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Focal adhesion kinase in netrin-1 signaling

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Netrins are a family of secreted molecules that are important for axonal outgrowth and guidance in the developing nervous system. However, the signaling mechanisms that lie immediately downstream of netrin receptors remain poorly understood. Here we report that the netrin receptor DCC (deleted in colorectal cancer) interacts with the focal adhesion kinase (FAK), a kinase implicated in regulating cell adhesion and migration. FAK was expressed in developing brains and was localized with DCC in cultured neurons. Netrin-1 induced FAK and DCC tyrosine phosphorylation. Disruption of FAK signaling abolished netrin-1-induced neurite outgrowth and attractive growth cone turning. Taken together, these results indicate a new signaling mechanism for DCC, in which FAK is activated upon netrin-1 stimulation and mediates netrin-1 function; they also identify a critical role for FAK in axon navigation.

Axonal pathfinding is essential for proper wiring in the brain during development. Axons are guided by attractive and repulsive cues that include netrins[1]. Netrins promote axonal outgrowth, guide neuronal growth cones and regulate neuronal branching in the developing nervous system[1–4]. Netrins act through two classes of receptors, DCC and UNC-5. The DCC family, which includes DCC and neogenin in vertebrates[5], UNC-40 in *Caenorhabditis elegans*[6] and frazzled in *Drosophila melanogaster*[7,8], is required for growth cone attraction. UNC-5, on the other hand, seems to mediate the repulsive effect of netrins[9–13]. DCCs have a large extracellular domain, a single transmembrane domain and a short cytoplasmic region with three conserved domains, namely P1, P2 and P3 (ref. 7). These P domains are important for DCC functions[12,14,15]. For example, the P3 domain in DCC is necessary for neurite outgrowth and attractive turning in *Xenopus laevis* spinal neurons[15] and for axonal projection of corticospinal tract fibers into the spinal cord in mice[16].

Intracellular mechanisms of netrin signaling have begun to be elucidated. Screens for suppressors of axonal outgrowth and guidance defects that are induced by expression of myristylated intracellular portions of UNC-40 or UNC-5 in *C. elegans* have identified several genes[17,18]. The proteins encoded by these genes include CED-10 (a Rac GTPase), UNC-34 (a mena homolog), UNC-115 (an actin-binding protein that contains a LIM domain) and UNC-44 (an ankyrin ortholog)[17,18]. In vertebrates, netrin-induced neurite outgrowth and/or attractive turning requires Rho family GTPases, phospholipase Cγ (PLCγ), phosphoinositol-3-kinase (PI3K) and extracellular regulated kinase (ERK)[19–24]. Because DCC and UNC-5 are transmembrane proteins without an obvious catalytic domain, it remains unclear how the intracellular signaling is initiated.

Here we present evidence that FAK is a downstream mediator in the netrin-DCC signaling pathway. FAK is a principal tyrosine kinase of focal adhesions, where cells adhere to the extracellular matrix (ECM)[25]. Perturbation of FAK signaling inhibits cell spreading and migration[26–28]. Although FAK is highly expressed in the developing nervous system[29], its function in neural development is poorly characterized. We demonstrate that DCC interacts with FAK. Netrin-1 activates FAK and stimulates DCC tyrosine phosphorylation in a manner that is dependent on FAK. Moreover, inhibition of FAK signaling in *X. laevis* spinal neurons blocks netrin-1-induced neurite outgrowth and the attractive growth cone turning response. These results identify an important role of FAK in netrin-1 signaling.

RESULTS

Interaction of FAK with the netrin receptor DCC

To better understand the function of FAK, we searched for its interacting proteins using the C terminus (FAK-Cterm; amino acids 800–1052) as bait in the yeast two-hybrid system. Using a mouse brain cDNA library in the screen, we isolated neogenin (**Fig. 1a**). The neogenin interaction with FAK did not require the P1 and P2 domains of neogenin, as their deletion had little effect on the binding (**Fig. 1a**). A mutant without the P3 domain, however, failed to interact with FAK (**Fig. 1a**), indicating that the P3 domain was necessary for FAK binding. Moreover, the P3 domain alone was sufficient to interact with FAK (**Fig. 1a**). The P3 domain of neogenin (amino acids 1480–1491) is highly homologous to DCC, with 90% amino acid identity (**Fig. 1c**). As expected, FAK also interacted with the P3 domain in DCC (**Fig. 1a**).

FAK-Cterm contains the proline-rich sequence and the FAT domain, both of which are important for FAK signaling[25]. To identify

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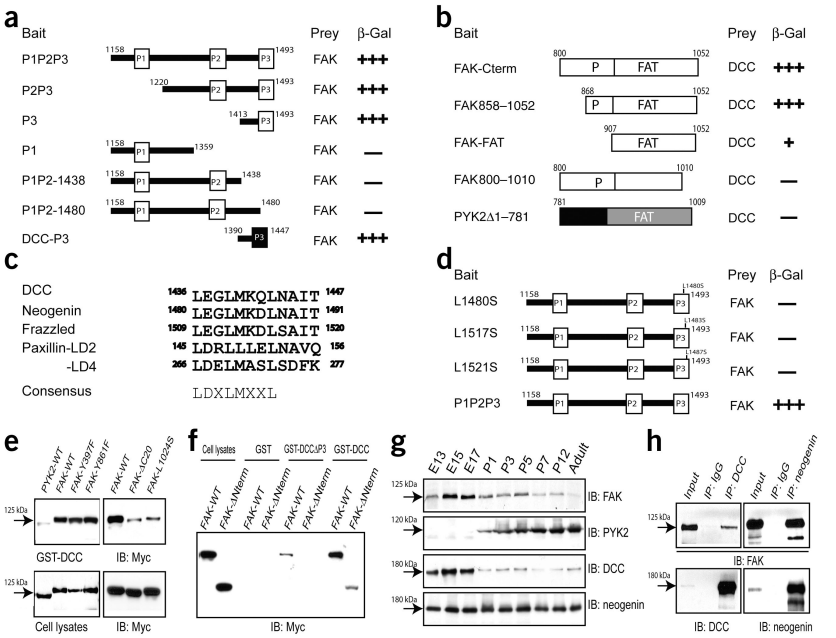

