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Netrin signal transduction and the guanine nucleotide exchange factor DOCK180 in attractive signaling

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Netrins are prototypical axon guidance cues whose attractive signaling requires the small GTPase Rac1. It remains unclear how Rac1 is regulated in the netrin pathway. DOCK180 is a member of a new family of guanine nucleotide exchange factors for Rho GTPases. Here we provide evidence implicating DOCK180 in netrin signal transduction. Netrin promoted the formation of a protein-protein interaction complex that included DOCK180 and the netrin receptor deleted in colorectal carcinoma (DCC). Inhibition of DOCK180 reduced activation of Rac1 by netrin. Both axon outgrowth and axon attraction induced by netrin were inhibited after DOCK180 knockdown in vertebrate neurons. The *in vivo* functional role of DOCK180 was demonstrated by its requirement for projection of commissural axons in the neural tube. These findings indicate that netrin stimulation recruits DOCK180 through DCC, which then activates small GTPases, suggesting an essential role for DOCK180 in mediating attractive responses by neurons to netrin-1.

During neural development, axon outgrowth and pathfinding are critical for neurons to establish their circuitry. The idea of axon guidance by diffusible cues was postulated by Ramon y Cajal in the 19th century[1]. Molecular studies of axon guidance have led to the identification of several families of guidance cues in the past 15 years (reviewed in refs. 2,3).

Netrins are a family of guidance cues whose functions are conserved from worms to mammals[2,4–6]. *Caenorhabditis elegans unc-6* was identified as a gene involved in axon guidance toward and away from the ventral midline of the body[7,8]. The vertebrate netrin-1 and netrin-2 were discovered as proteins that were capable of promoting axon outgrowth and the same molecules could also attract the commissural axons in the neural tube[4,5,9,10]. Netrins can be either attractants or repellents. DCC/UNC-40 can mediate both attractive and repulsive responses, whereas UNC-5 is only known to mediate repulsion[2,11–16]. Mammalian studies suggest that DCC can mediate attraction by itself and can mediate repulsion on association with UNC-5 (ref. 16). Genetic studies in worms suggest that either DCC or UNC-5 alone can also mediate repulsion[13,17]. Studies in *Drosophila* indicate that UNC-5 alone mediates short-range repulsion, whereas UNC-5 together with DCC mediates long-range repulsion[18].

Small GTPases of the Rho family are important for regulating the actin cytoskeleton and for controlling axon outgrowth and guidance. Rho GTPases are essential components in netrin-induced remodeling of the cytoskeleton[19,20]. Netrin-1 activates Cdc42 and Rac1 through DCC, and causes filopodia formation through Cdc42 and cell spreading through Rac1 in HEK293 cells and the neuroblastoma glioma cell line NG108-15 (ref. 19). Netrin-1 induced neurite outgrowth in N1E-115 neuroblastoma cells requires both Rac1 and Cdc42 activities[21]. Axon outgrowth from precerebellar neurons also requires Rac and Cdc42 (ref. 22). Genetic analysis in *C. elegans* indicates that Ced-10, a Rac-like GTPase, is required for axons to respond to the netrin homolog UNC-6 (ref. 23).

The activities of Rho GTPases are regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). Several mediators that are responsible for the regulation of Rho GTPases by axon guidance cues are GEFs for ephrins[24], GEFs for semaphorins[25,26] and GAPs for Slits[27]. Netrins are the only family of major axon guidance cues for which the molecules mediating their regulation of Rho GTPases remain unknown.

There are two distinct families of Rho GEFs: Dbl-homology (DH) domain–containing proteins and CZH (CDM-zizimin homology) proteins[28]. The conventional GEFs for Rho GTPase contain a DH domain that is responsible for nucleotide exchange. The newly defined GEFs are the CZH proteins[28–31], which include the two subfamilies CDM (Ced-5, DOCK180 and Myoblast City) proteins[32,33], which activate Rac1, and zizimin proteins, which activate Cdc42 (ref. 31). Currently, 11 mammalian proteins of the CDM family have been discovered[28]. The prototypical CDM family member DOCK180 was originally identified as a 180-kDa protein that biochemically interacts with the adaptor protein Crk[32]. The *C. elegans* ortholog of DOCK180, Ced-5, was identified as a protein required for cell migration and phagocytosis[33], whereas the *Drosophila* ortholog, Myoblast City, is essential for myoblast fusion, dorsal closure[34] and neural development[35].

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Human DOCK180 is involved in cytoskeletal reorganization[32]. DOCK180 has been implicated in integrin signaling through the CrkII-p130CAS complex[36]. In mammalian cells, the Crk-ELMO-DOCK180 complex activates Rac-dependent pathways[37,38]. DOCK180 directly interacts with Rac1 through a newly identified GEF domain (CZH2) and functions as a specific GEF for Rac[29,30]. The CZH2 domain (also called the DOCKER or DHR2 domain) interacts with a nucleotide-free GTPase and promotes its activation[28,32,36,39,40].

We have now obtained biochemical and functional evidence that DOCK180 is important for linking netrin stimulation to Rac1 activation. We found that DCC is directly associated with DOCK180. Inhibition of DOCK180 essentially eliminated netrin stimulation of Rac1 and partially reduced netrin stimulation of Cdc42. *In vitro*, DOCK180 was required for the neurite outgrowth–promotion activity and axon attraction by netrin-1. *In vivo*, knockdown of DOCK180 disrupted the projection of commissural axons in the neural tube. Taken together, these findings indicate a critical role for DOCK180 in mediating attractive responses to netrin and uncover an important component in netrin signal transduction.

