Rac GEF Dock4 interacts with cortactin to regulate dendritic spine formation

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ABSTRACT **In neuronal development, dendritic spine formation is important for the establishment of excitatory synaptic connectivity and functional neural circuits. Developmental deficiency in spine formation results in multiple neuropsychiatric disorders. Dock4, a guanine nucleotide exchange factor (GEF) for Rac, has been reported as a candidate genetic risk factor for autism, dyslexia, and schizophrenia. We previously showed that Dock4 is expressed in hippocampal neurons. However, the functions of Dock4 in hippocampal neurons and the underlying molecular mechanisms are poorly understood. Here we show that Dock4 is highly concentrated in dendritic spines and implicated in spine formation via interaction with the actin-binding protein cortactin. In cultured neurons, short hairpin RNA (shRNA)–mediated knockdown of Dock4 reduces dendritic spine density, which is rescued by coexpression of shRNA-resistant wild-type Dock4 but not by a GEF-deficient mutant of Dock4 or a truncated mutant lacking the cortactin-binding region. On the other hand, knockdown of cortactin suppresses Dock4-mediated spine formation. Taken together, the results show a novel and functionally important interaction between Dock4 and cortactin for regulating dendritic spine formation via activation of Rac.**

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INTRODUCTION

Dendritic spines are specialized protrusions found at dendrite postsynaptic regions of excitatory neurons in mammalian CNS (Harris and Kater, 1994), and spine morphology is directly linked to synaptic strength, stability, cell-surface receptor number, and neurotransmitter sensitivity (Kasai *et al.*, 2003). Abnormalities of spine morphology and density are associated with various neuropsychiatric disorders, such as autism spectrum disorders (ASDs), mental retardation, and schizophrenia (Lin and Koleske, 2010; Penzes *et al.*, 2011).

Filamentous actin (F-actin) is highly concentrated in dendritic spines and provides essential structural support for dendritic spine morphology (Matus *et al.*, 1982). Rho-family small GTPases, including Rho, Rac, and Cdc42, play key roles in the regulation of F-actin organization, and these proteins also contribute to the determination of spine morphology (Tashiro *et al.*, 2000). In particular, Rac1 and RhoA have antagonistic roles in the regulation of spine formation. Activation of Rac1 promotes spine formation, growth, and maintenance, whereas activation of RhoA causes spine retraction and decreases spine density (Newey *et al.*, 2005). Several modulators of actin cytoskeletal dynamics, including p21-activated kinase, LIM kinase, insulin receptor substrate p53 (IRSp53), WASP-family verprolin-homologous protein, and Arp2/3 complex, have been implicated in the downstream signaling of Rac in the regulation of spine morphology (Meng *et al.*, 2002; Choi *et al.*, 2005). Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) and are inactivated by GTPase-activating proteins (GAPs), and recent studies reported that GEFs and GAPs are involved in the regulation of dendritic spine formation (Tolias *et al.*, 2011). However, precise spatiotemporal regulation by different GEFs and GAPs is unclear.

Dock4 is a member of the Dock180-related Rho-GEF protein family and specifically activates Rac (Côté and Vuori, 2002; Meller *et al.*, 2005; Hiramoto *et al.*, 2006). *DOCK4* was originally identified as a gene deleted during tumor progression (Yajnik *et al.*, 2003), and recent studies report that *DOCK4* is a candidate gene for susceptibility to several neuropsychiatric disorders, including autism,

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Abbreviations used: ASD, autism spectrum disorder; DHR, Dock-homology region; DIV, days in vitro; F-actin, filamentous actin; GEF, guanine nucleotide exchange factor; SH3, Src-homology 3; shRNA, short hairpin RNA.

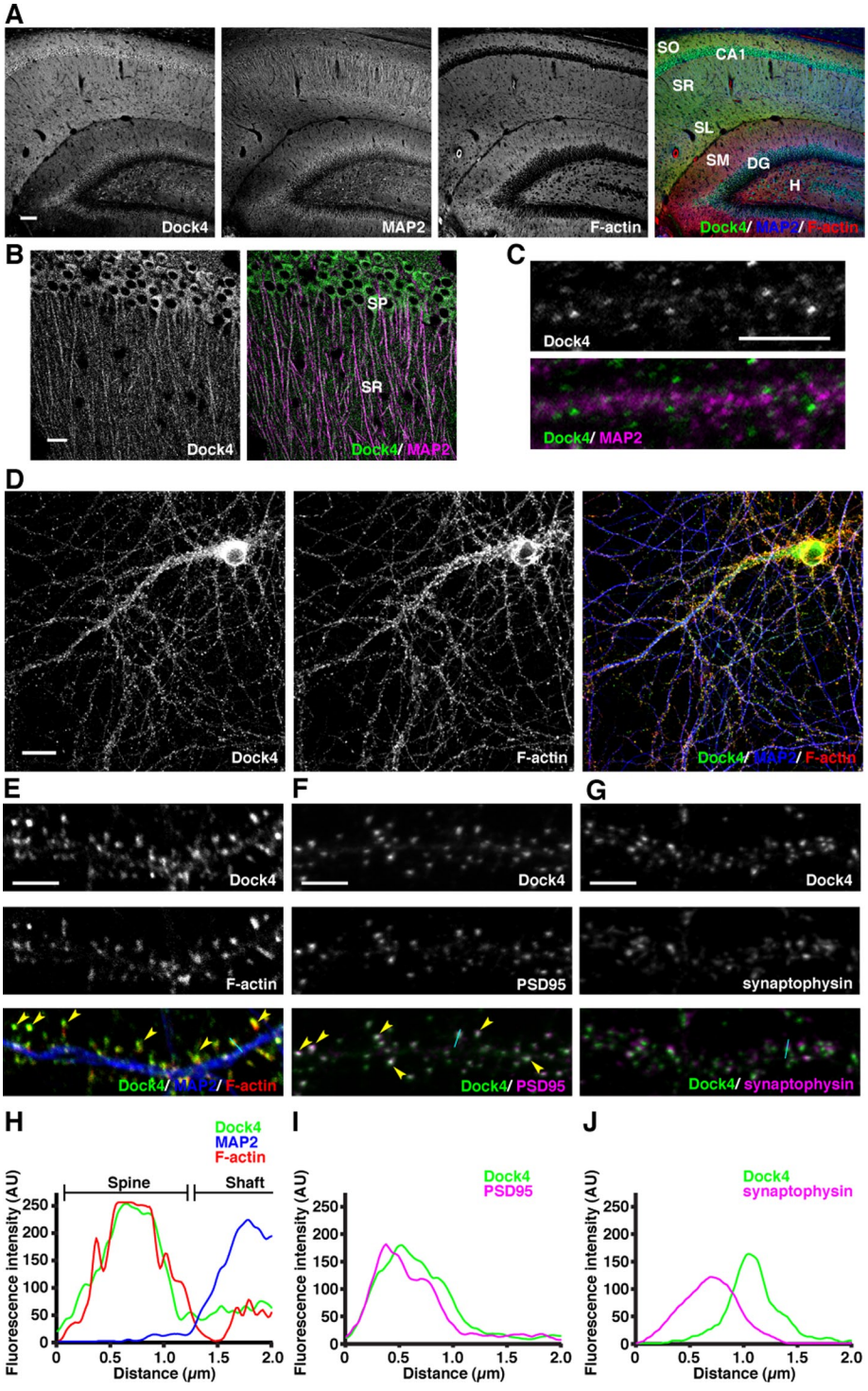

FIGURE 1: Subcellular localization of Dock4 in hippocampal neurons. (A–C) Frozen coronal sections of postnatal day 20 rat brain were incubated with anti-Dock4 antibody, anti-MAP2 antibody, and phalloidin to stain F-actin. (A) Low-magnification images of the hippocampus. Green, blue, and red indicate Dock4, MAP2, and F-actin staining, respectively. (B, C) High-magnification images of the pyramidal neurons of the CA1 region. Green and magenta indicate Dock4 and MAP2 staining, respectively. Scale bars, (A) 100 μm, (B) 20 μm, (C) 5 μm. DG, dentate gyrus; H, hilus; SL, stratum lacunosum; SM, stratum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. (D–G) Primary cultured rat hippocampal neurons were fixed at 15 DIV and then stained with indicated antibodies and phalloidin. (D, E) Green, blue, and red indicate Dock4, MAP2, and F-actin staining, respectively. (F) Green and magenta indicate Dock4 and PSD95 staining, respectively. (G) Green and magenta indicate Dock4 and synptophysin staining, respectively. Arrowheads indicate the dendritic spines where Dock4 colocalized with F-actin or PSD95, respectively. Scale bars, (D) 20 μm, (E–G) 5 μm. (H–J) Fluorescence intensity profiles along cyan lines drawn in E–G were measured in corresponding channels.

dyslexia, and schizophrenia (Maestrini *et al.*, 2010; Pagnamenta *et al.*, 2010; Alkelai *et al.*, 2012). Dock4 forms a complex with ELMO, and the ELMO–Dock4 complex serves as a functional GEF for Rac to promote cell migration in fibroblasts and breast cancer cells (Hiramoto *et al.*, 2006; Hiramoto-Yamaki *et al.*, 2010). On the other hand, Dock4 is highly expressed in rat brain, particularly in the hippocampus during late developmental stages and adulthood, and in cultured hippocampal neurons, Dock4 positively regulates dendritic growth and branching (Ueda *et al.*, 2008). However, its roles and their molecular mechanisms are largely unknown. In the present study, we provide evidence for a novel role of Dock4 in the regulation of dendritic spine development in hippocampal neurons. We also identify the actin-binding protein cortactin as a novel binding partner of Dock4. Cortactin is well known to regulate branching and stabilization of actin filaments (Weaver *et al.*, 2001) and is also implicated in spine morphogenesis and activity-dependent remodeling of spines in neurons (Hering and Sheng, 2003; Iki *et al.*, 2005; Chen and Hsueh, 2012). Our studies show that Dock4 localizes to dendritic spines and regulates spine formation through interaction with cortactin and activation of Rac.