Figure 1 FAK interaction with DCC and neogenin. (**a**) P3 domains in neogenin and DCC are essential for binding to FAK. (**b**) The FAT domain in FAK, but not PYK2, is essential for binding to DCC or neogenin. (**c**) Consensus sequences of LD motifs. (**d**) Mutations of the LD-like motif in neogenin block binding to FAK. In **a**, **b** and **d**, yeast cells were co-transformed with indicated plasmids encoding proteins fused with the Gal4 DNA binding domain (in bait) or with the Gal4 activation domain (in prey). Transformed yeast cells were seeded on Leu$^-$, Trp$^-$ and His$^-$ plates and were scored for β-gal activity: –, no blue after 24 h; +, blue after 4 h; +++, blue within 30 min. (**e**,**f**) *In vitro* interaction of DCC with FAK, PYK2 and FAK mutants by GST pull-down assay. Lysates of HEK293 cells transfected with indicated plasmids were incubated with GST, GST-DCC, or GST-DCCΔP3 fusion proteins that were immobilized on beads. Bound proteins were probed with anti-Myc. FAKΔC20 has a deletion of the C-terminal 20 amino acids; FAK-L1024S contains a point mutation of L1024 to S; FAKΔNterm has a deletion of the N-terminal 390 amino acids. IB, immunoblot antibody. (**g**) Expression of FAK, PYK2, DCC and neogenin in developing rat brains. Brain homogenates (20 μg of protein) from different developmental stages were subjected to western blot using indicated antibodies. E, embryonic; P, postnatal. Molecular weights are indicated on the left. (**h**) Interaction of FAK with DCC and neogenin in rat brains. E17 rat brain homogenates (500 μg of protein) were incubated with anti-DCC (monoclonal) or anti-neogenin (rabbit polyclonal) and resulting immunocomplexes were probed with an antibody to FAK (anti-PY397). The western blot included 5% of the input.

the region in FAK-Cterm that is necessary for interaction with DCC, we characterized several FAK mutants. Mutants containing the FAT domain (FAK-Cterm and FAK858–1052), or the FAT domain alone (FAK-FAT), interacted with DCC or neogenin (**Fig. 1b**, and data not shown). Partial deletion of the FAT domain (FAK800–1010) abolished the interaction (**Fig. 1b**). Thus the FAT domain seems to be both required and sufficient for the interaction with DCC. This interaction is specific, because DCC did not interact with proline-rich tyrosine kinase 2 (PYK2), another member of the FAK family with a homologous FAT domain (**Fig. 1b**).

The interaction between FAT and paxillin requires the LD motif in paxillin, which contains leucine and aspartate[30]. The dependence of the FAK-DCC interaction on the FAT domain led us to investigate whether the P3 domain contains a leucine-aspartate (LD) motif. Sequence analysis showed an LD-like motif in the last 12 amino acid residues of the P3 domain of DCC, neogenin and frazzled (**Fig. 1c**). Instead of aspartate, the second amino acid is glutamic acid, a favorable substitute (**Fig. 1c**). To determine whether the LD-like motif is involved in the interaction, we generated three mutants, each carrying a single amino acid mutation of one of the three key leucine residues, and assayed for FAK binding. None of the mutants was able to interact with FAK (**Fig. 1d**), suggesting a critical role of the LD-like motif. The FAK-neogenin–DCC interaction occurs not only in yeast cells but also in *in vitro* pull-down assays. Glutathione *S*-transferase (GST) fusion proteins containing the intracellular domain of DCC (GST-DCC) or neogenin (data not shown) were able to precipitate Myc-FAK, but not PYK2, from lysates of transfected cells (**Fig. 1e**). Mutations in the FAT domain (FAKΔC20 and FAK-L1024S) or deletion of the P3 domain (GST-DCCΔP3) markedly reduced the binding (**Fig. 1e**,**f**), in agreement with yeast two-hybrid results. In contrast, mutations in FAK tyrosine residues (FAK-Y397F and FAK-Y861F) had no effect on binding to DCC (**Fig. 1e**). Notably, GST-DCCΔP3 was still able to precipitate wild-type FAK (FAK-WT), albeit much less efficiently (**Fig. 1f**). It was, however, unable to precipitate FAKΔNterm, a mutant with deletion of the N terminus (**Fig. 1f**). These results suggest a P3-independent binding site in the N terminus of FAK.

We next examined the expression pattern of DCC, neogenin and FAK in developing brains. Western blot analysis showed that DCC was expressed abundantly in rat embryonic brains, and expression decreased as development progressed (**Fig. 1g**). In contrast, neogenin expression remained unchanged from embryonic day (E) 13 to adult (**Fig. 1g**). Notably, the expression pattern of FAK was similar to that of DCC: high during the embryonic stage but low after birth (**Fig. 1g**). PYK2, however, was undetectable in embryonic brains, but its expression increased after birth (**Fig. 1g**). The temporal correlation of FAK and DCC expression suggests a possible *in vivo* interaction between the two proteins. To test this, we prepared brain homogenates from rat embryos at E17, when FAK, DCC and neogenin are highly expressed. FAK was detected in immunoprecipitates using antibodies to DCC or neogenin (**Fig. 1h**), demonstrating that FAK associated with both DCC and neogenin in developing brains. The association seemed to be specific, as FAK was not detected in nonspecific IgG immunocomplexes (**Fig. 1h**). Taken together, our results indicate that FAK interacts with DCC and neogenin *in vitro*, in yeast and in rat brains.