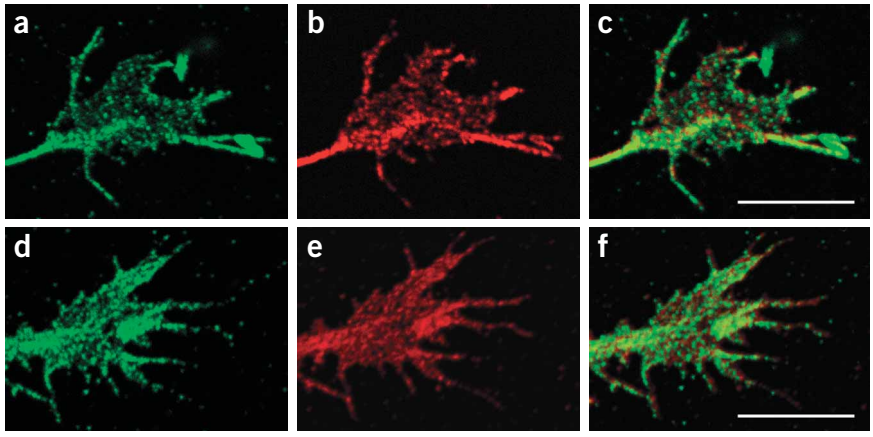

Figure 1 Subcellular localization of DOCK180, DCC and Rac1 in the growth cones of primary neurons. (**a**–**f**) Localization of DOCK180 (**a**,**d**), DCC (**b**) and Rac1 (**e**) in dissociated cortical neurons from E15 mouse embryos. (**c**) Merged image of **a** and **b**. (**f**) Merged image of **d** and **e**. Scale bar, 10 μm.

RESULTS

Localization of DOCK180 with DCC and Rac1 in primary neurons

DOCK180 is widely expressed in tissues, including the nervous system[28,30,32]. To determine whether DOCK180 is involved in netrin signaling, we examined the subcellular localization of DOCK180 in embryonic day (E) 15 mouse primary neurons from the neocortex and the spinal cord.

Immunocytochemical analysis with antibodies to DOCK180 and DCC revealed overlapping localization of DOCK180 and DCC in neocortical neurons in the soma, the axon and the growth cone (**Fig. 1a–c** and **Supplementary Fig. 1** online). DOCK180 was also localized with Rac1 (**Fig. 1d–f**). Similar results were obtained with primary neurons from the dorsal spinal cord of mouse embryos (data not shown).

A protein-protein interaction complex with DOCK180 and DCC

We tested for possible physical interaction between DCC and DOCK180 in both HEK293T cells and E15 mouse primary neurons. DOCK180 was readily detected in lysates that were immunoprecipitated with antibody to DCC from cells transfected with DCC, but not from control, untransfected cells, suggesting that DOCK180 formed a protein-protein interaction complex with DCC (**Fig. 2a**). Similar results were observed in experiments with antibody to DOCK180 for immunoprecipitation and antibody to DCC for Western analysis (**Fig. 2a**). In the control, no specific interaction was detected between normal mouse IgG and either DCC or DOCK180 (**Fig. 2a**).

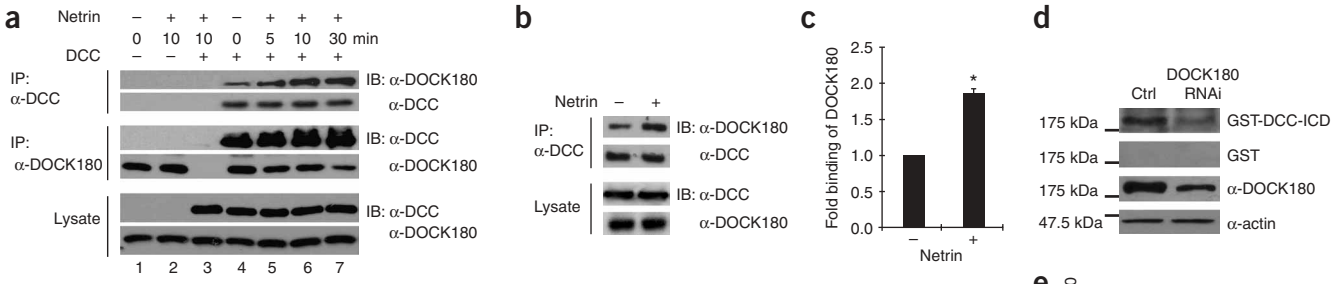

Figure 2 Interaction between DOCK180 and DCC. (**a**) Formation of a protein-protein interaction involving DOCK180 and DCC. HEK cells transfected with DCC or the vector were stimulated with conditioned media from control HEK cells or HEK cells that secreted netrin-1. Cell lysates were immunoprecipitated with antibody to DCC or DOCK180 (H-4) 48 h after transfection. IB, antibodies used for analyzing the immunoblots; IP, antibodies used for immunoprecipitation. Lane 3 contains the control immunoprecipitation using mouse IgG. Co-immunoprecipitation of DOCK180 and DCC required transfection of DCC into HEK cells. Loading controls are shown in the bottom panels. (**b**) Netrin-1 increased the interaction of DCC and DOCK180 in primary neurons. Lysates of dissociated neurons from E15 mouse neocortex were immunoprecipitated with antibody to DCC, and the immunoblots were analyzed with antibody to DOCK180. Endogenous DOCK180 and DCC formed a protein-protein interaction complex. Netrin increased this interaction. (**c**) Quantification of **b** from three independent experiments. The *y* axis shows density in arbitrary units; the netrin-treated group had a density 1.86 ± 0.06–fold greater than that of the control ($P < 0.05$). (**d**) Direct interaction of DOCK180 and DCC shown by far-western analysis. HEK293T cells were transfected with either DOCK180 shRNA or the control vector. GST-DCC-ICD or GST was used to probe the western blots. Only GST-DCC-ICD, not GST alone, bound to DOCK180. DOCK180 shRNA reduced the binding of GST-DCC-ICD to the DOCK180 band. Actin served as the loading control. (**e**) Quantification of GST-DCC-ICD binding to DOCK180. DOCK180 shRNA reduced the binding of DOCK180 to 36.80 ± 4.0% of the control (* indicates $P < 0.05$).