RESULTS

Dock4 is localized to dendritic spines in hippocampal neurons

We previously reported that Dock4 mRNA is highly expressed in rat hippocampus and the expression level of Dock4 protein is upregulated during late developmental stages, when accelerated dendritic growth and spinogenesis occur in cultured hippocampal neurons (Ueda *et al.*, 2008). To investigate the subcellular localization of Dock4 in hippocampal neurons, we first examined the distribution of Dock4 in the postnatal day 20 rat hippocampus. Immunofluorescence staining of tissue sections with anti-Dock4 antibody revealed that Dock4 was expressed in the hippocampal neurons in the cornu ammonis (CA) fields and the dentate gyrus (DG) region (Figure 1A, green). The same section was also labeled with MAP2 (blue) and F-actin (red). In the CA1 region of hippocampus, punctate Dock4 fluorescence (green) was observed along MAP2-positive

dendrites (magenta) of hippocampal neurons (Figure 1, B and C). Next we immunostained primary cultured rat hippocampal neurons at 15 d in vitro (DIV) and showed that Dock4 was widely distributed throughout the cell body and dendrites (Figure 1D). In particular, Dock4 fluorescence signals largely overlapped with those of F-actin and a postsynaptic marker PSD95 and were closely apposed to a presynaptic marker, synaptophysin (Figure 1, E–G). Fluorescence intensity analyses along cyan lines drawn in Figure 1, E–G, confirmed that the Dock4 fluorescence peak was entirely matched by the F-actin peak and the PSD95 peak but not the synaptophysin peak (Figure 1, H–J). Taken together, these results show the postsynaptic spine localization of Dock4 in hippocampal neurons.

Knockdown of Dock4 reduces dendritic spine density

To examine the physiological roles of Dock4 in dendritic spines, we performed RNA interference–mediated knockdown of Dock4 in cultured hippocampal neurons. We generated three short hairpin RNA (shRNA) expression vectors designed to target three different regions of mouse Dock4 cDNA (see *Materials and Methods*). Dock4 shRNA-1500 and -3685 (shDock4 #1 and #2, respectively) effectively reduced the level of Flag-tagged mouse Dock4 expressed in HEK293T cells, whereas Dock4 shRNA-114 had no effect. Therefore we used Dock4 shRNA-114 as a control shRNA (shControl; Figure 2A). Similarly, shDock4 #1 and #2 effectively reduced the intensity of endogenous Dock4 immunolabeling in cultured rat hippocampal neurons (green fluorescent protein [GFP]–positive neurons) compared with that of nearby untransfected neurons, but shControl did not (Figure 2, B and C). To examine the effects of Dock4 shRNAs on spine morphology, we cotransfected cultured hippocampal neurons with enhanced yellow fluorescent protein (EYFP) and shRNAs at 11 DIV and fixed and imaged them at 15 DIV. Expression of shControl did not affect the dendritic spine formation compared with that observed in neurons transfected with EYFP alone. However, expression of the effective Dock4 shRNAs impaired spine formation (Figure 2, D–G). Quantitative evaluations of protrusion density showed that expression of shControl had little effect on the number of dendritic protrusions (EYFP alone, 5.32 ± 0.23 spines and 0.88 ± 0.06 filopodia/10 μm; shControl, 5.59 ± 0.22 spines and 0.98 ± 0.06 filopodia/10 μm), whereas expression of shDock4s significantly decreased spine density (#1, 3.38 ± 0.16; #2, 3.59 ± 0.17; Figure 2H). The number of dendritic filopodia also tended to decrease in Dock4-knockdown neurons (#1, 0.77 ± 0.05; #2, 0.81 ± 0.06; Figure 2H). These results suggest that Dock4 is required for dendritic spine formation in hippocampal neurons.

Rac GEF activity is required for Dock4-mediated spine formation

Dock4 contains two conserved regions among the Dock180-related proteins, Dock-homology region 1 (DHR-1) and DHR-2 domains, and the DHR-2 domain of Dock4 catalyzes the nucleotide exchange activity for Rac (Côté and Vuori, 2002; Meller *et al.*, 2005; Hiramoto *et al.*, 2006). To examine whether the GEF activity toward Rac is required for Dock4-mediated spine formation, we generated shRNA-resistant constructs of Dock4 (Dock4-res; Figure 3A) to restore the expression of Dock4 in knockdown cells (Figures 2A and 3C). Coexpression of Dock4-res-WT in hippocampal neurons completely rescued the reduction of protrusion density by shDock4 #1 (shControl, 6.14 ± 0.23 spines and 1.11 ± 0.06 filopodia/10 μm; shDock4 #1, 3.54 ± 0.15 spines and 0.84 ± 0.05 filopodia/10 μm; shDock4 #1 + res-WT, 6.24 ± 0.24 spines and 1.01 ± 0.06 filopodia/10 μm; Figure 3, D–F and I), whereas coexpression of Dock4-res-AAA, which is defective in GEF activity toward Rac (Figure 3B; Hiramoto *et al.*, 2006), did not (3.27 ± 0.22 spines and 0.74 ± 0.06 filopodia/10 μm; Figure 3, G and I). These results suggest that Dock4 positively regulates dendritic spine formation through activation of Rac in hippocampal neurons.

Knockdown of ELMO2 reduces dendritic spine density

We previously reported that Dock4 interacts with ELMO through the N-terminal region, including Src-homology 3 (SH3) domain, and the ELMO–Dock4 complex serves as a functional GEF for Rac in intact cells (Hiramoto *et al.*, 2006). Among the ELMO subfamily proteins, ELMO2 is mainly expressed in the developing hippocampus, and Dock4 interacts with ELMO2 in cultured rat hippocampal neurons (Katoh *et al.*, 2006; Ueda *et al.*, 2008). To examine whether ELMO2 is also implicated in spine formation, we generated an effective shRNA against ELMO2 (shELMO2; Figure 4A) and evaluated the effect of ELMO2 knockdown on spine formation. Knockdown of ELMO2 in hippocampal neurons significantly reduced spine density (shControl, 5.44 ± 0.14 spines and 0.75 ± 0.06 filopodia/10 μm; shELMO2, 3.85 ± 0.17 spines and 0.67 ± 0.05 filopodia/10 μm; Figure 4, B and C). These results suggest that ELMO2 is also involved in dendritic spine formation in hippocampal neurons.

The C-terminal, proline-rich region is required for Dock4-mediated spine formation

To further investigate the regulatory mechanisms of Dock4 in dendritic spine formation, we next used a deletion mutant of Dock4 lacking the C-terminal, proline-rich region (Dock4-ΔC; Figure 3B). Coexpression of Dock4-res-ΔC failed to rescue the reduction of spine density by shDock4 #1 (4.12 ± 0.17). Instead it increased the number of dendritic filopodia (1.70 ± 0.09; Figure 3, H and I). Thus the C-terminal, proline-rich region of Dock4 plays a role in Dock4-mediated spine formation.

Dock4 binds to cortactin through the C-terminal, proline-rich region

Because the C-terminal, proline-rich region of Dock4 is critical for its function, we next screened Dock4-binding proteins by yeast two-hybrid system from adult rat brain cDNA library using the C-terminal region of Dock4 (amino acids 1602–1978) as bait and isolated a C-terminal fragment of cortactin (amino acids 403–546, cortactin-CT; Figure 5A) containing the SH3 domain as a potential binding partner. Cortactin is well known as a regulator of actin polymerization and stabilization in various cells (Wu and Parsons, 1993; Uruno *et al.*, 2001; Weaver *et al.*, 2001) and also known as a regulator of the dendritic spine formation in neurons (Hering and Sheng, 2003). To confirm the interaction between cortactin and Dock4, we expressed Flag-Dock4 in HEK293T cells and used a pull-down assay with recombinant glutathione *S*-transferase (GST)–fused cortactin or its mutants (Figure 5A). Wild-type cortactin (cortactin-WT) and its C-terminal fragment containing the SH3 domain (cortactin-SH3) bound Dock4, whereas a mutant lacking the SH3 domain (cortactin-ΔSH3) or containing a mutation in the SH3 domain (cortactin-W525K, which lost the ability to bind proline-rich ligands; Kinley *et al.*, 2003) did not (Figure 5B). Thus cortactin interacts with Dock4 through the SH3 domain. Dock180, another member of Dock family of proteins, also has a proline-rich region at the C-terminus (Côté and Vuori, 2002). To determine whether Dock180 also interacts with cortactin, were transiently transfected HEK293T cells with Myc-cortactin-CT and Flag-Dock4 or Flag-Dock180, together with their constitutive binding partner

