Dock3 induces axonal outgrowth by stimulating membrane recruitment of the WAVE complex

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Edited by Thomas C. Südhof, Stanford University School of Medicine, Palo Alto, CA, and approved March 16, 2010 (received for review December 18, 2009)

Atypical Rho-guanine nucleotide exchange factors (Rho-GEFs) that contain Dock homology regions (DHR-1 and DHR-2) are expressed in a variety of tissues; however, their functions and mechanisms of action remain unclear. We identify key conserved amino acids in the DHR-2 domain that are critical for the catalytic activity of Dock-GEFs (Dock1–4). We further demonstrate that Dock-GEFs directly associate with WASP family verprolin-homologous (WAVE) proteins through the DHR-1 domain. Brain-derived neurotrophic factor (BDNF)-TrkB signaling recruits the Dock3/WAVE1 complex to the plasma membrane, whereupon Dock3 activates Rac and dissociates from the WAVE complex in a phosphorylation-dependent manner. BDNF induces axonal sprouting through Dock-dependent Rac activation, and adult transgenic mice overexpressing Dock3 exhibit enhanced optic nerve regeneration after injury without affecting WAVE expression levels. Our results highlight a unique mechanism through which Dock-GEFs achieve spatial and temporal restriction of WAVE signaling, and identify Dock-GEF activity as a potential therapeutic target for axonal regeneration.

Dock family proteins | brain-derived neurotrophic factor | Fyn | axonal regeneration | optic nerve

The Rho-family GTPases (Rho-GTPases, including Rac1, Cdc42, and RhoA), which are best known for their roles in regulating the actin cytoskeleton, have been implicated in a broad spectrum of biological functions, such as cell motility and invasion, cell growth, cell survival, cell polarity, clearance of apoptotic cells, membrane protrusion, and axonal guidance (1, 2). Activation signals from Rac1 and Cdc42 are relayed to the actin-nucleating complex Arp2/3 by a family of proteins that includes Wiskott–Aldrich syndrome protein (WASP) and WASP family verprolin-homologous protein (WAVE) (3, 4). Rho-GTPase activation is mediated by guanine nucleotide exchange factors (GEFs), which share common motifs: the Dbl-homology (DH) domain, which mediates nucleotide exchange (5), and the pleckstrin homology (PH) domain, which targets proteins to membranes and mediates protein–protein interactions (6). Dock1 (Dock180)–related proteins are a new family of Rho-GEFs that lack the DH/PH domains. Instead, Dock family proteins are characterized by two evolutionarily conserved protein domains, termed Dock homology regions 1 and 2 (DHR-1 and DHR-2, respectively) (7). However, the precise functions of the DHR domains are poorly understood. In mammals, there are 11 Dock1-related proteins (Dock1–11). We previously reported that one of the new Rho-GEFs, Dock3, also known as MOCA (modifier of cell adhesion protein), is specifically expressed in the central nervous system (CNS) and induces membrane spreading by activating Rac1 (8).

Neurotrophins are a small family of evolutionarily well-conserved neuropeptides that function in neural cell survival, development, and vertebrate CNS function (9, 10). Neurotrophins, such as brain-derived neurotrophic factor (BDNF), bind to two classes of receptors, the Trk receptor tyrosine kinase family (TrkA, TrkB, TrkC) and the low-affinity receptor p75 ($p75^{NTR}$), leading to complex functional interactions (11, 12). Recent studies have demonstrated that neurotrophins play important roles during gastrulation, in the differentiation of hepatic stellate cells, and in neurulation, through the regulation of Rho-GTPases (9, 13, 14). Considering their relatively ubiquitous expression patterns, it is conceivable that Dock family members, in combination with neurotrophins, could be involved in the development and function of a variety of organs (15–17).

Here, we report that the atypical Rho-GEFs Dock1–4 share key amino acids within the DHR-2 domain that are critical for catalytic activity, and furthermore, that they bind directly to WAVE proteins through the DHR-1 domain. Moreover, we provide both in vitro and in vivo evidence that neuron-specific Dock3 plays a critical role in the membrane trafficking of WAVE proteins and also participates in axonal outgrowth in the adult CNS as an essential downstream component of BDNF-TrkB signaling.

Results

Identification of Critical Residues Involved in GEF Catalytic Activity. To identify residues critical for Rac1 activation, we constructed several Dock3 DHR-2 domain deletion mutants (Fig. 1*A*) and measured their catalytic activity with a GST-CRIB assay (Fig. 1*B*). All of the deletion mutants failed to activate Rac1 (Fig. 1*B*), indicating that amino acids 1358–1375 are required for Dock3-mediated GEF activity. We next introduced single alanine mutations into each of the 18 DHR-2 domain amino acids within full-length Dock3 and found that mutation at F1359, Y1360, G1361, Y1373, or V1374 completely abolished Rac1 activation (Fig. 1*C*) without affecting its ability to interact with Rac1 (Fig. 1*D*). Because the DHR-2 domain is highly conserved among Dock1–4 (Fig. 1*A*), the five single alanine substitutions corresponding to those found in Dock3 were introduced into Dock1, Dock2, and Dock4. All of the mutations drastically reduced Rac1 activation (Fig. S1), demonstrating that these five amino acids of the DHR-2 domain are critical for GEF catalytic activity in all Rac-specific Dock family members.