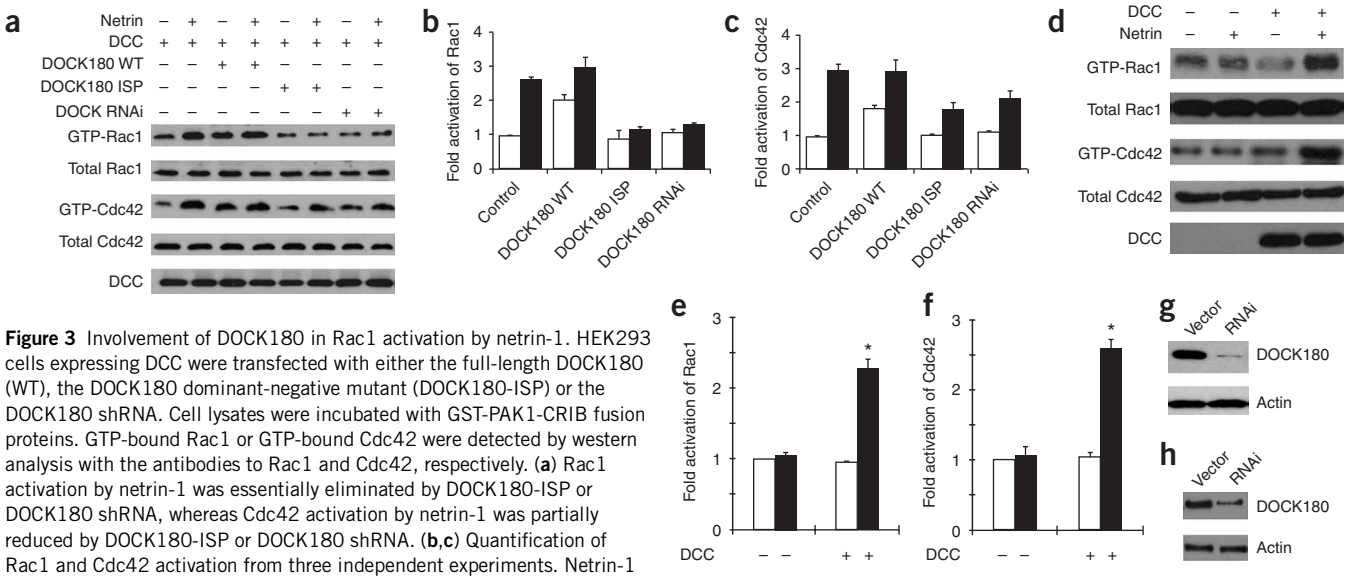

Figure 3 Involvement of DOCK180 in Rac1 activation by netrin-1. HEK293 cells expressing DCC were transfected with either the full-length DOCK180 (WT), the DOCK180 dominant-negative mutant (DOCK180-ISP) or the DOCK180 shRNA. Cell lysates were incubated with GST-PAK1-CRIB fusion proteins. GTP-bound Rac1 or GTP-bound Cdc42 were detected by western analysis with the antibodies to Rac1 and Cdc42, respectively. (**a**) Rac1 activation by netrin-1 was essentially eliminated by DOCK180-ISP or DOCK180 shRNA, whereas Cdc42 activation by netrin-1 was partially reduced by DOCK180-ISP or DOCK180 shRNA. (**b**,**c**) Quantification of Rac1 and Cdc42 activation from three independent experiments. Netrin-1 could not induce Rac1 activation in the presence of DOCK180-ISP or DOCK180 RNAi (**b**). The difference between the groups that received netrin (+) and those that did not (–) in the presence of DOCK180-ISP or DOCK180 shRNA was not statistically significant ($P > 0.1$). Netrin could still induce Cdc42 activation, but to a lesser extent (**c**). DOCK180-ISP blocked netrin-induced Cdc42 activation; the difference between the groups that received netrin (+) and those that did not (–) in the presence of DOCK180-ISP was not statistically significant ($P = 0.07$). DOCK180 shRNA partially blocked netrin-induced Cdc42 activation; the difference between the groups that received netrin (+) and those that did not (–) in the presence of DOCK180 shRNA was statistically significant ($P = 0.05$). (**d**–**f**) DCC-dependent activation of Rac and Cdc42 by netrin-1. (**g**,**h**) DOCK180 shRNA reduced DOCK180 protein levels in HEK cells (**d**) and in cortical neurons from E15 mouse embryos (**g**). The white and black bars in **b**, **c**, **e** and **f** represent the fold activation in the absence and presence of netrin-1, respectively.

We stimulated the HEK293T cells with netrin-1 before carrying out the immunoprecipitation experiments and found that netrin-1 increased the interaction of DOCK180 and DCC within 10 min (**Fig. 2a**). A previous study has shown that the neocortical axons are attracted to netrin-1 and that netrin-1–deficient mice have defects in several forebrain commissures[41]. To determine whether the endogenous DOCK180 and DCC proteins interact with each other, we carried out immunoprecipitation experiments with primary cortical neurons from E15 mouse embryos. We found that netrin-1 increased the interaction between endogenous DOCK180 and DCC proteins (**Fig. 2b**,**c**).

We used a modified far-western blotting procedure to test whether DCC and DOCK180 directly bind to each other. Briefly, lysate was prepared from HEK293T cells that normally expressed DOCK180, separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed by a purified fusion protein of the