Colocalization of DCC and FAK in cortical neurons

To determine whether FAK localizes with DCC in neurons, E17 rat cortical neurons were immunostained using DCC- and FAK-specific antibodies. More than 90% of the cultured cells derived from E17 rat cortex were neurons, because they expressed doublecortin (data not

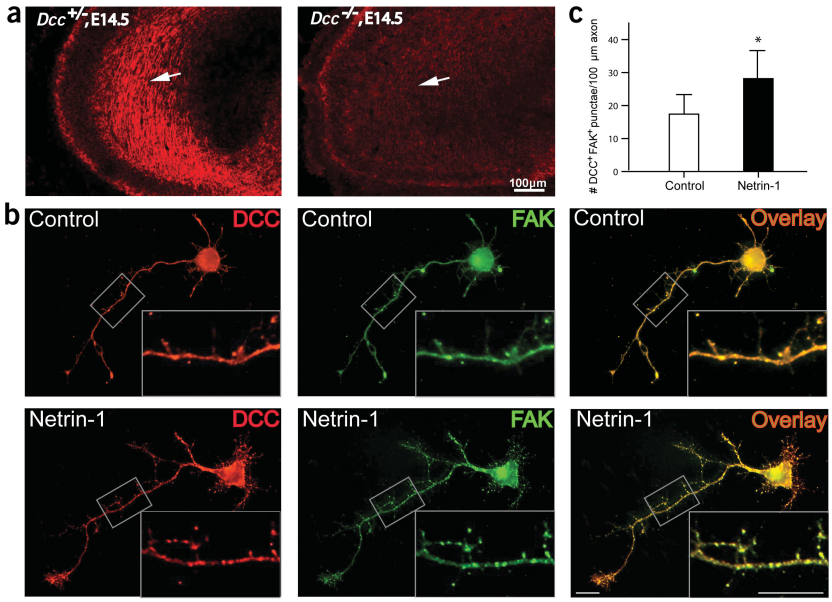

Figure 2 DCC and FAK localization in rat cortical neurons. (**a**) Images of immunostained DCC in olfactory bulb from DCC heterozygote ($Dcc^{+/-}$) and null ($Dcc^{-/-}$) embryos. Frozen sections of olfactory bulb derived from E14.5 $Dcc^{+/-}$ and $Dcc^{-/-}$ embryos were immunostained with anti-DCC (A20 goat polyclonal) and Cy3-conjugated secondary antibodies. Bar, 100 μm. (**b**) Images of immunostained DCC and FAK in cortical neurons. Rat cortical neurons (E17, DIV3) were stimulated with or without netrin-1 (200 ng/ml) for 30 min. They were fixed and were immunostained with A20 antibody as in **a** and an antibody to FAK (anti-PY397 rabbit polyclonal and FITC-conjugated secondary antibodies). Bars, 50 μm. (**c**) The number of punctae that coexpress DCC and FAK per 100 μm of axon is increased after netrin-1 incubation. Data shown are mean ± s.e.m. ($n = 5$), $^*P < 0.05$, different from control neurons (*t*-test).

shown), a marker for cortical neurons. The antibody to DCC (anti-DCC), a goat polyclonal antibody raised against an intracellular epitope of DCC, was specific in that it stained DCC in neuronal axons of olfactory bulb from DCC heterozygote ($Dcc^{+/-}$), but not homozygote ($Dcc^{-/-}$), embryos (**Fig. 2a**). Moreover, this antibody recognized a single band in cell lysates at the appropriate molecular weight and did not cross-react with neogenin (data not shown). Immunostaining showed that both DCC and FAK were detected in soma and neurites of cortical neurons (**Fig. 2b**). DCC immunoreactivity was distributed both at the plasma membrane (data not shown) and in an intracellular vesicle–like pool, as shown by the punctate staining (**Fig. 2b**), which is consistent with a recent report[31]. Merging of DCC and FAK images showed nearly completely overlapping staining of the two proteins (**Fig. 2b**). Upon netrin-1 stimulation, the number of DCC- and FAK-labeled punctae were significantly increased in neurites (**Fig. 2b,c**). These results indicate that DCC and FAK may be colocalized in rat cortical neurons, and netrin-1 stimulation may lead to the redistribution of DCC and FAK.

Netrin-1 induction of FAK tyrosine phosphorylation

Tyrosine phosphorylation of FAK is essential for its role in regulating cell adhesion and migration[25–29]. To determine whether FAK is involved in DCC signaling, we thus examined FAK tyrosine phosphorylation in neurons in response to netrin-1 stimulation. FAK was purified from lysates of netrin-1-stimulated cortical neurons (E17; cultured for 3 d *in vitro* (DIV3) by immunoprecipitation and was probed with RC20, an antibody to phosphotyrosine. FAK tyrosine phosphorylation increased in cortical neurons after netrin-1 stimulation (**Fig. 3a**). FAK is phosphorylated at multiple tyrosine residues, including Y397, Y577 and Y861, upon activation[32–34]. Phosphorylation of Y397 and Y577 leads to an increase in FAK catalytic activity, whereas phosphorylation of Y861 may regulate FAK binding to integrins[33,35]. We thus determined which tyrosine residues are phosphorylated upon netrin-1 stimulation by using antibodies that are specific for individual phosphotyrosine residues. Netrin-1 caused an increase in phosphorylation of Y397, Y577 and Y861 in a time-dependent manner (**Fig. 3b**). The phosphorylation peaked around 5–15 min, stayed elevated up to 30 min and started to decline around 60 min after stimulation (**Fig. 3c,d**). Although FAK phosphorylation was induced at multiple tyrosine residues, the induction of PY861 seemed to be most prominent (**Fig. 3d**). These results demonstrate that FAK is activated in response to netrin-1.

As the source of netrin-1, the above experiments used conditioned medium from HEK293 cells that expressed recombinant netrin-1. FAK activation by netrin-1 was probably due to the netrin-1 protein, because no effect was observed with conditioned medium from HEK293 cells that were transfected with an empty vector (**Fig. 3b**) or from HEK293 cells expressing slit-2, a repulsive guidance cue (**Fig. 3e**). To validate this further, we purified netrin-1 to apparent homogeneity (**Fig. 3f**) and studied its effect on FAK tyrosine phosphorylation. The purified netrin-1 increased FAK tyrosine phosphorylation in a dose-dependent manner (**Fig. 3g**), with an effective concentration that was similar to that required to stimulate neurite outgrowth in cortical explants[36].