Dock3 Regulates Axonal Outgrowth. To evaluate whether Dock3 induces axonal outgrowth, we transfected primary cultured hippocampal neurons with plasmid encoding Dock3. Overexpression of Dock3 significantly stimulated axonal outgrowth compared with the control (Fig. 2*A* and *B*). As neurotrophic factors such as BDNF may also regulate Rac1 activation (18), we next examined whether Dock3 and BDNF have a synergistic effect on axonal outgrowth. Overexpression of wild-type (WT) Dock3 increased BDNF-mediated

Author contributions: K.N. and T.H. designed research; K.N., C.H., C.T., and X.G. performed research; K.N., C.T., H.K., and L.F.P. contributed new reagents/analytic tools; K.N., C.H., X.G., L.F.P., and T.H. analyzed data; and K.N., L.F.P., and T.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0914514107/DCSupplemental.

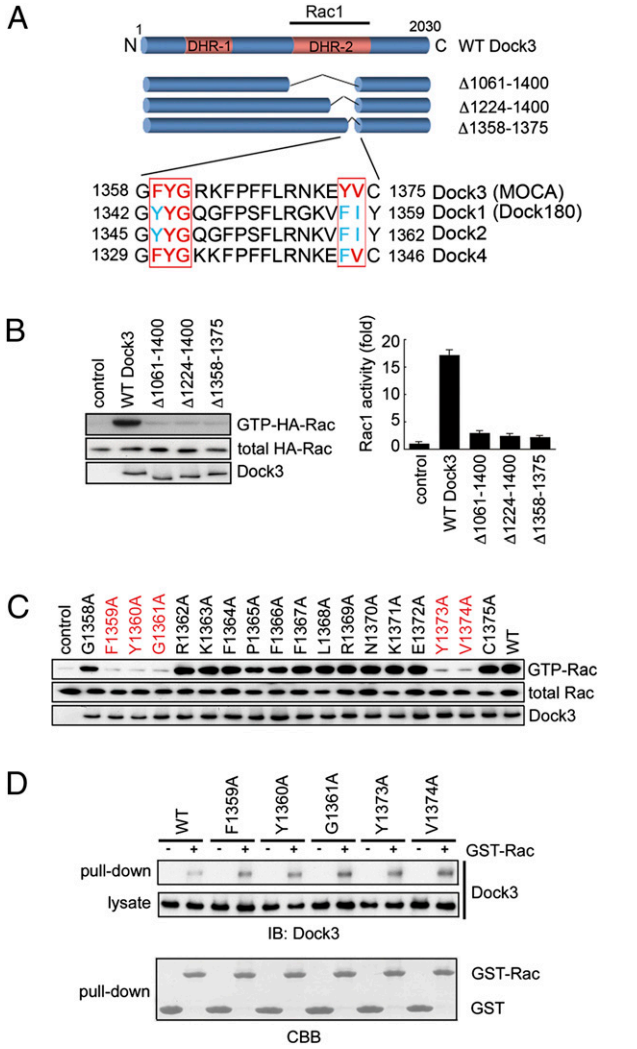

Fig. 1. Identification of residues critical for the catalytic activity of Dock family members. (*A*) Domain organization and sequence alignments of Dock1–4. (*B*) (*Left*) Cos-7 cells were transfected with Dock3 deletion mutants. Lysates were subjected to a GST-CRIB assay. (*Right*) Quantitation of GTP-Rac. (*C*) Cos-7 cells were transfected with the indicated alanine-substitution mutants of Dock3. Lysates were subjected to a GST-CRIB assay. (*D*) Cos-7 cells were transfected with the indicated alanine-substitution mutants of Dock3. Lysates were incubated with GST-Rac and glutathione-Sepharose. Bound proteins were subjected to immunoblot analysis with anti-Dock3 antibody. (*Lower*) Coomassie Brilliant Blue (CBB) staining of GST-fusion proteins used in this experiment.