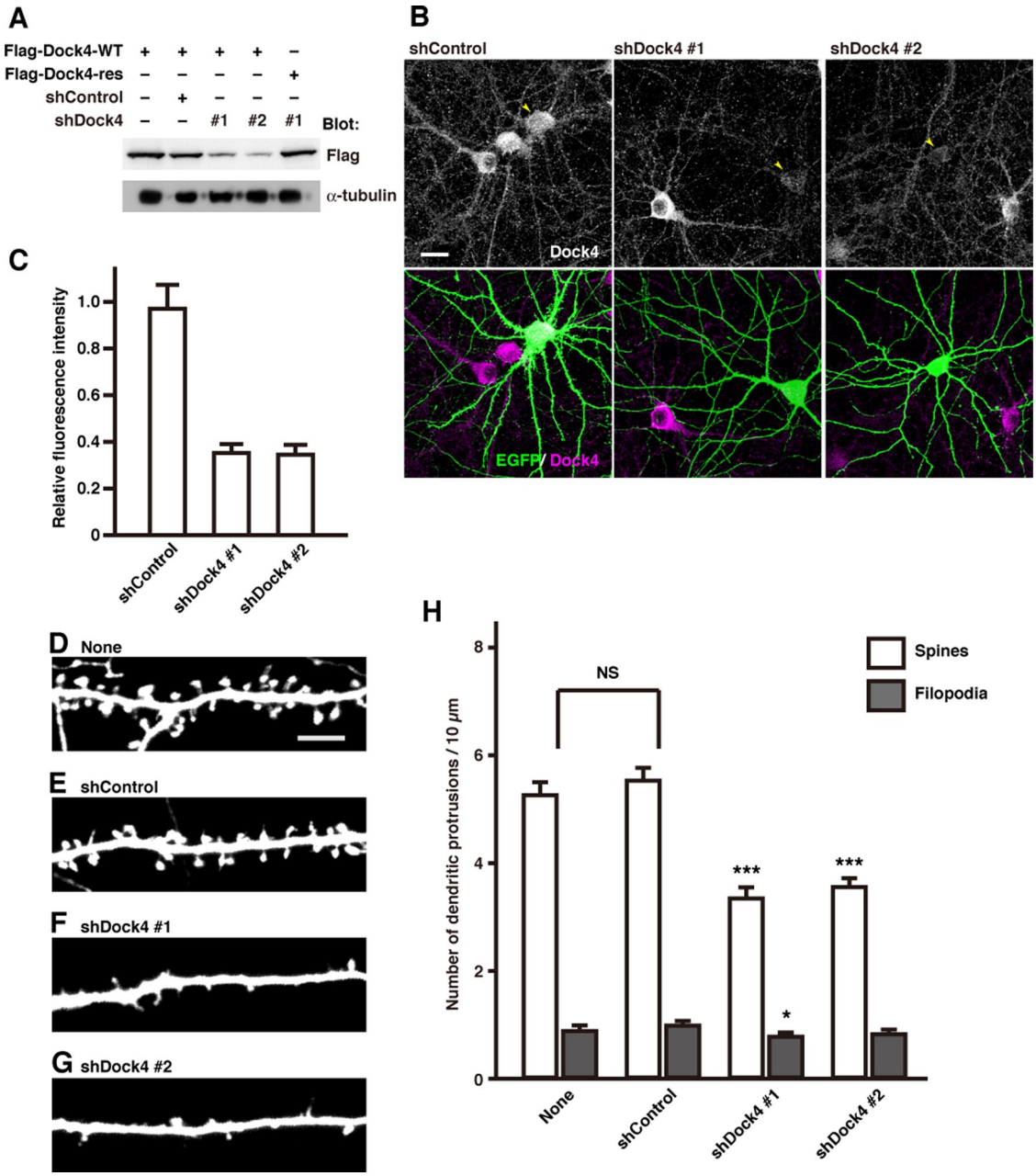

FIGURE 2: Knockdown of Dock4 suppresses dendritic spine formation. (A) Cell lysates from HEK293T cells cotransfected with indicated shRNAs (ineffective shControl or effective shDock4 #1, #2) and Flag-tagged, wild-type Dock4 (Dock4-WT) or shDock4 #1-resistant form of Dock4 (Dock4-res) were analyzed by immunoblotting with antibodies against Flag and α-tubulin. (B) Knockdown of endogenous Dock4 in hippocampal neurons. Primary cultured rat hippocampal neurons were cotransfected with EGFP and the indicated shRNA expression vector at 11 DIV. At 4 d after transfection, neurons were fixed and stained with antibody against Dock4 (magenta). Arrowheads indicate the transfected cells. Scale bar, 20 μm. (C) Quantification of the knockdown efficiency. Under the same conditions as those in B, the Dock4 fluorescence intensity in the cell bodies of the EGFP-positive transfected cells relative to that of nearby untransfected cells was measured. The data are mean ± SE of 10 cells. (D–G) Cultured hippocampal neurons were cotransfected with EYFP and the indicated shRNA expression vector at 11 DIV and then fixed at 15 DIV. Morphology of dendrites is shown by EYFP fluorescence. Scale bar, 5 μm. (H) Under the same conditions as those in D–G, the dendritic protrusions per 10 μm of dendrite were counted. The data are mean ± SE of 45 cells in three independent experiments. *$p < 0.05$; ***$p < 0.001$ (vs. shControl).

HA-ELMO2, and immunoprecipitated the cell lysates with anti-Myc antibody. Dock4 was coimmunoprecipitated with Myc-cortactin-CT, but Dock180 was not (Figure 5C). The reason for multiple bands in blots of Myc-cortactin-CT is unknown, but they were not detected in blots from cell lysates without Myc-cortactin-CT expression (data not shown). Among Dock180-related proteins, only Dock4 contains an amino acid sequence within the proline-rich region (amino acids 1796–1803: SPPVPPRP) that was similar to a predicted binding motif for the SH3 domain of cortactin (+PPXPXKP; Sparks *et al.*, 1996), which could explain the difference in the ability