Regulation of FAK tyrosine phosphorylation by DCC

To determine the role of DCC in netrin-1-induced FAK tyrosine phosphorylation, we reconstituted netrin-1 stimulation of FAK tyrosine phosphorylation in HEK293 cells. HEK293 cells do not express endogenous DCC or netrin-1 (data not shown), and thus netrin-1 had no effect on FAK tyrosine phosphorylation in the cells expressing FAK alone (**Fig. 4a**). Cotransfection with DCC rendered the cells able to respond to netrin-1 with an increase in FAK tyrosine phosphorylation (**Fig. 4a**), demonstrating a requirement for DCC. High expression of DCC increased FAK tyrosine phosphorylation in the absence of netrin-1, and under these conditions netrin-1 failed to elicit a further increase (**Fig. 4a**). Netrin-1 induction of FAK tyrosine phosphorylation was DCC specific, as netrin-1 failed to increase FAK tyrosine phosphorylation in cells expressing UNC5B (also known as UNC-5h2; **Fig. 4b,c**). Expression of neogenin also increased FAK tyrosine phosphorylation (**Fig. 4d**); however, induction of PY861 was much weaker than that of DCC and it could not be further increased by netrin-1 (**Fig. 4d**). To determine whether netrin-1-induced FAK phosphorylation depends on an interaction with DCC, HEK293 cells were transfected with various DCC

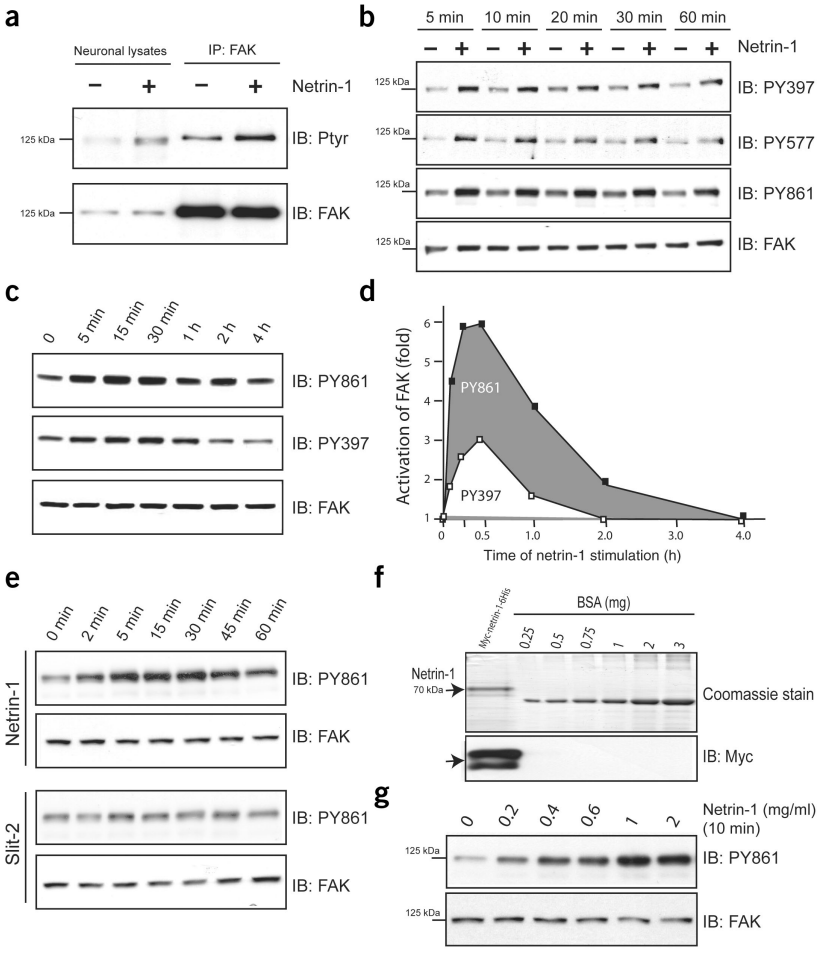

Figure 3 Netrin-1 induction of tyrosine phosphorylation of FAK in neurons. (**a**) Tyrosine phosphorylation of FAK in rat cortical neurons (E17, DIV3) in response to netrin-1. Cortical neurons were treated with netrin-1 (+) or control medium (–) for 20 min. Lysates (500 μg of protein) were incubated with anti-FAK for immunoprecipitation, and resulting immunocomplexes were subjected to western blotting using the antibody RC20 (Ptyr) or anti-FAK (as control). IB, immunoblot antibody. (**b**) Increase in FAK phosphorylation at Y397, Y577 and Y861 in netrin-1-stimulated neurons. (**c**) Time course of netrin-1 induction of PY861 and PY397 in neurons. (**d**) Quantification of netrin-1-induced FAK PY861 and PY397 in neurons. Shown is the relative FAK activity (PY397 and PY861) in response to netrin-1 (netrin-1/control; mean ± s.e.m.) from a representative experiment that was repeated three times with similar results. The amounts of PY397 and PY861 were determined through densitometry. (**e**) Induction of FAK PY861 by netrin-1 but not by slit-2. In **b**, **c** and **e**, rat cortical neurons (E17, DIV3) were stimulated with netrin-1(+), slit-2 or control (–) medium. Lysates (20 μg of protein) were subjected to western blot using antibodies as indicated. (**f**) Purification of netrin-1. Myc-His-tagged chicken netrin-1 was expressed in HEK293 cells. Lysates of high-salt membrane preparations were subjected to chromatography on a Ni-NTA affinity column. Purified netrin-1 was subjected to SDS-PAGE and subsequent staining with Coomassie blue. The amount of purified netrin-1 was quantified by comparison with a calibration curve of BSA. Purified netrin-1 was also immunoblotted with anti-Myc. (**g**) Dose-dependent effect of netrin-1 on FAK PY861 phosphorylation. Rat cortical neurons (E17, DIV3) were stimulated for 10 min by various concentrations of netrin-1 purified as in **f**. Neuronal lysates (20 μg of protein) were subjected to western blot using indicated antibodies.