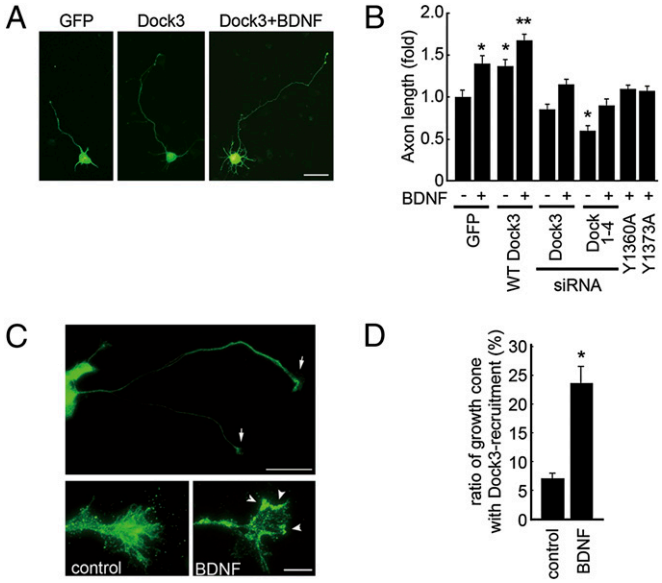

Fig. 2. Dock3 enhances BDNF-mediated axonal growth. (*A*) Hippocampal neurons were transfected with Dock3 and cultured in the presence or absence of BDNF for 3 days. (Scale bar, 20 μm.) (*B*) Hippocampal neurons transfected and treated as shown at the *x* axis were fixed at 3 days in vitro, and axon length was measured. $n = 30$ per experimental condition. Data are mean ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$. (*C*) Expression of Dock3 in retinal explant cultures. (*Upper*) Dock3 expression in growth cones (arrows). (*Lower*) High magnification of growth cones. Arrowheads indicate Dock3 recruitment to plasma membrane after BDNF treatment. (Scale bars, 50 μm in upper and 10 μm in lower.) (*D*) Ratio of growth cones in which Dock3 was recruited to plasma membrane. Data are mean ± SEM; $n = 20$ for each experimental condition. $*P < 0.01$.

axonal outgrowth (Fig. 2 *A* and *B*), whereas Dock3 siRNA for downregulating endogenous Dock3 inhibited the effect of BDNF (Fig. 2*B*). Combinatorial siRNA to Dock1–4 decreased the baseline axon length (Fig. 2*B*). In addition, Dock3^{Y1360A} and Dock3^{Y1373A} also inhibited the effect of BDNF (Fig. 2*B*). BDNF-mediated axonal outgrowth and Rac1 activation were observed in cultured neurons from WT and p75^{NTR} KO mice, but not from TrkB KO mice (Fig. S2). Trk receptor-specific inhibitor K252a and overexpression of dominant-negative Rac1 (Rac1 N17) suppressed BDNF-mediated axonal outgrowth (Fig. S2). Furthermore, we found that Dock3 is expressed in retinal ganglion cells (RGCs) and has a synergistic effect with BDNF on axonal outgrowth in cultured RGCs (Fig. S3). In retinal explant cultures, Dock3 protein was concentrated in growth cones (arrows in Fig. 2*C*) and was diffuse in cytoplasm (control panel in Fig. 2*C*). However, BDNF treatment rearranged Dock3 staining to the cell periphery (arrowheads in Fig. 2 *C* and *D*). These data demonstrate that Dock3 act in concert, upstream of Rac1, to promote axonal outgrowth through TrkB receptor signaling.

Interaction of Dock3 and Fyn at the Growth Cone. One consequence of the presence of BDNF in neurons is the activation of Fyn and its association with the TrkB receptor (19). Indeed, BDNF induced Fyn activation via phosphorylation at Y416 in primary cultured hipocampal neurons (Fig. 3*A*). Because the proline-rich motif of Dock3 binds to SH3 domain-containing proteins (8), we next examined whether Fyn, which also possesses an SH3 domain, interacts with Dock3. A GST-fusion protein of the proline-rich Dock3 C terminus (amino acids 1773–2028) coprecipitated Fyn in lysates of mouse brain as well as Fyn-transfected Cos-7 cells (Fig. 3*B*). In addition, a His-tag pull-down assay in Cos-7 cells transfected with His-tagged Dock3 together with WT Fyn, constitutively active Fyn^{Y528F}, or ΔSH3 Fyn^{Y528F} (20), confirmed the SH3 domain requirement and preferential binding to the active form of Fyn (Fig. 3*C*). In contrast, Fyn failed to bind to Elmo (21), which interacts with Dock1–4 and enhances their GEF activities (Fig. S4). Thus, the BDNF/TrkB-dependent activation and association with Fyn is specific. Consistent with the activated TrkB association with Fyn and interaction with Dock3, Dock3 translocated from cytosol to the membrane in SY5Y cells transfected with TrkB and Fyn^{Y528F} in the presence of BDNF, but not in cells transfected with TrkB and ΔSH3 Fyn^{Y528F} (Fig. 3*D*). In addition, BDNF-treated retinal explants exhibit colocalization of Dock3 and Fyn at the peripheral regions of the growth cone (arrowheads in Fig. 3*E*). These results are consistent with a model whereby BDNF-TrkB signaling induces the formation of a protein complex of Dock3 at the plasma membrane (Fig. 2 *C* and *D*). We further constructed a mutant Dock3 lacking Fyn binding domain (Δ1773–