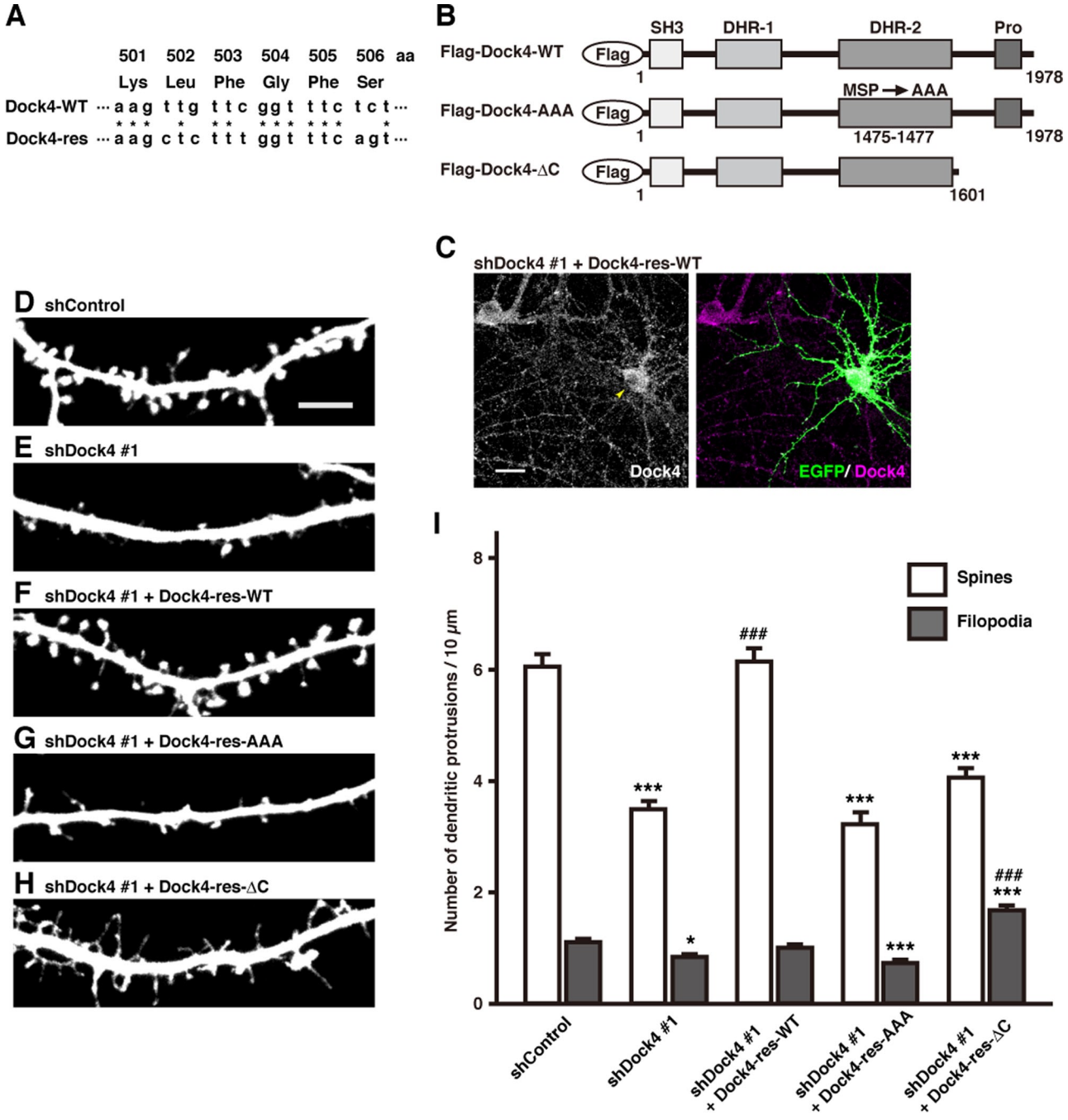

FIGURE 3: The GEF activity and the proline-rich region are required for Dock4-mediated spine formation. (A) Construction of shRNA-resistant Dock4 (Dock4-res) by silent mutagenesis. Sequence alignment of Dock4 and Dock4-res in the shDock4 #1 region is shown. Numbers indicate amino acid positions within the sequence, and asterisks indicate identical nucleotides. (B) Schematic diagram of Dock4 constructs used in this study. DHR, Dock-homology region; Pro, proline-rich region; SH3, Src-homology 3 domain. Numbers indicate amino acid positions in the sequence. (C) Primary cultured rat hippocampal neurons were cotransfected with EGFP, shDock4 #1, and Dock4-res-WT expression vector at 11 DIV. At 4 d after transfection, neurons were fixed and stained with antibody against Dock4 (magenta). Arrowheads indicate the transfected cells. Scale bar, 20 μm. (D–H) Cultured hippocampal neurons were cotransfected with EYFP and the indicated plasmids at 11 DIV and then fixed at 15 DIV. Morphology of dendrite segments is shown by EYFP fluorescence. Scale bar, 5 μm. (I) Under the same conditions as those in D–H, the dendritic protrusions per 10 μm of dendrite were counted. The data are mean ± SE of 45 cells in three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ (vs. shControl), $^{\#\#\#}p < 0.001$ (vs. shDock4 #1).

to bind to cortactin between Dock4 and Dock180. On the other hand, endogenous interaction between Dock4 and cortactin was observed in 16-DIV cultured rat hippocampal neurons by immunoprecipitation with anti-Dock4 antibody (Figure 5D). Furthermore, immunofluorescence showed that endogenous cortactin, which was highly concentrated at dendritic spines, as reported

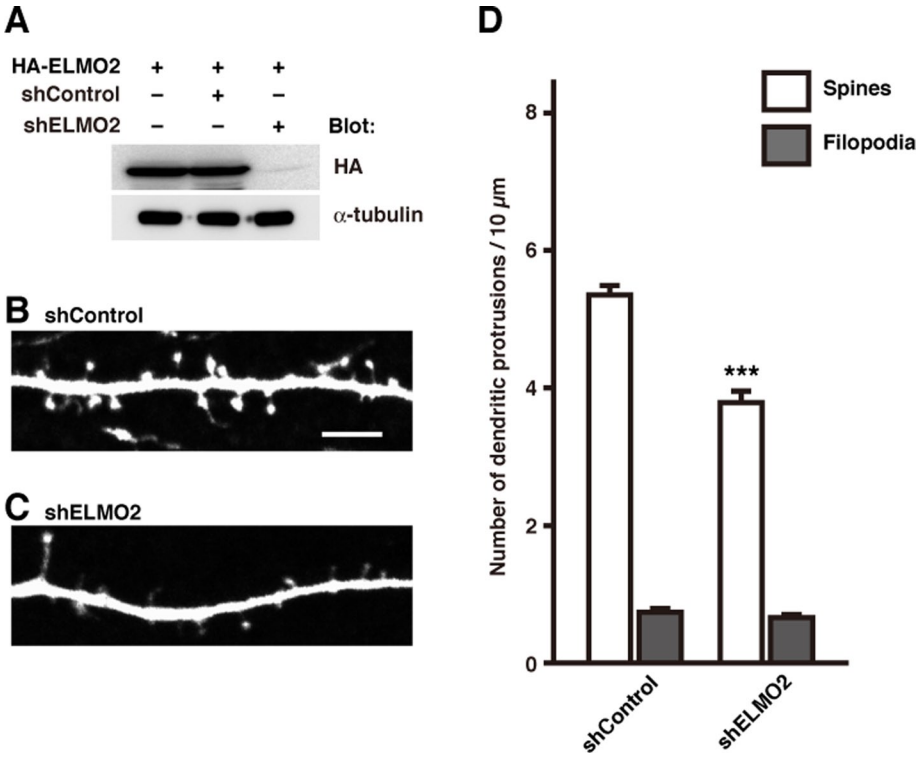

FIGURE 4: Knockdown of ELMO2 suppresses dendritic spine formation. (A) Cell lysates from HEK293T cells cotransfected with control or ELMO2 shRNA and HA-tagged ELMO2 were analyzed by immunoblotting with antibodies against HA and α-tubulin. (B, C) Cultured hippocampal neurons were cotransfected with EYFP and the indicated shRNA expression vector at 11 DIV and then fixed at 15 DIV. Morphology of dendrites is shown by EYFP fluorescence. Scale bar, 5 μm. (D) Under the same conditions as those in B and C, the dendritic protrusions per 10 μm of dendrite were counted. The data are mean ± SE of 45 cells in three independent experiments. ***$p < 0.001$.

previously (Hering and Sheng, 2003), colocalized with Dock4 (Figure 5, E and F).

Cortactin is necessary for Dock4-mediated spine formation

To determine whether cortactin is involved in Dock4-mediated spine formation, we generated an effective shRNA against cortactin (shCTTN; Figure 6, A and B) to investigate the effect of cortactin knockdown on spine formation. Knockdown of cortactin in hippocampal neurons significantly reduced the spine density (shControl, 6.32 ± 0.28 spines and 1.00 ± 0.06 filopodia/10 μm; shCTTN, 2.91 ± 0.18 spines and 0.91 ± 0.06 filopodia/10 μm; Figure 6, C, D, and G), as previously reported (Hering and Sheng, 2003). We next assessed the effect of Dock4 overexpression on spine formation in hippocampal neurons. Cultured hippocampal neurons were cotransfected with Dock4 and ELMO2 together with shCTTN or shControl, and protrusion density was analyzed. Overexpression of Dock4 and ELMO2 with shControl led to an increase in spine density compared with that observed in neurons expressing shControl alone (7.90 ± 0.20 spines and 0.85 ± 0.05 filopodia/10 μm; Figure 6, E and G). However, knockdown of cortactin completely suppressed the spine formation induced by overexpression of Dock4 and ELMO2. On the other hand, overexpression of Dock4 and ELMO2 in cortactin knockdown neurons increased filopodia density (3.53 ± 0.27 spines and 1.59 ± 0.08 filopodia/10 μm; Figure 6, F and G). Of interest, these phenotypes are similar to those observed in neurons coexpressing Dock4-res-ΔC with shDock4. Taken together, these results suggest that cortactin is required for Dock4-mediated spine formation.

The C-terminal, proline-rich region is required for spine localization of Dock4

To investigate the role of the C-terminal, proline-rich region of Dock4, we compared the distribution of Flag-Dock4-WT and Flag-Dock4-ΔC in dendrites of cultured hippocampal neurons. To minimize the effect of exogenous Dock4-WT or Dock4-ΔC on spine morphology, we fixed hippocampal neurons and stained them at 24 h after transfection (transfection at 14 DIV and fixation at 15 DIV). Flag-Dock4-WT was highly localized to spines and showed a similar distribution to that of endogenous Dock4, whereas Flag-Dock4-ΔC was observed in dendritic shafts (Figure 7, A and B). Fluorescence intensity analyses along magenta lines drawn in Figure 7, A and B, showed that the anti-Flag immunofluorescence peak of Dock4-WT was observed in the spine, whereas the peak of Dock4-ΔC was observed in the dendritic spine and shaft equally (Figure 7, C and D). On the other hand, cortactin localization to spines was unaltered by the expression of Dock4-ΔC. We also quantified the anti-Flag mean fluorescence intensity of the spine head and the dendritic shaft and expressed them as a ratio (spine/shaft fluorescence intensity). These quantitative analyses confirmed that Dock4-ΔC lost the specific localization to spines (Dock4-WT, 2.93 ± 0.23; Dock4-ΔC, 1.33 ± 0.21; Figure 7E). These results indicate that the C-terminal, proline-rich region of Dock4 is necessary for its localization to dendritic spines.

DISCUSSION

Dendritic spines are actin-rich structures, and regulation of the actin cytoskeletal reorganization is critical for dendritic spine formation and plasticity (Fischer *et al.*, 1998). The Rho-family GTPase Rac is well known as a molecular switch for signal transduction regulating the actin cytoskeleton in diverse cellular functions (Hall, 1998) and also as a key player in dendritic spine morphogenesis in excitatory neurons (Nakayama *et al.*, 2000; Tashiro *et al.*, 2000). In the present study, we show that the Rac activator Dock4 is localized in dendritic spines in hippocampal neurons and positively regulates spine formation. Our results suggest that both the Rac GFF domain and the C-terminal, proline-rich region of Dock4 play important roles in spine formation. We also find that the F-actin–binding protein cortactin interacts with the C-terminal, proline-rich region of Dock4 and is required for Dock4-mediated spine formation. Collectively our findings reveal a novel function of Dock4 in dendritic spines in hippocampal neurons.