mutants. Upon netrin-1 stimulation, PY861 was further increased (**Fig. 4d**). Unlike the wild type, DCCΔP3 and DCCΔP2P3 mutants, which lack the P3 domain and thus are unable to interact with FAK, failed to increase FAK Y861 phosphorylation even in the presence of netrin-1 (**Fig. 4d**), suggesting a requirement of the P3 domain in this event. Phosphorylation at Y397, however, did not require the P3 domain (**Fig. 4d**), suggesting differential regulation mechanisms between Y397 and Y861 phosphorylation. Furthermore, the deletion of the FAT domain, which abolishes the DCC-FAK interaction, increased basal FAK phosphorylation and prevented FAK Y861 and Y397 from being further phosphorylated in DCC-transfected cells (data not shown). Thus, the FAK-DCC interaction regulates FAK tyrosine phosphorylation.

We then investigated whether netrin-1 induction of FAK tyrosine phosphorylation in neurons depends on DCC. Neurons were isolated from DCC knockout (homozygote and heterozygote) and wild-type embryos. As observed with rat cortical neurons, neurons from DCC wild-type or heterozygote embryos responded to netrin-1 with an increase in FAK tyrosine phosphorylation (**Fig. 4e,f**). The induction of FAK Y861 phosphorylation was significantly attenuated in neurons from homozygote embryos (**Fig. 4e,f**), suggesting a role for DCC in FAK tyrosine phosphorylation by netrin-1. Netrin-1 was, however, still able to elicit FAK tyrosine phosphorylation in the mutant neurons (**Fig. 4e,f**), suggesting a DCC-independent mechanism.

Netrin-1-induced DCC tyrosine phosphorylation and FAK

Our finding that FAK interacts with DCC and becomes activated by netrin-1 suggests a role for tyrosine phosphorylation in netrin-1 signaling. To test this hypothesis, we investigated whether proteins become tyrosine phosphorylated in cortical neurons in response to netrin-1 stimulation. In comparison with controls, an increase in tyrosine phosphorylation was observed in three principal bands in netrin-1-stimulated rat cortical neurons (E17, DIV3): two at 125–130 kDa and one at 180 kDa (**Fig. 5a**). Because the molecular weight of DCC is around 180 kDa, we determined whether it became tyrosine phosphorylated in response to netrin-1. DCC was purified from lysates of netrin-1-stimulated cortical neurons by immunoprecipitation, and the immunoprecipitates were probed with RC20, an antibody to phosphotyrosine. Netrin-1 was indeed able to elicit tyrosine phosphorylation of DCC in rat cortical neurons (**Fig. 5b**). The 125-kDa protein was identified as FAK, whereas the 130-kDa protein seemed to be $p130^{cas}$ (data not shown).

We next investigated whether FAK is involved in netrin-1-induced tyrosine phosphorylation of DCC. To this end, we first asked whether

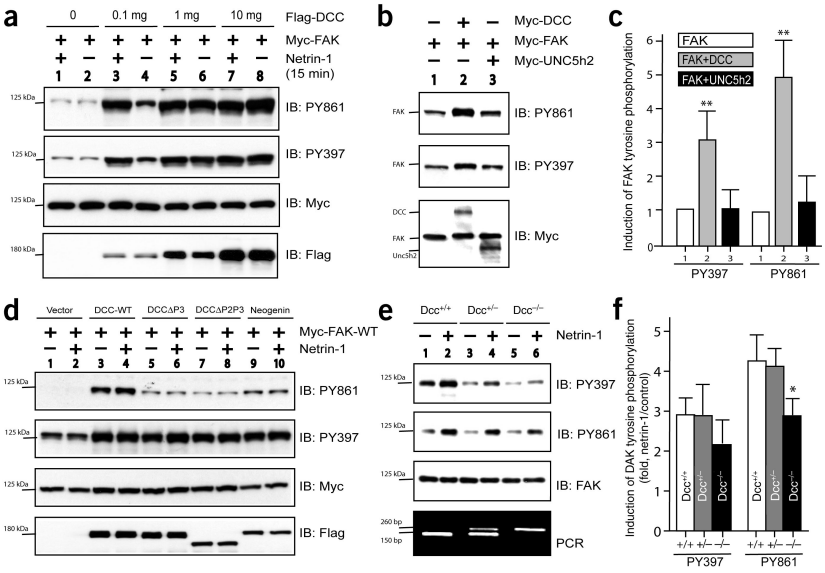

Figure 4 Regulation of FAK tyrosine phosphorylation by DCC. (**a**) Induction of FAK tyrosine phosphorylation by DCC expression. HEK293 cells were transfected with increasing concentrations of DCC. Cells were stimulated for 15 min with netrin-1 (+) or control medium (–) 24 h after transfection. Lysates were subjected to immunoblotting with indicated antibodies (IB). (**b**) Induction of FAK tyrosine phosphorylation by expression of DCC but not UNC5B. HEK293 cells were transfected with indicated plasmids. Cells were stimulated for 30 min with netrin-1 24 h after transfection. They were then lysed, and resulting lysates were subjected to immunoblotting. (**c**) Quantification of the effects of DCC and UNC5B on FAK PY397 and PY861. Shown is the relative FAK activity (PY397 and PY861) in response to DCC or UNC5B expression (DCC or UNC5B/control; mean ± s.e.m.) from a representative experiment that was repeated three times with similar results. The amounts of PY397 and PY861 were determined through densitometry. $^{**}P < 0.01$, different from cells expressing FAK alone (*t*-test). (**d**) Dependence of FAK Y861 phosphorylation on the P3 domain of DCC. HEK293 cells were transfected with indicated plasmids. Cells were stimulated with (+) or without (–) netrin-1 and were lysed. Resulting lysates were subjected to immunoblotting. (**e**) Reduction of netrin-1-induced FAK tyrosine phosphorylation in DCC mutant neurons. Cortical neurons derived from DCC wild-type and heterozygote and homozygote mutant embryos were stimulated with (+) or without (–) netrin-1 for 30 min and were lysed. Resulting lysates were subjected to immunoblotting. The bottom panel shows PCR genotyping. The 250-bp fragment was detected in homozygous embryos, whereas the 160-bp fragment was in wild-type mice. (**f**) Quantification of netrin-1-induced FAK PY397 and PY861 in DCC mutant neurons. Shown is the relative FAK activity (PY397 and PY861) in response to netrin-1 (netrin-1/control; mean ± s.e.m.; $n = 3$). The amounts of PY397 and PY861 were determined through densitometry. $^{*}P < 0.05$, different from DCC$^{+/+}$ neurons (*t*-test).