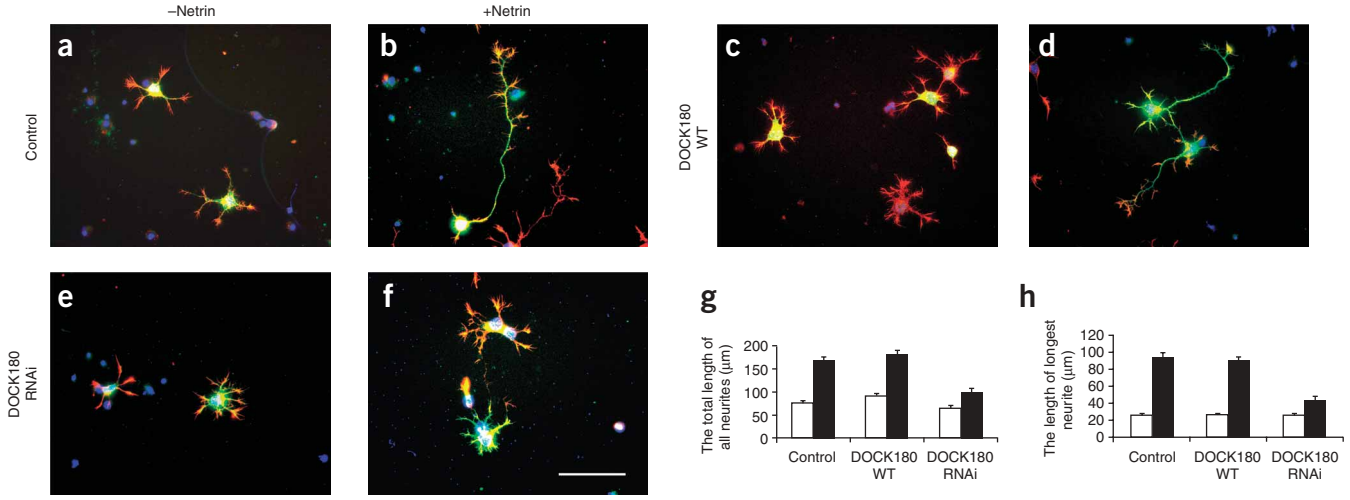

Figure 4 DOCK180 shRNA inhibited axon outgrowth induced by netrin. (**a**–**f**). Venus-YFP was transfected into cortical neurons from E15 mouse embryos with either the control vector (**a**,**b**), DOCK180 WT (**c**,**d**) or DOCK180 shRNA (**e**,**f**), and plated on PLL- and laminin-coated coverslips. Neurons were stimulated with either the purified netrin-1 protein (**b**,**d**,**f**) or the sham-purified control (**a**,**c**,**e**) for 40 h before being stained with rhodamine-phalloidin (red) and Hoechst. Only the neurites of YFP-positive neurons not in contact with other cells were measured and used in the statistical analyses. Scale bar, 50 μm. (**g**,**h**) Quantification of netrin-1–induced neurite outgrowth from cortical neurons. Both the average length of total neurites (**g**) and the length of the longest neurite (**h**) were analyzed. Data are means ± s.e.m. from three separate experiments. The white and black bars in **g** and **h** represent neurite length (**g**) or the length of the longest neurite (**h**) in the absence and presence of netrin-1, respectively.

intracellular domain (ICD) of DCC and GST (GST-DCC-ICD) or with GST as a control overnight at 4 °C. Bound GST-DCC-ICD was visualized by incubating the membrane with HRP-labeled antibody to GST. The same blots were analyzed by antibody to DOCK180 to show the position of the endogenous DOCK180. We found that GST-DCC-ICD bound to a band at the same position as DOCK180 on the blot (**Fig. 2d**, upper), but GST did not (**Fig. 2d**, middle). When an shRNA construct for DOCK180 was introduced into the HEK293T cells, the binding of GST-DCC-ICD to that band was significantly reduced (**Fig. 2d**,**e**, upper panel $P < 0.05$). These results demonstrate that DCC can bind to DOCK180 directly.

Role of DOCK180 in netrin activation of Rac1 and Cdc42

Netrin-1 activates Rac1 and Cdc42[2,19–23,42]. We tested whether DOCK180 was involved in netrin signaling. The amount of the active (and GTP bound) forms of Rac1 and Cdc42 present were measured by the GST pull-down assay, which used the Rac1- or Cdc42-binding domain of Pak as an affinity reagent to precipitate GTP-bound forms of GTPases[27]. As found previously[19,20], netrin-1 activated both Rac1 (**Fig. 3a**,**b**) and Cdc42 (**Fig. 3a**,**c**), and that the activation was dependent on DCC (**Fig. 3d–f**). We obtained a construct for DOCK180-ISP, a dominant-negative mutant for DOCK180 in which three contiguous residues at positions 1487–1489, Ile-Ser-Pro, were mutated into Ala-Ala-Ala. DOCK180-ISP could not bind to the nucleotide-free Rac or promote Rac-GTP loading[29]. We tested several shRNAs and selected one that effectively reduced DOCK180 protein in both HEK293T cells (**Fig. 3g**) and primary neurons (**Fig. 3h**). Using these reagents, we found that Rac1 activation by netrin was inhibited by both DOCK180-ISP and the shRNA (**Fig. 3a**,**b**). Cdc42 activation by netrin was partially inhibited by DOCK180-ISP and the shRNA (**Fig. 3a**,**c**). DOCK180-ISP and DOCK180 shRNA did not significantly reduce ($P > 0.1$) the amount of Rac1 and Cdc42 in the absence of DCC and netrin (**Supplementary Fig. 2** online).

Role of DOCK180 in netrin-induced axon outgrowth

To investigate whether DOCK180 is required in netrin-induced axon outgrowth, we used a DOCK180 shRNA construct (**Fig. 3h**). Primary cortical neurons from E15 mouse embryos were dissociated and transfected with Venus-YFP and either the control vector (**Fig. 4a**,**b**), wild-type DOCK180 (**Fig. 4c**,**d**) or the DOCK180 shRNA (**Fig. 4e**,**f**). After 40 h of culture in the absence or presence of netrin-1, neurons were fixed and stained with rhodamine-phalloidin (in red) for actin and Hoechst (in blue) for the nuclei. Transfected neurons were identified by the expression of Venus-YFP (in green). We detected cotransfection (**Supplementary Fig. 3** online).

In neurons transfected with the control vector (**Fig. 4a**,**b**), neurite outgrowth was stimulated by netrin-1. Without netrin treatment, the length of the longest neurite from each neuron was 27.02 ± 1.83 μm (mean ± s.e.m.) and the total length of all neurites from each neuron was 77.47 ± 2.92 μm (**Fig. 4g**,**h**). With netrin treatment, the length of the longest neurite from each neuron was 94.33 ± 5.34 μm and the total length of all neurites from each neuron was 168.92 ± 7.30 μm (**Fig. 4g**,**h**).