Recent studies showed that several Rac GEFs are localized in dendritic spines and involved in spine morphogenesis (Tolias *et al.*, 2011). Among them, Kalirin-7 and Tiam1 form complexes with *N*-methyl-D-aspartate (NMDA) receptors, and their GEF activities are enhanced by calcium/calmodulin-dependent kinase II

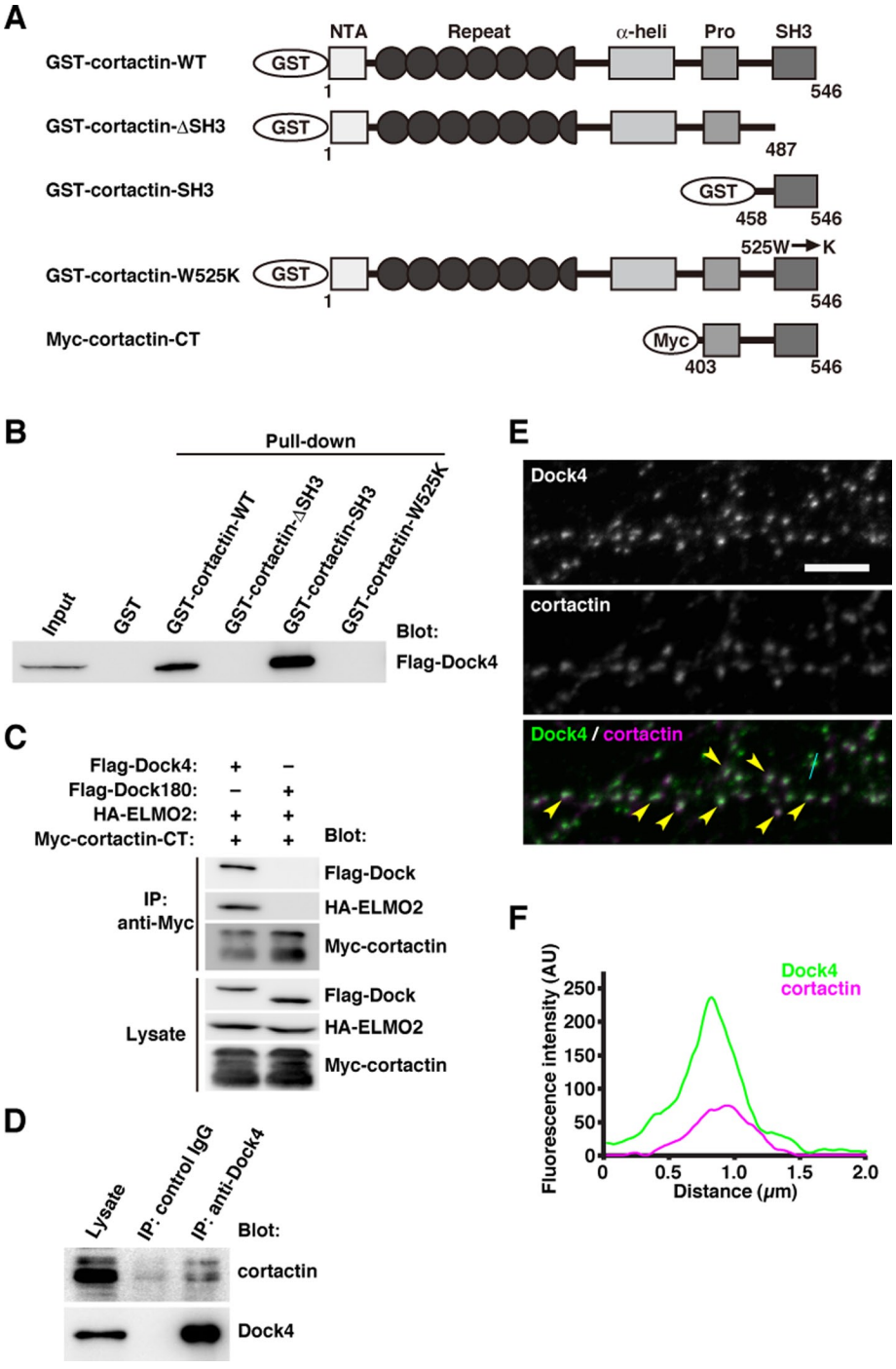

FIGURE 5: Cortactin is a novel binding partner of Dock4. (A) Schematic diagrams of cortactin constructs used in this study. NTA, N-terminal acidic region; Repeat, tandem repeat of 37–amino acid actin-binding domain; α-heli, α-helical region. Numbers indicate amino acid position in the sequence. (B) Cell lysates from HEK293T cells transfected with Flag–Dock4 were used in pull-down assay with GST or GST-fused cortactin proteins. (C) Cell lysates from HEK293T cells transfected with the indicated plasmids were immunoprecipitated with anti-Myc antibody, and bound proteins and lysates were analyzed by immunoblotting. (D) Cell lysates from cultured hippocampal neurons at 16 DIV were immunoprecipitated with anti-Dock4 antibody or control immunoglobulin G, and bound proteins and total cell lysates were analyzed by immunoblotting with anti-Dock4 and anti-cortactin antibodies. (E) Cultured hippocampal neurons at 15 DIV were fixed and double stained with anti-Dock4 (green) and anti-cortactin (magenta) antibodies. Arrowheads indicate the dendritic spines where cortactin colocalized with Dock4. Scale bar, 5 μm. (F) Fluorescence intensity profiles along cyan line drawn in E were measured in corresponding channels.

(CaMKII)–mediated phosphorylation after glutamate stimulation of the NMDA receptor (Xie *et al.*, 2007; Tolias *et al.*, 2005). Kalirin-7 and Tiam1 are also translocated into the postsynaptic region by interacting with the EphB receptor after ephrinB ligand stimulation and enhance Rac activity in the spines (Penzes *et al.*, 2003; Tolias *et al.*, 2007). βPIX is recruited to spines by G-protein–coupled receptor kinase–interacting protein 1, and its GEF activity is also enhanced by CaMKI-mediated phosphorylation (Zhang *et al.*, 2003; Saneyoshi *et al.*, 2008). On the other hand, Dock4 forms a complex with the actin-binding protein cortactin in dendritic spines and regulates spine formation via activation of Rac. Therefore Dock4 and other Rac GEFs are targeted to dendritic spines by different systems and may generate spatiotemporally diverse regulation of Rac activity.

Cortactin binds to F-actin via the central tandem repeat region and to Arp2/3 complex via the N-terminal acidic region and promotes polymerization, branching, and stabilization of the actin cytoskeleton (Weed *et al.*, 2000; Uruno *et al.*, 2001; Weaver *et al.*, 2001). Cortactin is highly enriched in dendritic spines, where it colocalizes with F-actin and positively regulates spine formation (Hering and Sheng, 2003; Chen and Hsueh, 2012). Dock4 also colocalizes with F-actin and cortactin in spines, and Dock4-mediated spine formation requires cortactin. Cortactin interacts with the C-terminal, proline-rich region of Dock4, and deletion of this region causes a dramatic change in the localization of Dock4. Therefore the interaction with cortactin may play an important role in spine localization of Dock4. However, we could not provide direct evidence of the involvement of cortactin in spine targeting of Dock4 because knockdown of cortactin results in the loss of dendritic spines. On the other hand, previous studies reported that cortactin interacts with various signaling molecules, such as N-WASP, WIP, Fgd1, ZO-1, and dynamin, through the C-terminal SH3 domain and contributes to diverse cellular processes, including cell migration, adhesion, and endocytosis (Daly, 2004; Ammer and Weed, 2008; Kirkbride *et al.*, 2011). Thus it is possible that cortactin functions as a scaffold protein to recruit these signaling molecules to the actin cytoskeleton. Of interest, the intracellular localization of cortactin is regulated by the activation of Rac1 in fibroblasts (Weed *et al.*, 1998). On the basis of these findings, we hypothesize that Dock4 may be recruited to the F-actin–rich region in dendritic spines via interaction with