FAK is required by using mouse fibroblasts that did not express the FAK gene (*Ptk2*$^{-/-}$) that were then transfected with *Dcc*. Tyrosine phosphorylation of DCC was increased in control *Ptk2*$^{+/+}$ fibroblasts upon netrin-1 stimulation (**Fig. 5c**). In *Ptk2*$^{-/-}$ fibroblasts, however, netrin-1 was unable to induce DCC tyrosine phosphorylation (**Fig. 5c**), suggesting the necessity of FAK in this event. We then examined whether FAK is sufficient to induce DCC tyrosine phosphorylation using HEK293 cells, which express low amounts of FAK but do not express DCC (data not shown). Transfected Flag-DCC was not tyrosine phosphorylated even in the presence of netrin-1 (data not show) but became tyrosine phosphorylated in FAK-cotransfected cells in response to netrin-1 (**Fig. 5d**). Notably, cotransfection of K454A, a FAK kinase-dead mutant[28], also caused an increase in DCC tyrosine phosphorylation by netrin-1 (**Fig. 5d**). In contrast, mutation of Y397, a docking site for a Src kinase when phosphorylated, abolished induced DCC tyrosine phosphorylation (**Fig. 5d**). These results indicate that FAK tyrosine kinase activity is dispensable, at least in part, with regard to netrin-1-induced DCC tyrosine phosphorylation and that FAK may function as a scaffold protein by recruiting a Src kinase.

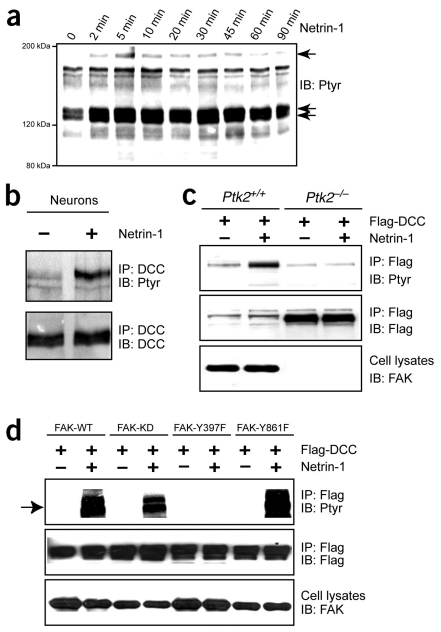

Figure 5 FAK-dependent tyrosine phosphorylation of DCC in response to netrin-1. (**a**) Identification of proteins that became tyrosine phosphorylated in netrin-1-stimulated rat cortical neurons (E17, DIV3). Neurons were treated with netrin-1 for indicated times and were lysed. Lysates (20 μg of protein) were subjected to western blot using the phosphotyrosine (Ptyr) antibody RC20. Arrows indicate three principal bands with an increase in phosphotyrosine. No apparent change in phosphotyrosine of proteins below 80 kDa was observed (data not shown). (**b**) DCC became tyrosine phosphorylated in netrin-1-stimulated rat cortical neurons (E17, DIV3). Neuronal lysates (500 μg of protein) were incubated with anti-DCC, and immunoprecipitated (IP) DCC was subjected to immunoblot (IB) using the antibody RC20 and anti-DCC. (**c**) Netrin-1 induction of DCC tyrosine phosphorylation depends on FAK. *Ptk2*$^{+/+}$ and *Ptk2*$^{-/-}$ fibroblasts were transfected with Flag-DCC and were stimulated with netrin-1. Flag-DCC was immunoprecipitated and probed with RC20. (**d**) Netrin-1 induction of DCC tyrosine phosphorylation requires FAK Y397 phosphorylation but not kinase activity. HEK293 cells were co-transfected with indicated plasmids. Immunoprecipitated DCC was probed with RC20 (Ptyr) and other indicated antibodies. FAK-KD is a kinase-dead mutant whose K545 is mutated to A.