Netrin-1 promotion of axon outgrowth was significantly reduced in neurons transfected with the DOCK180 shRNA ($P < 0.01$) (**Fig. 4f**); the length of the longest neurite from each neuron was increased from 26.89 ± 1.99 μm without netrin to 44.14 ± 4.54 μm with netrin, and the total length of all neurites per neuron was increased from 66.03 ± 3.18 μm without netrin to 99.66 ± 6.07 μm with netrin (**Fig. 4g**,**h**). When we compared the neurite outgrowth

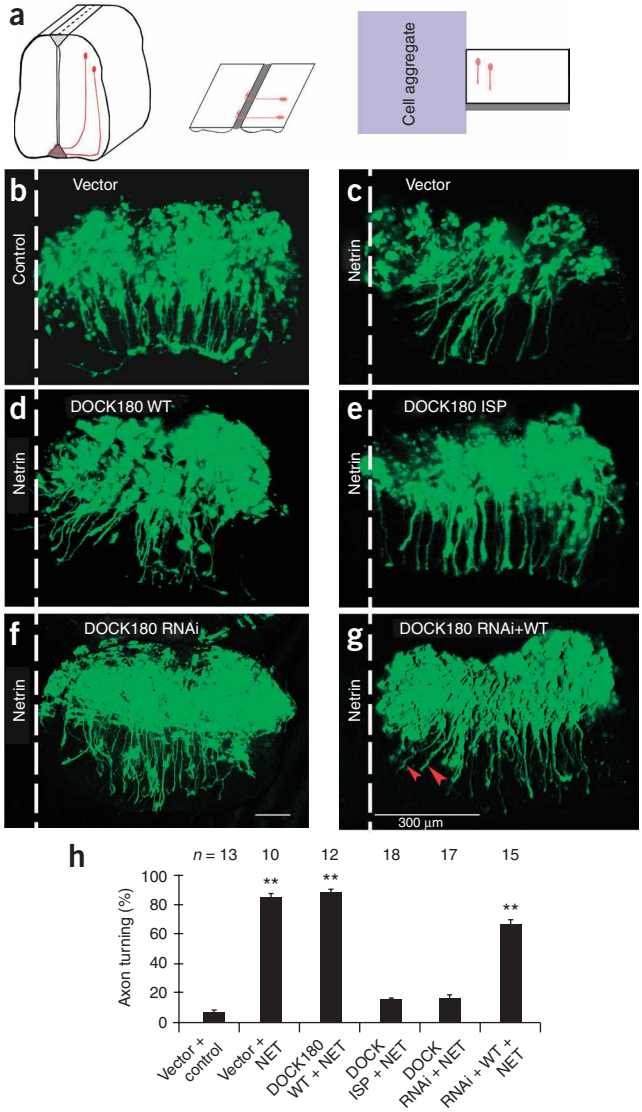

Figure 5 Inhibition of netrin-1–induced turning of commissural axons by DOCK180-ISP and DOCK180 siRNA. (**a**) A diagram showing the chicken open-book preparation. Electroporation of Venus-YFP allowed visualization of axons[43]. (**b**) Axons expressing YFP projected toward the floor plate and not toward the aggregates of control HEK293T cells. (**c**,**d**) Axons expressing YFP (**c**) or YFP and DOCK180 (**d**) turned toward netrin-1. (**e**) The majority of axons expressing YFP and DOCK180-ISP projected toward the floor plate and not toward netrin-1. (**f**) DOCK180 siRNA blocked netrin-1–induced attraction of commissural axon. Scale bar, 100 μm. (**g**) Transfection of DOCK180 siRNA with wild-type DOCK180 rescued the DOCK180 siRNA phenotype. Scale bar, 300 μm. (**h**) Quantification. Turning percentages were calculated by dividing the number of axons turning toward netrin-1 with the total number of axons within 300 μm of the HEK293T aggregates. Axons with turning angles >5° were counted. Data are mean ± s.e.m. *n* indicates the number of explants tested. NET, netrin-secreting HEK293T cells. The turning percentages were 6.6 ± 1.0% for vector + HEK (**b**), 85.0 ± 2.6% for vector + NET (**c**), 88.9 ± 1.3% for wild-type DOCK180 + NET (**d**), 15.3 ± 0.6% for DOCK180-ISP + NET (**e**), 16.5 ± 1.2% for DOCK180 siRNA (**f**) and 67.2 ± 2.9% for DOCK180 siRNA + wild type (**g**). ** indicates $P < 0.0001$ between **b** and **c** groups; $P < 0.0001$ between **c** and **e** groups; $P < 0.0001$ between **c** and **f** groups; $P < 0.0001$ between **f** and **g** groups (Student's *t*-test).

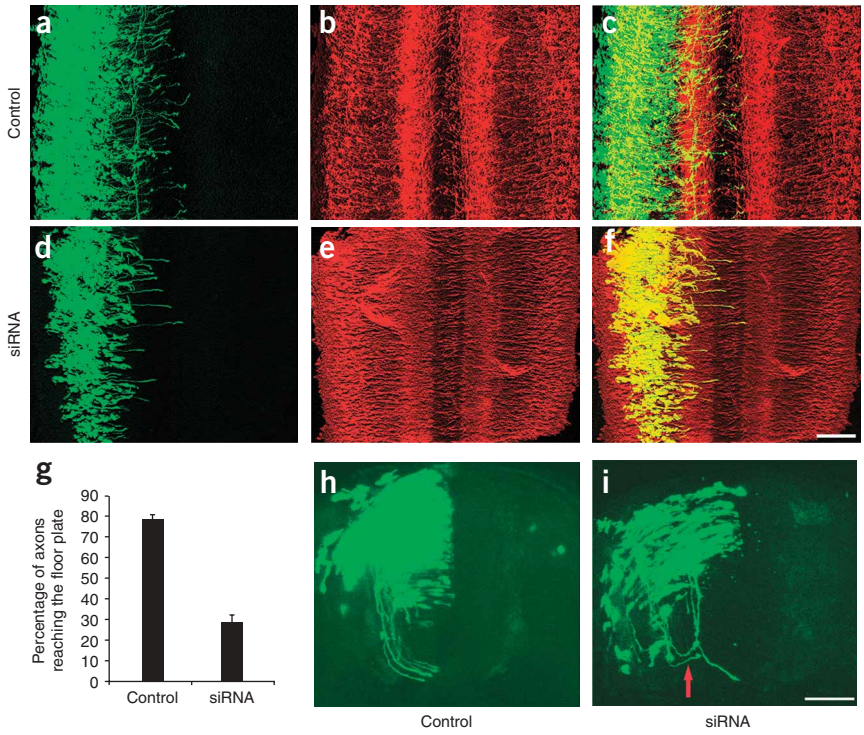

Figure 6 DOCK180 siRNA caused *in vivo* misguidance of commissural axons. (**a**–**f**) Venus-YFP alone (**a**–**c**) or Venus YFP with DOCK180 siRNA (**d**–**f**) were introduced into the chicken neural tube by electroporation at stage 12–15. The lumbosacral region of spinal cord was isolated at stage 22–23 and immunostained with antibody to axonin-1. Panels **a** and **d** are YFP images, **b** and **e** are images of immunostaining with antibody to axonin-1, and **c** and **f** are merged images. (**g**) Quantification of the percentage of axons reaching the floor plate. We observed that 78.8 ± 1.6% of commissural axons expressing Venus-YFP alone reached the floor plate, as did 28.8 ± 3.4% of commissural axons expressing Venus-YFP and DOCK180 siRNA ($P < 0.0001$, Student's *t*-test). (**h**,**i**) The chick neural tube was electroporated with Venus-YFP alone (**h**) or Venus-YFP and DOCK180 siRNA (**i**). The effect of DOCK180 siRNA on commissural axon projection was observed in the transverse sections. The red arrow points to two misguided axons. Scale bar, 100 μm.