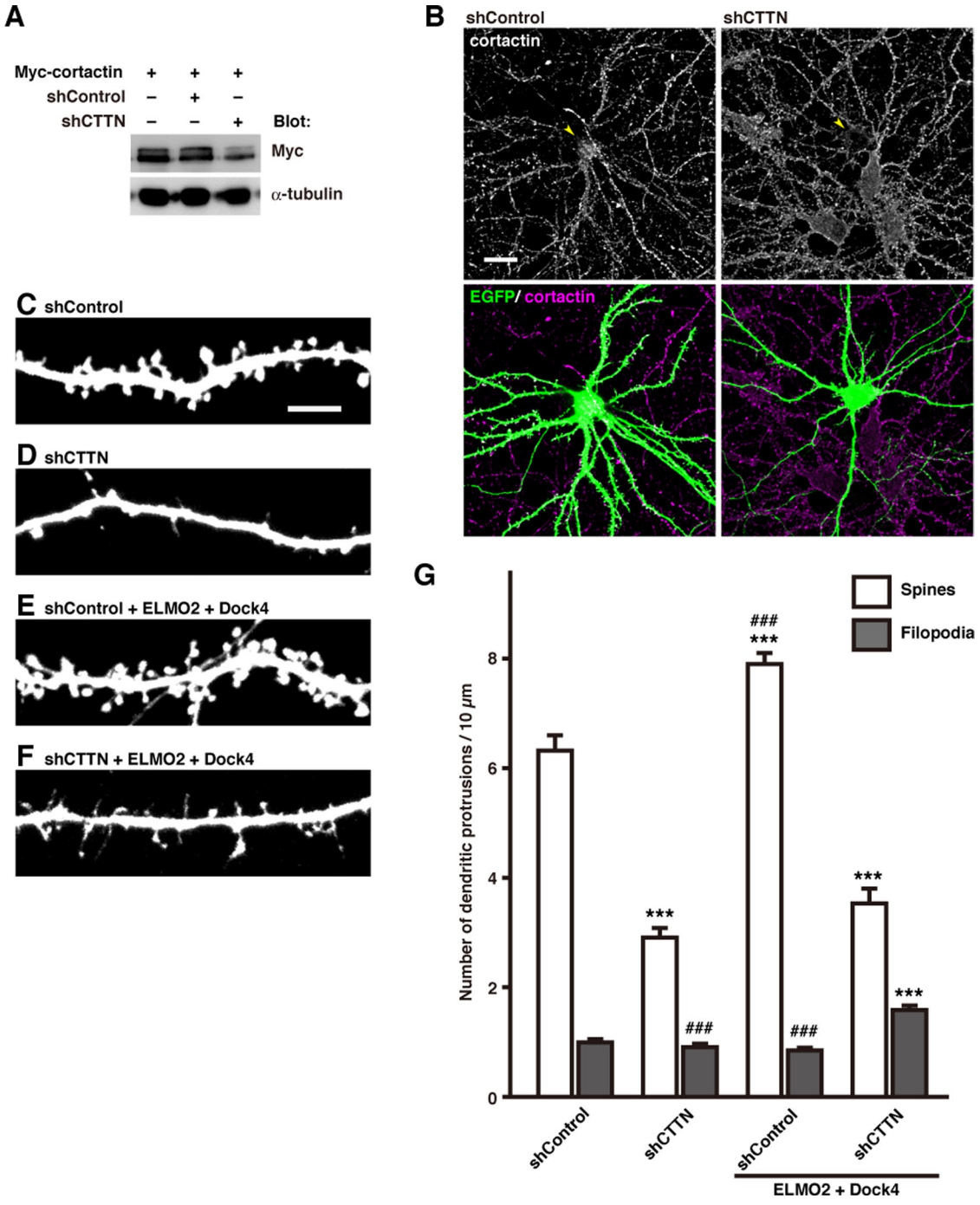

FIGURE 6: Cortactin is required for Dock4-mediated spine formation. (A) Cell lysates from HEK293T cells cotransfected with control or cortactin shRNAs and Myc-tagged cortactin were analyzed by immunoblotting with antibodies against Myc and α-tubulin. (B) Knockdown of endogenous cortactin in hippocampal neurons. Primary cultured rat hippocampal neurons were cotransfected with EGFP and the indicated shRNA expression vector at 11 DIV. At 4 d after transfection, neurons were fixed and stained with antibody against cortactin (magenta). Arrowheads indicate the transfected cells. Scale bar, 20 μm. (C–F) Cultured hippocampal neurons were cotransfected with EYFP and the indicated plasmids at 11 DIV and then fixed at 15 DIV. Morphology of dendrite segments is shown by EYFP fluorescence. Scale bar, 5 μm. (G) Under the same conditions as those in C–F, the dendritic protrusions per 10 μm of dendrite were counted. The data are mean ± SE of 45 cells in three independent experiments. ***$p < 0.001$ (vs. shControl), ###$p < 0.001$ (vs. shCTTN + ELMO2 + Dock4).

cortactin and promote actin polymerization via activation of Rac, which in turn recruits additional cortactin with Dock4 to the actin cytoskeleton, providing a positive feedback mechanism regulating the actin cytoskeleton by cortactin and Dock4 in dendritic spines.

Thin and long dendritic protrusions found in immature neurons are called dendritic filopodia. They are believed to be precursors of dendritic spines and important for initiation of synaptogenic contacts (Ziv and Smith, 1996). In this study, expression of a mutant of

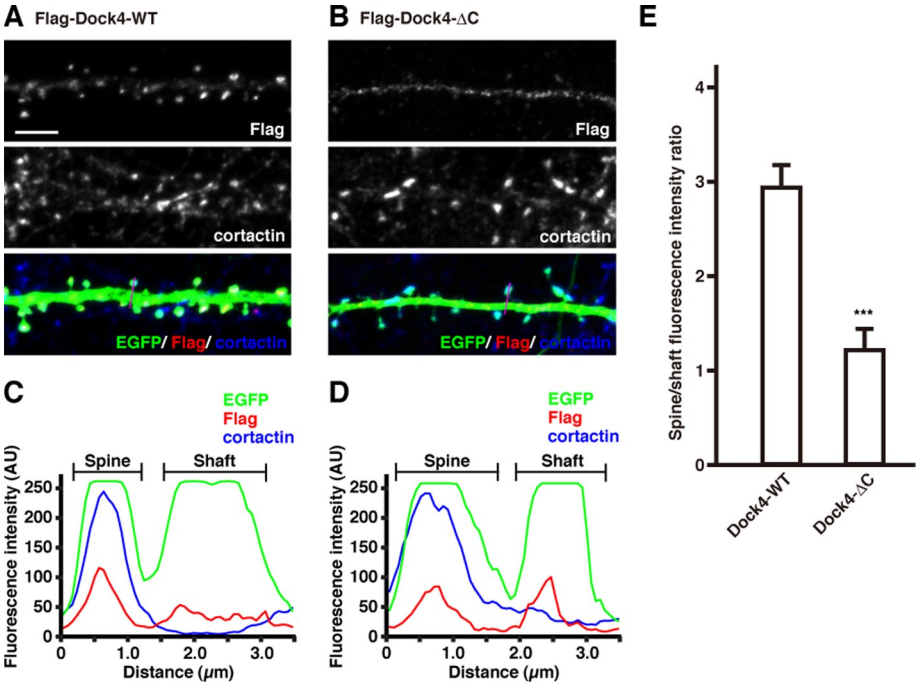

FIGURE 7: The proline-rich region is necessary for spine targeting of Dock4. (A, B) Primary cultured hippocampal neurons were cotransfected with ELMO2 and Flag-tagged Dock4-WT (A) or Dock4-ΔC (B) together with EGFP at 14 DIV and then fixed and stained at 15 DIV with antibody against Flag (magenta). Scale bar, 5 μm. (C, D) Fluorescence intensity profiles along magenta lines drawn in A and B were measured in corresponding channels. (E) Under the same conditions as those in A and B, relative anti-Flag fluorescence intensity ratio of spine/shaft normalized by EGFP fluorescence intensity was measured. The data are mean ± SE of 372 spines from 10 cells (Dock4-WT) and 270 spines from 10 cells (Dock4-ΔC). ***$p < 0.001$.

Dock4 lacking the cortactin-binding region in Dock4-knockdown neurons or overexpression of wild-type Dock4 in the absence of cortactin results in a decrease in the number of mushroom-shaped mature spines and an increase in the number of dendritic filopodia. These observations suggest that interaction between Dock4 and cortactin is necessary for spine maturation and/or mature spine maintenance. This is supported by a previous report that mutants of cortactin lacking the C-terminal region, which contains the Dock4-binding domain, significantly reduced the spine head width, even though these mutants can localize to dendritic spines (Hering and Sheng, 2003). On the other hand, it remains unclear whether Dock4 also regulates spine initiation. From the result that expression of Dock4 without cortactin interaction increases filopodia density, it is possible that Dock4 also regulates spine initiation by a cortactin-independent manner. Further investigations are required to determine at what stages (e.g., initiation, maturation, stabilization, maintenance, and plasticity) Dock4 is involved in spine formation and to understand how Dock4 regulates spine morphology in hippocampal neurons during development.

DOCK4 has been reported to be a susceptibility gene for several neuropsychiatric disorders, such as ASDs, dyslexia, and schizophrenia, by family-based, genome-wide association studies (Maestrini *et al.*, 2010; Pagnamenta *et al.*, 2010; Alkelai *et al.*, 2012). These disorders are believed to be associated with the disturbance of neuronal connectivity (Penzes *et al.*, 2011). ASDs make up a behaviorally defined syndrome characterized by deficits in social interaction, disruption of verbal and nonverbal communication, and the presence of repetitive, stereotyped behaviors (Lord *et al.*, 2000); they become established usually around 2–3 yr of age (Cox *et al.*, 1999). On the other hand, schizophrenia is a mental disorder characterized by auditory and visual hallucinations, paranoia, delusions, blunted affect, avolition, and social withdrawal; it typically emerges in late adolescence or early adulthood (Lewis and Lieberman, 2000). Anatomical studies of hippocampal neurons have reported reduction in dendritic complexity and decrease in spine volume and number in individuals with autism and schizophrenia, respectively (Raymond *et al.*, 1996; Kolomeets *et al.*, 2005). Because Dock4 is involved in regulation of spine density in addition to its role in dendritic growth and branching in hippocampal neurons (Ueda *et al.*, 2008), dysfunction of Dock4 in dendrites during development might contribute to the pathogenesis of those psychiatric disorders. Further studies might allow us to better understand the biological mechanisms underlying these disorders and provide insights for new therapies.