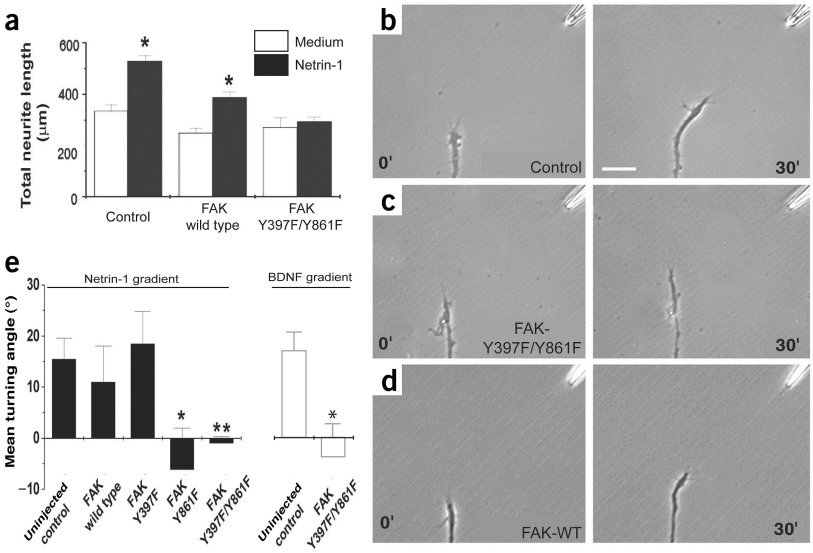

Figure 6 Requirement of FAK tyrosine phosphorylation for netrin-1-induced neurite outgrowth and attractive turning. (**a**) Effects of FAK and the Y397F/Y861F mutant on netrin-1-stimulated neurite outgrowth in *X. laevis* spinal neurons. mRNAs of FAK or the Y397F/Y861F mutant were injected into one of the *X. laevis* blastomeres at the two-cell stage together with the GFP mRNA. Spinal neurons derived from injected embryos (stage 20) were stimulated with or without netrin-1. Total neurite length was measured in individual GFP-positive neurons from different injections. Data are mean ± s.e.m. from at least three separate experiments. $^{*}P < 0.05$, different from control neurons expressing GFP (*t*-test). (**b**) Images of a growth cone at the onset (left) and the end (right) of a 30-min exposure to a netrin-1 gradient. Bar, 20 μm. (**c**,**d**) Similar to conditions in **b**, except that the neuron expressed FAK-Y397F/Y861F (**c**) and wild-type FAK (**d**). In **b**, **c** and **d**, neurons of injected embryos (stage 20) were cultured for 16–18 h. (**e**) Mean turning angles of all growth cones examined. Data shown are mean ± s.e.m. from 10–20 neurons. $^{*}P < 0.05$, $^{**}P < 0.01$, different from controls (Kolmogorov-Smirnov test).

FAK tyrosine phosphorylation and netrin-1 functions

X. laevis spinal neurons express endogenous FAK and have been extensively used as a model to study neurite outgrowth and growth cone turning[12,14,15,19,22]. To investigate the role of FAK in netrin-1 function, we determined whether perturbation of FAK tyrosine phosphorylation affects netrin-1-induced neurite outgrowth and growth cone turning in *X. laevis* spinal neurons. To manipulate FAK signaling, messenger RNAs encoding FAK or its mutants were injected, together with green fluorescent protein (GFP) mRNA, into one of the blastomeres at the two-cell stage[22]. Neurons expressing injected constructs were identified by GFP fluorescence. Immunocytochemistry using antibodies to Myc (anti-Myc; for Myc-FAK) indicated that the GFP fluorescence faithfully reflected the expression of the exogenous protein (data not shown). *X. laevis* spinal neurons showed increased neurite extension in the presence of netrin-1 (10 ng/ml in the culture medium, overnight; **Fig. 6a**). This event was, however, significantly inhibited in neurons expressing FAK-Y397F/Y861F but not FAK-WT, suggesting the necessity of FAK tyrosine phosphorylation in this event (**Fig. 6a**). Notably, FAK-WT and FAK-Y397F/Y861F had no significant effect on basal neurite extension in spinal neurons (**Fig. 6a**).

To determine whether FAK tyrosine phosphorylation is required for netrin-1-induced growth cone turning in *X. laevis* spinal neurons, netrin-1 was applied using a pipette to generate a gradient (5 μg/ml in the pipette)[22]. Growth cones of control neurons turned toward netrin-1 within 30 min (**Fig. 6b**,**e**)[22]. The response seemed to be based on basal neurite extension, because netrin-1, when applied under these conditions, induces only an acute growth cone turning response but not neurite extension[22]. As a control, wild-type FAK had little effect on the netrin-1-induced turning response (**Fig. 6d**,**e**). The response was, however, blocked in neurons expressing FAK-Y397F/Y861F (**Fig. 6c**,**e**), suggesting that FAK tyrosine phosphorylation is involved in this event. Of note is that the growth cone turning response induced by brain-derived neurotrophic factor (BDNF), a factor known to be a chemoattractant for growth cones of *X. laevis* spinal neurons that shares similar signaling pathways to netrin-1 (refs. 19,22), was also blocked in neurons expressing FAK-Y397F/Y861F (**Fig. 6e**). In light of the observation that FAK-Y397F/Y861F had no significant effect on basal neurite outgrowth (**Fig. 6a**), these results indicate that FAK tyrosine phosphorylation may be required not only for netrin-1 but also for the BDNF-induced attractive turning response. Further analyses showed that FAK-Y861F blocked the netrin-1-induced turning response, whereas the FAK Y397 mutant, whose phosphorylation was independent of P3, had no effect (**Fig. 6e**), demonstrating the specificity of the inhibitory effect. Taken together, these results suggest potential differential functions of FAK Y861 and Y397 phosphorylation in netrin-1 signaling and show a critical role for FAK tyrosine phosphorylation in netrin-1-induced neurite extension and attractive turning.

DISCUSSION

Here we present evidence for the involvement of FAK in mediating netrin-1-induced neurite outgrowth and guidance. FAK interacts with DCC and becomes activated in response to netrin-1. Inhibition of FAK tyrosine phosphorylation blocks netrin-1 function. In our working model, FAK serves as a mediator immediately downstream of DCC for netrin-1 signaling. These results provide new leads regarding how guidance cues regulate the cytoskeleton and cross-talk with the integrin signaling pathway.