induced by netrin from neurons transfected with the control vector to that induced from neurons transfected with the shRNA, the difference was statistically very significant ($P < 0.0001$). DOCK180 shRNA did not affect basal neurite outgrowth. Overexpression of wild-type DOCK180 did not significantly increase axon outgrowth ($P < 0.01$; **Fig. 4c**,**d**,**g**,**h**). DOCK180-ISP was also able to reduce axon outgrowth induced by netrin-1 (data not shown). Taken together, these results indicate that DOCK180 is required for netrin induction of axon outgrowth from primary neurons.

Role of DOCK180 in axon attraction by netrin

An important function of netrin is to attract the commissural axons in the neural tube[4,9,10,41]. To determine whether DOCK180 is involved in axon attraction by netrin, we used the open-book assay with commissural axons from chicken embryos[43].

Venus-YFP was introduced into the neural tube of chick embryos at stages 12–15 by electroporation, along with either the control vector, wild-type DOCK180, DOCK180-ISP or an siRNA against the chicken DOCK180 (**Fig. 5**). An explant of the neural tube was isolated and the dorsal midline was cut open and laid out as an 'open book' (**Fig. 5a**). The electroporated half of the spinal cord (visualized by green fluorescence) was then isolated and cultured with an aggregate of control HEK293T cells or HEK293T cells that stably secreted netrin-1 (**Fig. 5a**). Commissural axons transfected with the control vector turned toward netrin-1 when the neural tube explants were cultured with HEK293T cells secreting netrin-1 (**Fig. 5c**,**h**), whereas axons projected straight toward the floor plate when the neural tube explants were cultured with control HEK293T cells (**Fig. 5b**,**h**).

The wild-type DOCK180 did not affect attraction by netrin (**Fig. 5d**,**h**). DOCK180-ISP significantly inhibited the attractive response of the commissural axons to netrin-1 ($P < 0.0001$; **Fig. 5e**,**h**). DOCK180 siRNA significantly inhibited commissural axons turning toward netrin-1 ($P < 0.0001$; **Fig. 5f**,**h**). We took advantage of the differences in the chicken and human DOCK180 sequences and used the wild-type human DOCK180 construct to rescue the RNAi phenotype. The wild-type DOCK transgene rescued the RNAi phenotype (**Fig. 5g**,**h**). These results indicate that DOCK180 is important in axon attraction by netrin-1.

Because there is only neogenin, but no DCC, in the chicken, we tested whether neogenin interacted with DOCK180 by carrying out immunoprecipitation with HEK293T cells transfected with Flag-tagged neogenin and found an association between DOCK180 and neogenin (**Supplementary Fig. 4** online). Thus, DOCK180 could interact with DCC in mice and with neogenin in chickens.

Role of DOCK180 in commissural axon projection *in vivo*

The results described here indicate that DOCK180 is required for biochemical signaling by netrin and for *in vitro* functions of netrin in neurite outgrowth and axon attraction. To determine the *in vivo* role of DOCK180, we examined the effect of DOCK180 siRNA on commissural axon projection in chick embryos.

Venus-YFP alone or Venus-YFP and DOCK180 siRNA were introduced by electroporation into the neural tube of chick embryos at stage 12. Embryos were allowed to develop until stage 23 and the lumbosacral segments of the spinal cord were isolated. The explants were immunostained with the antibody to axonin-1 that recognized the commissural axons (**Fig. 6**)[44]. More than 90% of axons that expressed Venus-YFP were commissural axons marked by antibody to axonin-1 (**Fig. 6a–c**).

By stage 23, 78.8 ± 1.6% of the commissural axons expressing Venus-YFP alone reached the floor plate (**Fig. 6a–c**,**g**). In contrast, only 28.8 ± 3.4% of the commissural axons transfected with the DOCK180 siRNA reached the floor plate (**Fig. 6d–f**). DOCK180 siRNA significantly inhibited the projection of commissural axons ($P < 0.0001$; **Fig. 6d–g**). To further examine whether the knockdown of DOCK180 disrupted the commissural axon pathfinding *in vivo*, we electroporated either Venus-YFP alone or Venus YFP and DOCK180 siRNA into chick spinal cord and prepared transverse sections of the chick spinal cord at stage 23 (**Fig. 6h**,**i**). Commissural axons expressing Venus-YFP projected normally toward the floor plate (**Fig. 6h**), whereas commissural axons expressing Venus-YFP and the DOCK180 siRNA were misguided (**Fig. 6i**),

further supporting a role for DOCK180 in the projection of commissural axons *in vivo*.

DISCUSSION

Our results have established that DOCK180 is important for mediating netrin signal transduction. Our biochemical data indicate that DOCK180 is required for Rac1 activation by netrin and is also involved in Cdc42 activation by netrin. Our *in vitro* and *in vivo* functional data demonstrate that DOCK180 is essential for both the neurite outgrowth–promotion activity of netrin and the axon attraction activity of netrin. These results have bridged a gap in our understanding of the signal transduction pathway of the prototypical attractant netrin.