MATERIALS AND METHODS

Antibodies and reagents

A rabbit polyclonal antibody against Dock4, which was used for immunoprecipitation, was obtained as described previously (Hiramoto *et al.*, 2006; Supplemental Figure S1A). The other antibodies were purchased commercially: a mouse monoclonal antibody against Dock4 used for immunoblotting and immunostaining (R6Y; Supplemental Figure S1B), a mouse monoclonal anti-Myc antibody (9E10), and a rabbit polyclonal antibody against cortactin (H-191; Santa Cruz Biotechnology, Santa Cruz, CA); a mouse monoclonal antibody against cortactin (4F11; Millipore, Billerica, MA); rabbit monoclonal antibodies against PSD95 (EP2652Y) and synaptophysin (YE269) and a rabbit polyclonal antibody against MAP2 (Abcam, Cambridge, MA); mouse monoclonal antibodies against Flag (M2) and α-tubulin (B-5-1-2; Sigma-Aldrich, St. Louis, MO); a rat monoclonal antibody against hemagglutinin (HA; 3F10; Roche, Indianapolis, IN); secondary antibodies conjugated to horseradish peroxidase (HRP; Dako, Carpinteria, CA); and secondary antibodies conjugated to Alexa Fluor 488, 555, and 647 (Invitrogen, Carlsbad, CA). F-actin was visualized with rhodamine-phalloidin (Invitrogen).

Plasmid constructs

The expression plasmid encoding Flag-tagged human Dock180 (pCXN2) was a gift from M. Matsuda (Kyoto University, Kyoto, Japan). EYFP expression vector (pCAG) was a gift from J. Miyazaki (Osaka University, Osaka, Japan) and T. Saito (Chiba University, Chiba, Japan). Flag-tagged mouse Dock4-WT, Dock4-AAA (M1475A, S1476A, P1477A), Dock4-ΔC (amino acids 1–1601), and HA-tagged rat ELMO2 were generated as described previously (Katoh and Negishi, 2003; Hiramoto *et al.*, 2006). EGFP was inserted into pCXN2. Mouse cortactin was obtained by reverse transcription PCR from mouse brain and subcloned into pCMV-Myc (Clontech, Mountain View, CA) or pGEX-4T-2 (GE Healthcare, Piscataway, NJ). Cortactin-ΔSH3 (amino acids 1–487), cortactin-SH3 (amino acids 458–546), and cortactin-W525K were generated by PCR-mediated

mutagenesis and subcloned into pGEX-4T-2. All shRNAs used in this study were expressed by using an shRNA expression vector, pSilencer-hygro (Ambion, Austin, TX). The shRNAs for Dock4 were designed to target 19 or 21 nucleotides of the mouse transcript (shControl, nucleotides 114–132, 5′-GTGCGACGGCTGGTACAGA-3′; shDock4 #1, nucleotides 1500–1518, 5′-GAAGTTGTTCGGTTTCTCT-3′; shDock4 #2, nucleotides 3685–3705, 5′-GCCTACACTCTCCTGTTGTAT-3′). The shRNA for ELMO2 was designed to target 19 nucleotides of the mouse transcript (shELMO2, nucleotides 1998–2016, 5′-GGATATGTCCAGCGAGCTA-3′). The shRNA for cortactin was designed based on an shRNA sequence in a previous report (Hering and Sheng, 2003; shCTTN, nucleotides 330–348, 5′-GCACTGCTCACAAGTGGAC-3′). The Dock4 shRNA–resistant constructs, Dock4-res, were obtained by introducing silent mutations within the target sequence of shDock4 #1 (nucleotides 1500–1518 were replaced with 5′-GAAGCTCTTTGGTTTCAGT-3′).

Immunohistochemistry

All rats used in this study were purchased from Japan SLC (Hamamatsu, Japan) and treated in accordance with the guidelines of the Animal Care and Use Committee of Graduate School of Biostudies at Kyoto University. Postnatal day 20 Wistar rats were anesthetized and transcardially perfused. Isolated brains were fixed with 4% paraformaldehyde and then saturated with 30% sucrose in phosphate-buffered saline (PBS). Frozen brains were sliced in 30-µm-thick coronal sections using a cryostat (CM3050S; Leica, Wetzlar, Germany). Sections were washed with PBS, permeabilized with 0.3% Triton X-100 in PBS for 15 min, and then blocked with PBS containing 2% goat serum and 0.15% Triton X-100 for 1 h at room temperature. The sections were incubated with the primary antibodies for 24 h at 4°C. After washing with PBS containing 0.1% Tween 20, the sections were incubated with the appropriate secondary antibodies and rhodamine–phalloidin for 24 h at 4°C. Then they were washed with PBS containing 0.1% Tween 20 and mounted with ProLong Gold Antifade Reagents (Invitrogen). Confocal images were acquired using a laser-scanning confocal imaging system (FLUOVIEW FV1000-D; Olympus, Tokyo, Japan) and a microscope equipped with spectral system (IX81-S; Olympus) with a 10×/numerical aperture (NA) 0.40 dry or a 60×/NA 1.35 oil objective (Olympus). The images were arranged and labeled using Photoshop (Adobe, San Jose, CA).

Cell culture and transfection

Primary hippocampal neurons were prepared from the hippocampi of embryonic day 19 (E19) Wistar rats as described previously (Ueda *et al.*, 2008) and seeded on 24-well culture plates containing glass coverslips (circular, 13 mm in diameter; Matsunami Glass, Osaka, Japan) coated with poly-L-lysine (Sigma-Aldrich) at a density of 3×10^4 cells in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and cultured under humidified air containing 5% CO_2 at 37°C for 5 h. Then culture medium was replaced with Neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen), 0.5 mM GlutaMAX (Invitrogen), 50 U/ml penicillin, and 0.05 mg/ml streptomycin, and neurons were cultured under humidified air containing 5% CO_2 at 37°C. Transient transfections were carried out at 11 or 14 DIV using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

HEK293T cells were cultured in DMEM containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin under humidified air containing 5% CO_2 at 37°C, and transient transfections were carried out using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions.

Pull-down assay, immunoprecipitation, and immunoblotting

For GST pull-down assays, recombinant GST-fusion proteins were purified from *E. coli* as described previously (Katoh *et al.*, 1998). HEK293T cells transfected with Flag-tagged Dock4 were lysed with the ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Cell lysates were then centrifuged for 10 min at 16,000 × *g* at 4°C. The supernatants were incubated for 10 min at 4°C with 10 µg GST or GST-fused cortactin proteins and subsequently with glutathione–Sepharose beads for 1 h at 4°C. After washing with ice-cold cell lysis buffer, the bound proteins were analyzed by SDS–PAGE and immunoblotting.

For immunoprecipitation, HEK293T cells cotransfected with the indicated plasmids, or primary cultured hippocampal neurons were lysed for 10 min with ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). After centrifugation for 10 min at 16,000 × *g*, the supernatants were incubated with indicated antibodies for 1 h, followed by incubation with protein G–Sepharose (GE Healthcare) for 1 h. Then the beads were washed with the cell lysis buffer, and the bound proteins were analyzed by SDS–PAGE and immunoblotting.

For immunoblot analysis, proteins were separated by SDS–PAGE and were electrophoretically transferred onto polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 3% low-fat milk in Tris-buffered saline and then incubated with primary antibodies. The primary antibodies were detected with HRP-conjugated secondary antibodies and chemiluminescence detection kits (Chemi-Lumi One [Nacalai Tesque, Kyoto, Japan] or ECL Prime [GE Healthcare]). Images were captured using a LAS3000 analyzer (Fujifilm, Tokyo, Japan).

Immunofluorescence microscopy

Primary cultured hippocampal neurons on coverslips were fixed with 4% paraformaldehyde in PBS for 15 min. After residual paraformaldehyde had been quenched with 50 mM NH_4Cl in PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated with 10% FBS in PBS for 30 min to block nonspecific antibody binding. Then cells were incubated with primary antibodies in Can Get Signal Immunostain Immunoreaction Enhancer SolutionA (Toyobo, Osaka, Japan) for 1 h. After wash with PBS, cells were incubated with the appropriate Alexa-conjugated secondary antibodies and rhodamine–phalloidin for 1 h, washed with PBS, and mounted in ProLong Gold Antifade Reagents (Invitrogen). The Z-stacked images were acquired using a laser-scanning confocal imaging system (FLUOVIEW FV1000-D; Olympus) and a microscope equipped with spectral system (IX81-S; Olympus) with a 60×/NA 1.35 oil objective (Olympus; five planes with 0.52-µm step width per stack; Figures 1, 5, and 7) or a laser-scanning confocal imaging system (EZ-C1 version 3.20 software; Nikon, Melville, NY) and a microscope (Eclipse TE2000-U; Nikon) with a 60×/NA 1.40 oil objective (Nikon) and a digital camera (DXM1200C; Nikon; six planes with 0.3-µm step width per stack; Figures 2, 3, 4, and 6). The images were arranged and labeled using Photoshop (Adobe).

Image analysis and quantification

Quantification of dendritic protrusion density was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Protrusions were manually counted along >250 µm of total dendrite segments per neuron. In this study, dendritic spines were defined as protrusions with mushroom-shaped heads or stubby-shaped

structures, and dendritic filopodia were defined as headless protrusions. At least 45 neurons per experimental group were collected from three independent experiments, and statistical differences for multiple groups were assessed by one-way analysis of variance and Tukey's post hoc test using SPSS 16.0 software (IBM, Armonk, NY).

Quantification of spine/shaft fluorescence intensity ratio was performed using Photoshop software. Averages of anti-Flag immunofluorescence intensity of >1 μm^2 of the spine head (to eliminate immature spines) and the dendritic shaft areas and EGFP fluorescence intensity of the same areas were measured. Then anti-Flag fluorescence values were normalized with EGFP fluorescence values as a volume marker, and spine/shaft ratios were calculated.