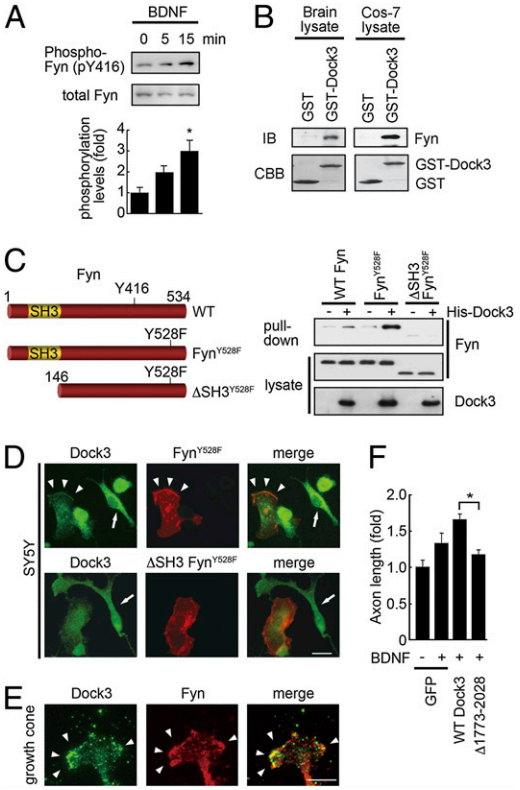

Fig. 3. BDNF induces direct binding of Dock3 and Fyn in the growth cone. (*A*) Hippocampal neurons were transfected with WT Fyn and stimulated with BDNF for the indicated times. Lysates were subjected to immunoblot analysis with anti-Fyn^{pY416} or total Fyn antibodies. (*Lower*) expression levels of phosphorylated Fyn. Data are mean ± SEM of three independent experiments. *$P < 0.05$. (*B*) Lysates from mouse brain and Cos-7 cells transfected with WT Fyn were incubated with a GST-fusion protein of the Dock3 proline-rich domain. Bound proteins were subjected to immunoblot (IB) analysis with an anti-Fyn antibody. (*Lower*) Coomassie Brilliant Blue (CBB) staining of the GST-fusion proteins used in this experiment. (*C*) (*Left*) Schematic diagram of the WT and mutant Fyn constructs used in this assay. (*Right*) Lysates from Cos-7 cells transfected as shown at the top of the images were subjected to a His-tag pull-down assay with antibodies against Fyn and Dock3. (*D*) SY5Y cells were transfected with TrkB and Fyn^{Y528F} or ΔSH3 Fyn^{Y528F} and treated with BDNF. Arrowheads indicate colocalization of Dock3 (green) and WAVE1 (red) at the peripheral region. Arrow indicates a nontransfected cell. (Scale bar, 20 μm.) (*E*) Colocalization of endogenous Dock3 (green) and Fyn (red) in growth cone of retinal explant cultures after BDNF treatment. (Scale bar, 10 μm.) (*F*) Hippocampal neurons transfected and treated as shown at the *x* axis were fixed at 3 days in vitro and axon length measured. $n = 30$ for each experimental condition. Data are mean ± SEM of three independent experiments. *$P < 0.05$.