A number of studies have recently shown that DCC interacts with the Src family tyrosine kinase Fyn and the focal adhesion kinase (FAK), and that these kinases are essential for attractive signaling by netrin[43,45,46]. Our recent work showed that $p130^{CAS}$ is a critical component in the netrin pathway, functioning downstream of Fyn and FAK and upstream of Rac1 (ref. 47). Netrin-1 not only induces tyrosine phosphorylation of $p130^{CAS}$, but also increases the binding of $p130^{CAS}$ to FAK and Fyn[47]. Inhibition of p130CAS by either a dominant-negative mutant or RNAi decreases netrin activation of Rac1 (ref. 47). $p130^{CAS}$ RNAi inhibits the attraction of commissural axons in the spinal cord by netrin-1 and causes defects in commissural axon projection in the embryo[47]. However, none of those components can act as a GAP or a GEF in regulating Rac1 or Cdc42 activity. Our present findings indicate that DOCK180 is crucial for netrin signaling.

There are at least 22 small GTPases in the Rho family[28]. Rho GTPases cycle between active and inactive states through the binding of guanine nucleotides. DOCK180 is a GEF specific for Rac1 (ref. 29). Our conclusion that DOCK180 is a key GEF for the regulation of Rac1 activity by netrin is supported by the phenotypes of a dominant-negative mutant of DOCK180 and DOCK180 shRNA (**Fig. 3**). To our surprise, we also found that Cdc42 activation by netrin was also inhibited by the DOCK180 dominant-negative mutant and by DOCK180 shRNA (**Fig. 3**), although the inhibition of Cdc42 was partial even when the inhibition of Rac1 was essentially complete, at least in HEK293T cells. Although it can not be ruled out that the DOCK180 dominant-negative mutant inhibits GEFs other than DOCK180, it is unlikely that the DOCK180 shRNA could target other GEFs. A simple explanation for our results is that Cdc42 activation by netrin also requires DOCK180, either directly as a regulator of endogenous Cdc42 or indirectly if Rac1 is required for full activation of Cdc42 activation by netrin. Cross-talk between Rho GTPases has been found in fibroblasts and in the context of axon guidance. The involvement of Cdc42-specific GEFs such as zizimin1 (refs. 28,31) in axonal guidance remains to be tested.

A steric-inhibition model for regulating the DOCK180 family of GEFs has been proposed[48]. At the basal state, the N-terminal SH3 domain of DOCK180 binds to the distant catalytic Docker domain, which blocks Rac access to the Docker domain and negatively regulates DOCK180 function[48]. Binding of the PxxP motif of the ELMO protein to the SH3 domain of DOCK180 could disrupt the SH3 and Docker interaction, thus allowing Rac access to the Docker domain and increasing the GEF activity of DOCK180 (ref. 48). In the netrin pathway, DOCK180 can bind to DCC directly (**Fig. 3c**). This could provide a direct pathway for netrin stimulation of DOCK180 activity.

An alternative pathway for netrin stimulation of DOCK180 is through $p130^{CAS}$ and ELMO. DCC can form complexes with Fyn, FAK and $p130^{CAS}$ (refs. 43,45,46). Netrin can stimulate $p130^{CAS}$ phosphorylation[47]. Phosphorylated $p130^{CAS}$ forms a complex with the GEF complex DOCK180 and ELMO, which can activate Rac1 (refs. 36,48,49). Therefore, netrin stimulation can activate DOCK180 either through DCC binding to DOCK180 or through the $p130^{CAS}$, ELMO and DOCK180 complex. These two possibilities are not mutually exclusive.

In worms and flies, DOCK180 is also involved in axon guidance[35,50], and it would be interesting to investigate whether it (or another GEF) is used in netrin signaling.

METHODS

Materials. We used antibodies to DOCK180 (from E. Kiyokawa, International Medical Center of Japan), DOCK180 (H-4), DOCK180 (C-20), Fyn, actin and FAK (Santa Cruz and Transduction Lab), and Rac1, Cdc42 and DCC (BD Biosciences). We also used antibodies to phosphotyrosine (4G10, Upstate Biotechnology) and axonin-1 (from E.T. Stoeckli, Biocenter, Sweden). Netrin-1 protein was purified from HEK293T cells that stably secreted a Myc-tagged netrin-1 by immunoprecipitation using the anti-Myc affinity matrix (Covance).

The DOCK180 constructs were generously provided by K.S. Ravichandran (University of Virginia). Flag-tagged DOCK180 and DOCK180-ISP have been described previously[29]. An shRNA for DOCK180 was generated by us with the following complementary oligonucleotides inserted into the mU6pro vector: 5′-TTT GTA TGT GGA TCG AGA GAA CCT TC AAG AGA GGT TCT CTC GAT CCA CAT ATT TTT-3′ and 5′-CTA GAA AAA TAT GTG GAT CGA GAG AAC CTC TCT TGA AGG TTC TCT CGA TCC ACA TA-3′ (targeted against 5′-TAT GTG GAT CGA GAG AAC C-3′ of human and mouse DOCK180). This region was not homologous to any DOCK180 family member or to any other known gene. An siRNA 27-mer duplex for chicken and mouse DOCK180 was synthesized by Dharmacon with the sequence 5′-UAA UCA GUA UGC AGA UAU GCU AAA CAA-3′ (sense) and 5′-UUG UUU AGC AUA UCU GCA UAC UGA UUA-3′ (antisense).

Culturing primary neurons. Dissociated primary neurons were cultured essentially as described[43], but with some modifications. Embryos were removed from timed-pregnant mice at E15. Frontal brains were dissected in cold Hank's buffered salt solution medium (Gibco). The cortices were cut into small pieces with scissors and trypsinized for 15 min at 37 °C. The dissociated neurons were resuspended in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and 20 U ml^{-1} of penicillin/streptomycin, and plated on poly-L-lysine (PLL, 100 μg ml^{-1})-coated dishes or coverslips overnight at 37 °C in a 5% CO_2 incubator. Cells were then used for immunoprecipitation, western blotting or immunostaining.

Immunocytochemistry. To determine the subcellular localization of DOCK180, DCC and Rac1 proteins, we fixed dissociated primary neurons from E15 embryonic cortices for 10 min in 4% prewarmed paraformaldehyde solution (127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1mM EGTA, 10 mM PIPES) and permeabilized and blocked with 0.1% Triton X-100 and 3% BSA in PBS for 1 h. Cells were then incubated with antibodies to DOCK180 (rabbit, 1:200), DCC (mouse, 1:500) or Rac1 (mouse, 1:200) overnight at 4 °C in a humidified chamber. After being washed three times with PBS, primary antibodies were visualized with secondary antibodies separately (antibody to rabbit-Cy2 for DOCK180 at 1:200, antibody to mouse-Cy3 for DCC and Rac1 at 1:200). Images were taken under the confocal microscope (Olympus IX70 Fluoview).

