., Sattler, R., Zhang, X., Haganir, R. L., Kambampati, V., Mains, R. E., and Eipper, B. A. (2001) The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. *Neuron* **29**, 229–242
 73. Buchsbaum, R. J., Connolly, B. A., and Feig, L. A. (2003) Regulation of p70 S6 kinase by complex formation between the Rac guanine nucleotide exchange factor (Rac-GEF) Tiam1 and the scaffold spinophilin. *J. Biol. Chem.* **278**, 18833–18841
 74. Rajagopal, S., Ji, Y., Xu, K., Li, Y., Wicks, K., Liu, J., Wong, K. W., Herman, I. M., Isberg, R. R., and Buchsbaum, R. J. (2010) Scaffold proteins IRSp53 and spinophilin regulate localized Rac activation by T-lymphocyte invasion and metastasis protein 1 (TIAM1). *J. Biol. Chem.* **285**, 18060–18071
 75. Ryan, X. P., Alldritt, J., Svenningsson, P., Allen, P. B., Wu, G. Y., Nairn, A. C., and Greengard, P. (2005) The Rho-specific GEF Lfc interacts with neurabin and spinophilin to regulate dendritic spine morphology. *Neuron* **47**, 85–100
 76. Sarrouilhe, D., di Tommaso, A., Métaayé, T., and Ladeveze, V. (2006) Spinophilin: from partners to functions. *Biochimie* **88**, 1099–1113
 77. Iossifov, I., Ronemus, M., Levy, D., Wang, Z., Hakker, I., Rosenbaum, J., Yamrom, B., Lee, Y. H., Narzisi, G., Leotta, A., Kendall, J., Grabowska, E.,

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- Ma, B., Marks, S., Rodgers, L., Stepansky, A., Troge, J., Andrews, P., Bekritsky, M., Pradhan, K., Ghiban, E., Kramer, M., Parla, J., Demeter, R., Fulton, L. L., Fulton, R. S., Magrini, V. J., Ye, K., Darnell, J. C., Darnell, R. B., Mardis, E. R., Wilson, R. K., Schatz, M. C., McCombie, W. R., and Wigler, M. (2012) De novo gene disruptions in children on the autistic spectrum. *Neuron* **74**, 285–299
78. Edwards, A. C., Aliev, F., Bierut, L. J., Bucholz, K. K., Edenberg, H., Hesselbrock, V., Kramer, J., Kuperman, S., Nurnberger, J. I., Jr., Schuckit, M. A., Porjesz, B., and Dick, D. M. (2012) Genome-wide association study of comorbid depressive syndrome and alcohol dependence. *Psychiatr. Genet.* **22**, 31–41
79. Gass, J. T., and Olive, M. F. (2012) Neurochemical and neurostructural plasticity in alcoholism. *ACS Chem. Neurosci.* **3**, 494–504
80. Ota, K. T., and Duman, R. S. (2013) Environmental and pharmacological modulations of cellular plasticity: role in the pathophysiology and treatment of depression. *Neurobiol. Dis.* **57**, 28–37