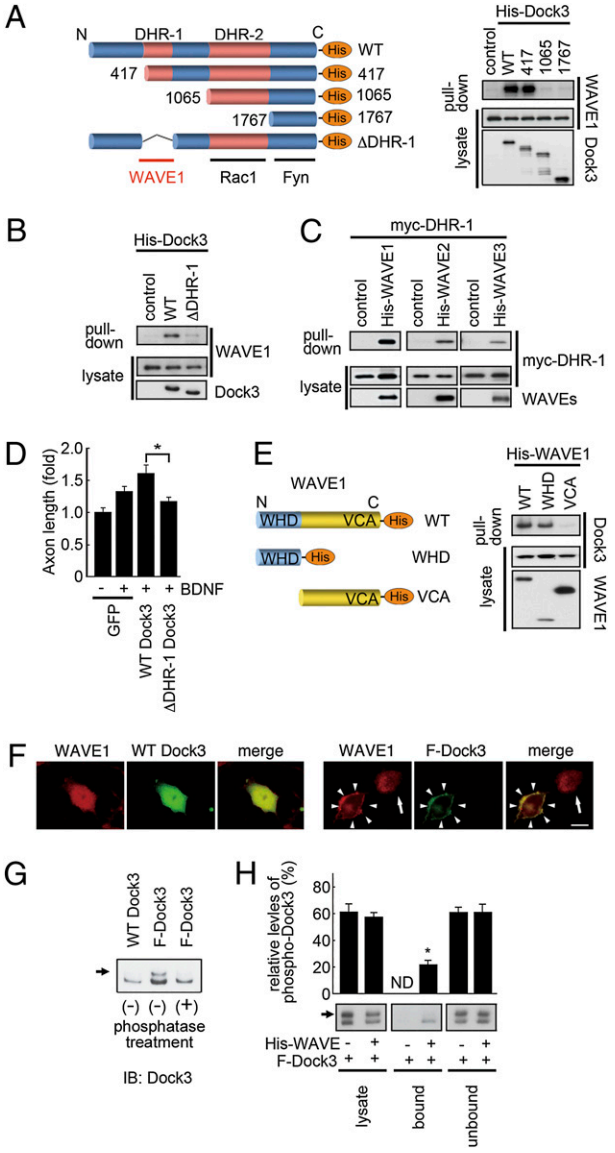

Fig. 4. Phosphorylation of Dock3 regulates its interaction with WAVE. (*A*) (*Left*) schematic diagram of the WT and mutant Dock3 constructs used in this assay. (*Right*) Lysates from Cos-7 cells transfected as shown at the top of the images were subjected to a His-tag pull-down assay with antibodies against WAVE1 and Dock3. (*B*) Lysates from Cos-7 cells transfected as shown at the top of the images were subjected to a His-tag pull-down assay with antibodies against WAVE1 and Dock3. (*C*) Lysates from Cos-7 cells transfected as shown at the top of the images were subjected to a His-tag pull-down assay with antibodies against myc-tag, WAVE1, WAVE2, and WAVE3. (*D*) Hippocampal neurons transfected and treated as shown at the *x* axis were fixed at 3 days in vitro, and axon length was measured. $n = 30$ for each experimental condition. Data are mean ± SEM of three independent experiments. *$P < 0.05$. (*E*) (*Left*) Schematic diagram of the WT and mutant WAVE1 constructs used in this assay. (*Right*) Lysates from Cos-7 cells transfected as shown at the top of the images were subjected to a His-tag pull-down assay with antibodies against Dock3 and WAVE1. (*F*) SY5Y cells transfected with WT Dock3 or F-Dock3 were probed with antibodies against WAVE1 (red) and Dock3 (green). Arrowheads indicate WAVE1 protein at the peripheral region. The arrow indicates a nontransfected cell. (Scale bar, 10 μm.) (*G*) Lysates from Cos-7 cells transfected with WT Dock3 or F-Dock3 were subjected to immunoblot analysis with an anti-Dock3 antibody. Arrow indicates phosphorylated Dock3. (*H*) (*Upper*) Relative expression levels of phosphorylated Dock3 to total Dock3. (*Lower*) Lysates from Cos-7 cells transfected as shown at the bottom of the images were subjected to a His-tag pull-down assay. Bound and unbound proteins were subjected to immunoblot analysis with an anti-Dock3 antibody. Arrow indicates phosphorylated Dock3.

2028) and found that this mutant failed to stimulate BDNF-induced axonal outgrowth (Fig. 3*F*).

Direct Interaction of Dock3 and WAVE Proteins. Because WAVE proteins play a major role in Rac-induced actin dynamics, including actin nucleation and polymerization (3, 4), we speculated that they could be involved in Dock3-mediated axonal outgrowth. To investigate the possibility that WAVE1 can directly bind to Dock3, we performed a His-tag pull-down assay using WT Dock3 or several Dock3 truncation mutants. We found that Dock3 directly bound to WAVE1 at the DHR-1 domain (Fig. 4*A*). We next constructed a mutant that lacks the DHR-1 domain (ΔDHR-1) and determined that this mutant also failed to bind to WAVE1 (Fig. 4*B*). We further confirmed that the DHR-1 domain of Dock3 was required to mediate WAVE1–3 binding (Fig. 4*C*). Indeed, this interaction between Dock and WAVE proteins can be generalized (Fig. S5). In addition, we

demonstrated that ΔDHR-1 failed to stimulate BDNF-induced axonal outgrowth in hippocampal neurons (Fig. 4*D*). We next prepared both WT and two mutant forms of WAVE1 constructs and found that one mutant that lacks the WAVE-homology domain (WHD) failed to bind to WT Dock3 (Fig. 4*E*). Based on the preceding data, we hypothesized that membrane targeting of Dock3 may regulate the intracellular localization of WAVE. To assess this possibility, we transfected SY5Y cells with WT Dock3 and subjected them to immunocytochemistry. Overexpression of WT Dock3 did not change the intracellular localization of WAVE1 (Fig. 4*F*). In contrast, overexpression of farnesylated Dock3 (F-Dock3, a membrane-targeted form of Dock3) translocated WAVE1 from the cytoplasm to the peripheral regions (arrowheads in Fig. 4*F*). Taken together, these results suggest that the DHR-1 domain of Dock family proteins mediates direct binding to WAVE proteins leading to colocalization at the cell periphery and axonal outgrowth.

We also found a strong enhancement of the Dock3 upper mobility band (arrow in Fig. 4*G*) in Cos-7 cells transfected with F-Dock3 but not with WT Dock3 (26 ± 6% and 2 ± 1% of total Dock3, respectively). Consistent with the upper mobility band representing a phosphorylated form of Dock3, the mobility shift was eliminated by phosphatase treatment (Fig. 4*G*). We next examined the effect of Dock3 phosphorylation on its ability to bind WAVE. Cos-7 cells were transfected with His-tagged WAVE1 and F-Dock3 and subjected to a pull-down assay. Immunoblot analysis revealed that WAVE1 binds to the unphosphorylated form of Dock3 more effectively than to the phosphorylated form (Fig. 4*H*). Thus, the Dock3/WAVE1 complex may dissociate upon Dock3 phosphorylation, leading to spatially restricted actin dynamics through WAVE signaling.