Yeast two-hybrid screening

A rat cDNA library fused to the GAL4 activation domain of the pACT2 vector (Clontech) was screened using pGBKT/Dock4-CT (amino acids 1602–1978) as bait in the yeast strain AH109 according to the manufacturer's instructions. Interaction between the bait and library proteins activates transcription of the reporter gene *HIS3*, *Ade*, and *lacZ*. From 8.7×10^6 transformants, 323 colonies grew on selective medium lacking histidine and adenine and were also positive for β-galactosidase activity. One of those was found to encode the C-terminal 144 amino acids of cortactin. For the β-galactosidase filter assay, colonies of yeast transformants were transferred onto Hybond-N filter papers (Amersham Biosciences, Piscataway, NJ) and permeabilized in liquid nitrogen. Each filter was placed on a Whatman No. 2 filter paper that had been presoaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 37.5 mM β-mercaptoethanol) containing 0.33 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside and was incubated at 30°C for 5–8 h.

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REFERENCES

Alkelai A, Lupoli S, Greenbaum L, Kohn Y, Kanyas-Sarner K, Ben-Asher E, Lancet D, Macciardi F, Lerer B (2012). *DOCK4* and *CEACAM21* as novel schizophrenia candidate genes in the Jewish population. Int J Neuropsychopharm 15, 459–469.

Ammer AG, Weed SA (2008). Cortactin branches out: roles in regulating protrusive actin dynamics. Cell Motil Cytoskeleton 65, 687–707.

Chen YK, Hsueh YP (2012). Cortactin-binding protein 2 modulates the mobility of cortactin and regulates dendritic spine formation and maintenance. J Neurosci 32, 1043–1055.

Choi J *et al.* (2005). Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. J Neurosci 25, 869–879.

Côté JF, Vuori K (2002). Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. J Cell Sci 115, 4901–4913.

Cox A, Klein K, Charman T, Baird G, Baron-Cohen S, Swettenham J, Drew A, Wheelwright S (1999). Autism spectrum disorders at 20 and 42 months of age: stability of clinical and ADI-R diagnosis. J Child Psychol Psychiatry 40, 719–732.

Daly RJ (2004). Cortactin signalling and dynamic actin networks. Biochem J 382, 13–25.

Fischer M, Kaech S, Knutti D, Matus A (1998). Rapid actin-based plasticity in dendritic spines. Neuron 20, 847–854.

Hall A (1998). Rho GTPases and the actin cytoskeleton. Science 279, 509–514.

Harris KM, Kater SB (1994). Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. Annu Rev Neurosci 17, 341–371.

Hering H, Sheng M (2003). Activity-dependent redistribution and essential role of cortactin in dendritic spine morphogenesis. J Neurosci 23, 11759–11769.

Hiramoto K, Negishi M, Katoh H (2006). Dock4 is regulated by RhoG and promotes Rac-dependent cell migration. Exp Cell Res 312, 4205–4216.

Hiramoto-Yamaki N, Takeuchi S, Ueda S, Harada K, Fujimoto S, Negishi M, Katoh H (2010). Ephexin4 and EphA2 mediate cell migration through a RhoG-dependent mechanism. J Cell Biol 190, 461–477.

Iki J, Inoue A, Bito H, Okabe S (2005). Bi-directional regulation of postsynaptic cortactin distribution by BDNF and NMDA receptor activity. Eur J Neurosci 22, 2985–2994.

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003). Structure-stability-function relationships of dendritic spines. Trends Neurosci 26, 360–368.

Katoh H, Aoki J, Ichikawa A, Negishi M (1998). p160 RhoA-binding kinase ROKα induces neurite retraction. J Biol Chem 273, 2489–2492.

Katoh H, Fujimoto S, Ishida C, Ishikawa Y, Negishi M (2006). Differential distribution of ELMO1 and ELMO2 mRNAs in the developing mouse brain. Brain Res 1073–1074, 103–108.

Katoh H, Negishi M (2003). RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. Nature 424, 461–464.

Kinley AW, Weed SA, Weaver AM, Karginov AV, Bissonette E, Cooper JA, Parsons JT (2003). Cortactin Interacts with WIP in regulating Arp2/3 activation and membrane protrusion. Curr Biol 13, 384–393.

Kirkbride KC, Sung BH, Sinha S, Weaver AM (2011). Cortactin: a multifunctional regulator of cellular invasiveness. Cell Adh Migr 5, 187–198.

Kolomeets NS, Orlovskaya DD, Rachmanova VI, Uranova NA (2005). Ultrastructural alterations in hippocampal mossy fiber synapses in schizophrenia: a postmortem morphometric study. Synapse 57, 47–55.

Lewis DA, Lieberman JA (2000). Catching up on schizophrenia: natural history and neurobiology. Neuron 28, 325–334.

Lin YC, Koleske AJ (2010). Mechanisms of synapse and dendrite maintenance and their disruption in psychiatric and neurodegenerative disorders. Annu Rev Neurosci 33, 349–378.

Lord C, Risi S, Lambrecht L, Cook EH Jr, Leventhal BL, DiLavore PC, Pickles A, Rutter M (2000). The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. J Autism Dev Disord 30, 205–223.

Maestrini E *et al.* (2010). High-density SNP association study and copy number variation analysis of the *AUTS1* and *AUTS5* loci implicate the *IMMP2L-DOCK4* gene region in autism susceptibility. Mol Psychiatry 9, 954–968.

Matus A, Ackermann M, Pehling G, Byers HR, Fujiwara K (1982). High actin concentrations in brain dendritic spines and postsynaptic densities. Proc Natl Acad Sci USA 79, 7590–7594.

Meller N, Merlot S, Guda C (2005). CZH proteins: a new family of Rho-GEFs. J Cell Sci 118, 4937–4946.

Meng Y *et al.* (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron 35, 121–133.

Nakayama AY, Harms MB, Luo L (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. J Neurosci 20, 5329–5338.

Newey SE, Velamoor V, Govek EE, Van Aelst L (2005). Rho GTPases, dendritic structure, and mental retardation. J Neurobiol 64, 58–74.

Pagnamenta AT *et al.* (2010). Characterization of a family with rare deletions in *CNTNAP5* and *DOCK4* suggests novel risk loci for autism and dyslexia. Biol Psychiatry 68, 320–328.

Penzes P, Beeser A, Chernoff J, Schiller MR, Eipper BA, Mains RE, Huganir RL (2003). Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. Neuron 37, 263–274.

Penzes P, Cahill ME, Jones KA, VanLeeuwen JE, Woolfrey KM (2011). Dendritic spine pathology in neuropsychiatric disorders. Nat Neurosci 14, 285–293.

Raymond GV, Bauman ML, Kemper TL (1996). Hippocampus in autism: a Golgi analysis. Acta Neuropathol 91, 117–119.

Saneyoshi T, Wayman G, Fortin D, Davare M, Hoshi N, Nozaki N, Natsume T, Soderling TR (2008). Activity-dependent synaptogenesis: regulation by a CaM-kinase kinase/CaM-kinase I/βPIX signaling complex. Neuron 57, 94–107.

Sparks AB, Rider JE, Hoffman NG, Fowlkes DM, Quilliam LA, Kay BK (1996). Distinct ligand preferences of Src homology 3 domains from Src, Yes,

Abl, Cortactin, p53bp2, PLCγ, Crk, and Grb2. Proc Natl Acad Sci USA 93, 1540–1544.
Tashiro A, Minden A, 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.
Tolias KF, Bikoff JB, Burette A, Paradis S, Harrar D, Tavazoie S, Weinberg RJ, Greenberg ME (2005). The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines. Neuron 45, 525–538.
Tolias KF, Bikoff JB, Kane CG, Tolias CS, Hu L, Greenberg ME (2007). The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development. Proc Natl Acad Sci USA 104, 7265–7270.
Tolias KF, Duman JG, Um K (2011). Control of synapse development and plasticity by Rho GTPase regulatory proteins. Prog Neurobiol 94, 133–148.
Ueda S, Fujimoto S, Hiramoto K, Negishi M, Katoh H (2008). Dock4 regulates dendritic development in hippocampal neurons. J Neurosci Res 86, 3052–3061.
Uruno T, Liu J, Zhang P, Fan Yx, Egile C, Li R, Mueller SC, Zhan X (2001). Activation of Arp2/3 complex-mediated actin polymerization by cortactin. Nat Cell Biol 3, 259–266.
Weaver AM, Karginov AV, Kinley AW, Weed SA, Li Y, Parsons JT, Cooper JA (2001). Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr Biol 11, 370–374.
Weed SA, Du Y, Parsons JT (1998). Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. J Cell Sci 111, 2433–2443.
Weed SA, Karginov AV, Schafer DA, Weaver AM, Kinley AW, Cooper JA, Parsons JT (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. J Cell Biol 151, 29–40.
Wu H, Parsons JT (1993). Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. J Cell Biol 120, 1417–1426.
Xie Z, Srivastava DP, Photowala H, Kai L, Cahill ME, Woolfrey KM, Shum CY, Surmeier DJ, Penzes P (2007). Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. Neuron 56, 640–656.
Yajnik V *et al*. (2003). *DOCK4*, a GTPase activator, is disrupted during tumorigenesis. Cell 112, 673–684.
Zhang H, Webb DJ, Asmussen H, Horwitz AF (2003). Synapse formation is regulated by the signaling adaptor GIT1. J Cell Biol 161, 131–142.
Ziv NE, Smith SJ (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17, 91–102.