Immunoprecipitation and western analysis. Primary cortical neurons were lysed with MLB lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 10 mM $MgCl_2$ and protease inhibitor mixture; Roche Molecular Biochemicals) as described previously[43]. Lysates were immunoprecipitated with specific antibodies for 2 h and incubated with protein A/G beads for 3 h or overnight at 4 °C.

HEK293 cells were transfected with the calcium phosphate method and lysed 48 h after transfection in MLB lysis buffer. Extracts were centrifuged and supernatants were incubated with the primary antibody for 2 h at 4 °C,

and then with protein A/G Sepharose beads. Precipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed with specific antibodies.

Far-western analysis. The direct interaction of DOCK180 with the intracellular domain of DCC (DCC-ICD) was analyzed by a far-western blotting procedure. HEK293T cells transfected with a vector or the DOCK180 shRNA were lysed in the MLB lysis buffer and boiled in 1× SDS loading buffer. Lysates were separated by SDS-PAGE and transferred onto the nitrocellulose membrane. The membrane was pre-incubated in 1× PBST (0.05% Tween-20 in 1× PBS) for 2 h and 3% BSA in 1× PBST for another 2 h before incubation with the purified GST-DCC-ICD protein or the control GST overnight at 4 °C. Bound GST-DCC-ICD was visualized by incubating the membrane with antibodies to GST that were conjugated to HRP, following the instructions of the enhanced chemiluminescence kit (Amersham).

Rac1 and Cdc42 activity assays. Approximately 2 × 10^5 HEK293T cells expressing DCC were transfected with wild-type DOCK180, DOCK-ISP, or DOCK shRNA (5 μg per group) by the calcium phosphate method. We stimulated cells 48 h after transfection with the purified netrin-1 protein (500 ng ml^{-1}) or the sham-purified control for 5 min. Cells were lysed with the MLB lysis buffer and centrifuged for 15 min at 14,000*g* at 4 °C. To measure the levels of active GTP-bound GTPase (Rac1, Cdc42), we coupled 30 μg of the GST-CRIB of PAK to glutathione-Sepharose beads (Amersham Biosciences) at 4 °C for 30 min before each of the pull-down assays. Supernatants after centrifugation were incubated with Sepharose bead–associated GST-PAK for 45 min at 4 °C. Beads were washed three times with the lysis buffer and the bound small GTPase proteins were separated by 15% SDS-PAGE. Western blots were analyzed with antibodies to Rac1 and Cdc42 (ref. 27).

Axon outgrowth assay. To analyze neurite outgrowth, E15 cortical neurons were dissociated as described above. The suspending neurons (4 × 10^6 neurons per group) were mixed with Venus-YFP and control vector, Venus-YFP and DOCK180 WT or Venus-YFP and DOCK180 RNAi constructs, and immediately placed in the nucleofection cuvette (Amaxa Biosystems). After using the O-005 program for nucleofection, cells were diluted in prewarmed DMEM medium (containing 10% FCS) and plated on coverslips coated with PLL (50 μg ml^{-1}) and laminin (5 μg ml^{-1}) at 50,000 cells per well. Neurons were cultured in DMEM with 10% FCS and penicillin/streptomycin at 37 °C with 5% CO_2 for 2 h. Cells were then cultured in serum-free culture medium (DMEM with B27 and penicillin/streptomycin) containing the purified netrin-1 (250 ng ml^{-1}) or the sham-purified control at 37 °C with 5% CO_2 for 40 h. Cells were then fixed with 4% PFA for 10 min and stained with phalloidin (Molecular Probes). Nuclei were visualized with the Hoechst dye 33342.

Chicken spinal cord axon turning assay. Our electroporation procedure was similar to one described previously[43]. DNA or siRNA (4 mg ml^{-1}), together with Venus-YFP (1 mg ml^{-1}), was injected into the central canal of the spinal cord of chicken embryos *in ovo* at stages 12–15. The electroporation program was 25 mV, 50 ms, 5 pulses (BTX, ECM830). Embryos were dissected at stages 20–s21 and the half spinal cord showing fluorescence was isolated. The explanted spinal cord was cultured with aggregates of control or netrin-1 –secreting HEK293T cells for 24 h. Axon turning was counted when the angle of turning toward the HEK293T aggregate was more than 5°. The percentage of turning axons was calculated by dividing the numbers of fluorescent axons turning toward the HEK293T cell aggregate with the total numbers of fluorescent axons within 300 μm of the HEK293T cell aggregates. Images were collected with a confocal microscope.

Analysis of commissural axon projection *in vivo*. Our procedures for chick embryo collection and electroporation are described above. Chick embryos were killed between stages 22–23 after electroporation. This stage was chosen for analysis because it is the time when the commissural axons in the lumbosacral region cross the midline[44,50]. The lumbosacral region of the spinal cord was isolated and examined under a fluorescent microscope. Samples expressing YFP were chosen for opening at the roof plate (open-book preparation). Whole-mount immunostaining of the spinal cord was carried out after fixation. Briefly, samples were permeabilized in 0.5% Triton X-100 for 15 min and blocked for 30 min in the blocking buffer (PBS containing 5% goat serum and 0.1% Triton). Tissues were incubated with the antibody to axonin-1 (rabbit, 1:1,000 dilution) in the blocking buffer at 37 °C for 2 h. After being rinsed with 1× PBS three times, tissues were incubated with the secondary antibody (antibody to rabbit-Cy3, 1:200 dilution) at 37 °C for 2 h. The lumbosacral region of the spinal cord in the open-book preparation was mounted in Gel/Mount (Biomed). Images were taken under a confocal microscope (Olympus IX70 Fluoview).

To observe commissural axon turning *in vivo*, the lumbosacral region of the spinal cord expressing YFP at stage 23 was collected. Transverse sections of 200 μm were collected and mounted. Stacked images were collected with a confocal microscope and analyzed.

Note: Supplementary information is available on the Nature Neuroscience website.

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