Promoting Axonal Outgrowth in Adult CNS by Modulating Dock3 Activity. To investigate the effects of Dock3 in vivo, we generated transgenic (Tg) mice overexpressing WT Dock3 under control of the actin promoter. Tg mice showed high expression levels of Dock3 in many tissues, especially in the optic nerve (~5.6-fold) and retina (~2.3-fold; Fig. S6*A*), but the structure of such tissues were normal (Fig. S6 *B* and *C*). Therefore, we prepared retinal explants from WT and Tg mice and examined the effect of Dock3 on axon length. Quantitative analysis revealed that axon length was clearly greater in Tg mice than in their WT littermates (Fig. 5*A*). Furthermore, Dock3 and BDNF exerted synergistic effects on axonal growth, as observed in vitro (Fig. 2 *A* and *B*). These results suggest that axonal outgrowth is promoted in Dock3 Tg mice. To explore this possibility, we used the optic nerve microcrush model and investigated the extent of axonal outgrowth 2 weeks after injury by staining with GAP43 antibody (22). WT mice subjected to nerve crush showed >40 axons extending 200 μm from the injury site, and half this number extended 500 μm. In contrast, Tg mice had, on average, >80 axons extending 200 μm and >60 axons extending 500 μm beyond the lesion site (Fig. 5*B*). Immunohistochemical analysis of Dock3 and WAVE1 in injured optic nerves revealed that their coexpression was hardly detectable in WT mice, but was clearly increased in regenerating axons in Tg mice (Fig. 5*C*). We next examined the levels of Dock3 and WAVE1 proteins in optic nerves by immunoblotting (Fig. S7). Dock3 protein was reduced in injured WT optic nerves (decreased to ~10% of control), but this reduction was less severe in Tg mice. In both WT and Tg mice, WAVE1 protein was less abundant after the injury (reduced to ~60%). Whereas in the membrane fraction of crushed optic nerves, Dock3 and WAVE1 expression levels in Tg mice were significantly higher than WT mice (Fig. 5*D* and *E*). We also examined the expression of TrkB and Fyn in injured optic nerves, but their expression levels in Tg mice were not significantly altered compared with those in WT mice (Fig. S8). Thus, overexpression of Dock3 does not affect the expression levels of its binding partners, but may induce axonal outgrowth through effective membrane recruitment of WAVE1.

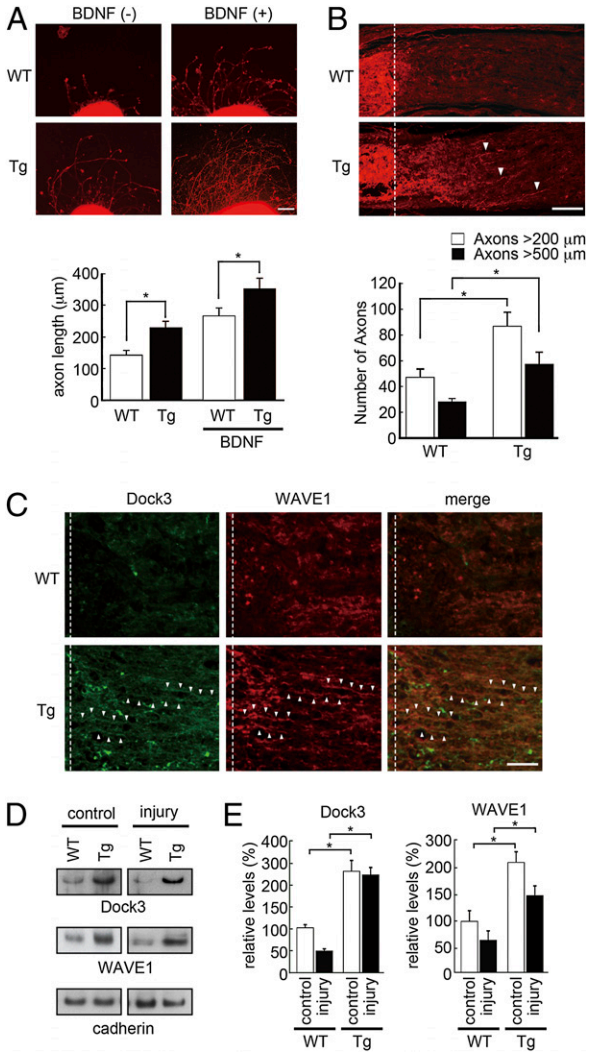

Fig. 5. Overexpression of Dock3 induces axonal regeneration in vivo. (*A*) (*Upper*) Retinal explants from WT and Dock3 Tg mice cultured in the presence or absence of BDNF were fixed and labeled with phalloidin at 2 days in vitro. (*Lower*) Axon length was measured and quantified. Data are mean ± SEM, $n > 80$ axons for each group. *$P < 0.05$. (Scale bar, 100 μm.) (*B*) (*Upper*) GAP43-labeled axons in the optic nerve proximal to the injury site (dotted line) 2 weeks after nerve surgery. Arrowheads indicate regenerating axons. (*Lower*) Regenerating axons from lesion site were measured and quantified. Data are mean ± SEM for six independent experiments. *$P < 0.05$. (Scale bar, 50 μm.) (*C*) Immunostaining of the optic nerves proximal to the injury site (dotted line). Arrowheads indicate colocalization of Dock3 (green) and WAVE1 (red) in the regenerating axons. (Scale bar, 20 μm.) (*D*) Immunoblot analysis of Dock3 and WAVE1 in the membrane fraction of optic nerve 2 weeks after nerve surgery. (*E*) Expression levels of Dock3 and WAVE1 in the membrane fraction of normal and injured optic nerves were quantified. Data are presented as means ± SEM of three independent experiments. *$P < 0.05$.

Discussion

In the present study, we found that Dock1–4, which are atypical members of the Rho-GEF family, share several conserved amino acids in their DHR-2 domains that are required for GEF activity. In addition, Dock1–4 bind directly to WAVE1–3 via their DHR-1 domains, and this is disrupted when Dock1–4 become phosphorylated. We further showed that Dock3 forms a protein complex with Fyn and WAVE1 at the plasma membrane downstream of TrkB. Finally, we demonstrated that overexpression of Dock3 increases WAVE1 expression levels in the plasma membrane

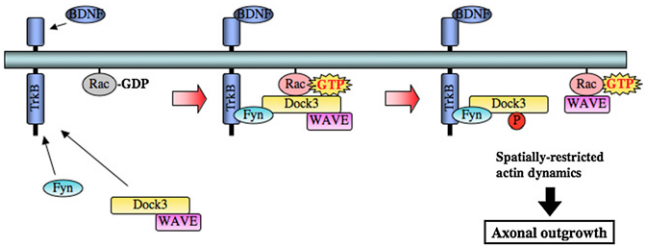

Fig. 6. Proposed role of Dock3 in BDNF-mediated axonal outgrowth. BDNF-TrkB signaling induces Fyn phosphorylation and stimulates membrane recruitment of the Dock3/WAVE complex. Dock3 is then able to activate Rac-GDP (inactive form). Rac-GTP (active form) and WAVE are dissociated from phosphorylated Dock3 and stimulate actin reorganization, leading to axonal outgrowth.

and promotes optic nerve regeneration in vivo. Taken together, our results suggest a unique mechanism for axonal outgrowth through which Dock-GEFs recruit WAVE proteins to specific sites within cells and induce spatially restricted actin dynamics in the CNS (Fig. 6).

BDNF/TrkB signaling is important for a variety of CNS developmental processes including synaptic pruning (23, 24). In addition, Rho-GTPase signaling can affect both spine structure and synaptic function (25, 26). In this study, we found that treatment of hippocampal neurons with BDNF enhances Rac1 activation and neurite outgrowth. It is therefore possible that Dock-GEFs mediate multiple BDNF/TrkB functions, including the establishment of long-term potentiation. On the other hand, loss of Dock3 leads to sensorimotor impairments and structural changes, including axonal swellings, but had no effects on life span or fertility (15). These relatively mild phenotypes are reasonable, considering the redundant catalytic sequences found in the DHR-2 domains of Dock1–4. Recent studies have revealed that a Val66Met polymorphism in human BDNF is involved in the pathogenesis of attention-deficit/hyperactivity disorder (ADHD) (27–29). Interestingly, a pericentric inversion breakpoint in the *DOCK3* gene has been described in ADHD patients (30). Thus, further study is required to determine the full implications of the Dock-GEFs in BDNF-associated development, adult physiology, and disease.

Dock3 overexpression induced optic nerve regeneration after injury without affecting expression levels of its binding partners. For example, WAVE1 expression level after optic nerve injury was decreased to ~60% in both WT and Dock3 Tg mice. However, WAVE1 expression was increased in the membrane fraction and was coexpressed with Dock3 in the regenerating axons. Thus, the tight regulation of Rac1-GDP/Rac1-GTP cycling and effective membrane recruitment of WAVE proteins by Dock3, rather than prolonged activation of Rac1 or WAVE proteins (31–33), may be required for axonal growth via actin polymerization. Our present findings support the notion that neurons have to intrinsically upregulate the necessary growth-associated molecules to extend an axon (34, 35). On the other hand, recent studies have shown that glial scarring and several myelin inhibitors block axonal growth following CNS injury (35, 36). Thus, overexpression of Dock-GEFs may have a synergistic effect in combination with suppression of glial scarring and myelin-associated inhibitory signaling.

Lack of axonal regeneration in the adult CNS is one of the most important issues to be resolved in various neurodegenerative disorders. For example, glaucoma is characterized by a slow progressive degeneration of optic nerve axons. Thus, Dock/WAVE complexes and their related binding partners may be possible therapeutic targets in multiple forms of glaucoma that otherwise lead to severe visual impairment. We are presently investigating these issues by crossing Dock3 Tg mice with glutamate transporter knockout mice, which are the first animal models of normal tension glaucoma (37). Thus, further studies are required to explore the full potential of Dock-GEFs that could be used for effective regeneration therapy.

Materials and Methods

Experimental Animals. Experiments were performed using $p75^{NTR}$ knockout and TrkB × hGFAP-cre knockout (TrkB KO) mice in accordance with the Tokyo Metropolitan Institute for Neuroscience *Guidelines for the Care and Use of Animals*. The TrkB deletion was confirmed in the hippocampus of the TrkB KO mice (23). A Tg construct containing the CAG promoter, *Dock3* coding sequence (GenBank accession no. NM_153413), and a poly-adenylation signal was used to generate Dock3 Tg mice. The founder mice were generated by injecting the transgene into fertilized C57BL/6 eggs. RGCs were retrogradely labeled with Fluoro-Gold (Fluorochrome) as previously described (38).

Cell Culture. Primary cultured hippocampal neurons (39) and retinal explants (22) were prepared from E16 mice. In some experiments, they were stimulated with BDNF (5 or 50 ng/mL; Alomone Labs).

Plasmids and siRNA Transfection. WAVE1–3 plasmids were provided by T. Takenawa (40). Elmo2 and GST-CRIB plasmids were provided by H. Kato and M. Negishi (21). Rac and Dock1 plasmids were provided by M. Matsuda (41). Dock3, Fyn, and WAVE1 fragments were PCR-amplified from full-length cDNAs and expressed as His-tagged proteins. Alanine substitutions were generated with the PrimeSTAR Mutagenesis Basal Kit (Takara). RNA oligomers containing 21 nucleotides for RNA interference for Dock1–4 were synthesized in the sense and antisense directions (Dock1: 5′-GGAAGUCACCACAACGCU UUU-3′; Dock2: 5′-GCAUCUCA CGCUACAGAUUUU-3′; Dock3: 5′-GCAGAUCA GUGAACGGUUUU-3′; Dock4: 5′-GCAAGAGUGUGGCCAGAAAUU-3′) (JBioS). Transfection of siRNAs and plasmids was performed using the Nucleofector System (Amaxa) or Lipofectamine Plus (Invitrogen).

Pull-Down Assay. His-tagged proteins were purified from Cos-7 cell lysates with TALON resin (BD Biosciences) for 20 min at 4 °C with gentle agitation. The protein levels of Rac-GTP were measured by affinity precipitation using the GST-CRIB of PAK1. Bacterial GST-fusion proteins were incubated with lysates from Cos-7 cells or mouse brains. The precipitated samples were subjected to SDS-PAGE followed by immunoblot analysis (8) with the following antibodies: Fyn, phospho-Fyn (Tyr416) and WAVE1 were obtained from BD Biosciences; WAVE2 and WAVE3 were obtained from Santa Cruz.

Immunostaining. SY5Y cells, retinal explants, sections of retina, and optic nerves were incubated with anti-Dock3 (1:200), anti-WAVE1 (1:200), and anti-GAP43 (1:1,000; Chemicon) antibodies. Cy2-conjugated donkey antirabbit IgG or Cy3-conjugated donkey antimouse IgG were used as secondary antibodies. F-actin was visualized with rhodamine-labeled phalloidin (Invitrogen).

Optic Nerve Injury. Mice were anesthetized with sodium pentobarbital before optic nerve crush. Optic nerves were exposed intraorbitally and crushed about 0.5–1.0 mm from the posterior pole of the eyeball with fine surgical forceps for 5 s (22). Fourteen days after surgery, axonal outgrowth was quantified by counting GAP43-positive axons that crossed a virtual line parallel to the lesion site at 200 μm and 500 μm distal to the lesion site. Protein expression levels in the lesion site were measured by immunoblot analysis with antibodies against Dock3 and WAVE1–3. Membrane fraction was prepared as previously described (42).

Image Analysis and Statistics. To measure axon length, the longest axon of each neuron was traced and calculated using National Institutes of Health ImageJ (version 1.38). Approximately 50 neurons with axons were scanned using a DP70 CCD camera (Olympus). Data are presented as mean ± SEM. For statistical analyses, a two-tailed Student's *t* test was used. Values of $P < 0.05$ were regarded as statistically significant.

ACKNOWLEDGMENTS. We are grateful to T. Takenawa (Kobe University) and H. Kato, M. Negishi, and M. Matsuda (Kyoto University) for reagents. We thank A. Kimura, K. Nakamura, and K. Nishimura for technical assistance, and R. McKay for comments on the manuscript. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K.N., C.H., and T.H.); Ministry of Health, Labour, and Welfare of Japan; Uehara Memorial Foundation; Naito Foundation; Suzuken Memorial Foundation; Daiwa Securities Health Foundation; Takeda Science Foundation; and Japan Medical Association (to T.H.).

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