The P3 domain in DCC is essential for netrin-1-induced attractive turning, neurite outgrowth and the slit-2-induced silencing response of netrin-1(refs. 14,15). HGF-induced attractive turning and neurite outgrowth are blocked in *X. laevis* spinal neurons when the P3 domain is deleted from the Met-DCC chimeric protein[15]. DCC^{Kanga} mice, which are generated by spontaneous deletion of the P3 domain, show abnormal projection of corticospinal tract fibers into the spinal cord[16]. The identification of FAK as a binding partner of the DCC-P3 domain indicates that FAK may be a mediator that is immediately downstream of DCC for netrin-DCC signaling. Upon integrin engagement, FAK becomes phosphorylated on Y397, which recruits SH2 domain–containing proteins including Src family kinases and

PLCγ[37,38]. It is probably phosphorylation of FAK Y397 by netrin-1 that recruits these signaling molecules to the DCC complex, providing a mechanism by which netrin-1/DCC activates Src kinases and PLCγ. On the other hand, FAK Y861 is a principal site of phosphorylation in v-Src-transformed cells. Although the exact function of Y861 phosphorylation remains to be characterized, a recent observation suggests a role for Y861 phosphorylation in regulating FAK binding to integrins[35]. Thus, netrin-1 induction of FAK Y861 phosphorylation may alter its binding to integrins, which could be important for netrin-1-induced growth cone turning.

Our results also identify FAK as a possible link between guidance cue receptors and cytoskeletal reorganization. It is known that FAK functions as a scaffold protein to assemble multiprotein complexes at cell adhesion sites. Signaling proteins are recruited to adhesion sites in a manner that is dependent on FAK tyrosine phosphorylation; FAK also binds to cytoskeleton-associated proteins including paxillin or Hic5 and talin[38]. These proteins interact directly or indirectly with the actin cytoskeleton. In light of these observations, we propose that FAK, by means of bridging DCC and cytoskeleton-associated proteins, assembles a multiprotein complex in neurites to regulate cytoskeletal reorganization that is necessary for neurite outgrowth and growth cone turning. The complex contains not only cytoskeletal proteins but also signaling proteins that are implicated in regulating cytoskeleton reorganization including RhoGAP proteins (Graf and PSGAP) and $p130^{Cas}$ (refs. 39,40). This FAK-centered signaling complex may be not only required for netrin-1 functions but also involved in other chemoattractant-induced turning responses, such as that of BDNF.

Along the navigation path, growth cones encounter many environmental cues in the ECM or on the cell surface in addition to classic attractive and repulsive cues. For example, neural cell adhesion molecule (N-CAM) and L1, cell adhesion molecules of the immunoglobulin superfamily, are permissive signals for neurite outgrowth or turning. They promote neuronal axon outgrowth and fasciculation. Stimulation of N-CAM or L1 by homophilic binding or by antibodies that recognize extracellular determinants activates FAK[41]. In addition, FAK associates with N-CAM140, a transmembrane isoform of N-CAM in migratory growth cones, and is required for N-CAM-induced neurite outgrowth. Thus, FAK may represent a convergence point between permissive and instructive guidance cues. This notion is also supported by the observation that laminin-1, a principal ECM glycoprotein in neurons, activates FAK and regulates netrin-1 function[42]. It is of note that immunoglobulin superfamily transmembrane proteins may associate with integrins to coordinately regulate cell-cell and cell-ECM adhesions, and DCC is a protein that contains multiple immunoglobulin domains and thus may interact with integrins. Notably, netrins that are related to laminin β1 in structure also contain an RGD sequence, a motif identified in many ECM proteins for integrin binding[43]. Although this hypothesis needs to be tested by further experiments, our results indicate that there may be cross-talk between netrin-1–DCC signaling and integrin signaling, at the very least at the level of FAK. Indeed, netrin-1 binds to integrins[44], and many signaling proteins known to be regulated by integrins are involved in netrin-1 function, including Src family kinases[45,46], PLCγ[19], and Rho family GTPases[21]. Thus, coordinated stimulation of the two pathways may be important for cytoskeletal reorganization and subsequent neurite outgrowth and growth cone turning. FAK may act not only as a mediator of neurite outgrowth and guidance but also as a platform for cross-talk among signaling pathways, which provides a mechanism for complex behaviors of axons in response to guidance cues and matrix factors.

The exact mechanisms by which FAK becomes activated remain unknown. Our results indicate that recruitment of FAK to the plasma membrane and formation of DCC dimers or clusters may be critical for netrin-1-induced FAK tyrosine phosphorylation. First, DCC forms clusters in netrin-1-treated neurons, which correlates with FAK tyrosine phosphorylation (data not shown). Second, overexpression of DCC, which leads to DCC dimerization or oligomerization, induces FAK tyrosine phosphorylation, even in the absence of netrin-1. Third, treatment of neurons with an antibody that blocks DCC function, which could induce DCC clustering, also increases FAK tyrosine phosphorylation (data not shown). In agreement with this notion are reports that integrin clustering, induced either by ligand occupation or antibodies, is sufficient to induce FAK tyrosine phosphorylation[47,48]. Taken together, these observations support a role for DCC dimerization or clustering in FAK activation. However, it has not escaped our attention that netrin-1-induced FAK tyrosine phosphorylation is not completely eliminated in $Dcc^{-/-}$ neurons, suggesting that in addition to DCC, other receptors are involved in this event. We speculate that integrins may contribute in part to netrin-1-induced FAK tyrosine phosphorylation.

METHODS

Reagents and animals. Monoclonal antibodies were purchased from Santa Cruz Biotechnology (anti-Myc), Oncogene Science (anti-DCC), Sigma Chemical Co. (anti-Flag) and Transduction Labs (anti-FAK, anti-PYK2 and RC20). Polyclonal antibodies were purchased from Biosources International, Inc. (anti-PY397, anti-PY577 and anti-PY861) and Santa Cruz Biotechnology (anti-DCC, A20). Rabbit polyclonal anti-neogenin antibody was generated using GST-neogenin (amino acids 1158–1527) as an antigen. Chicken netrin-1 cDNA (kindly provided by M. Tessier-Lavigne; Stanford University) was subcloned into pcDNA3-Myc/His to generate Myc-netrin-1-6His for purification. Stable HEK293 cells expressing human netrin-1 were provided by J.Y. Wu (Washington University). Unless otherwise indicated, conditioned medium containing ~200 ng/ml human netrin-1 was used for stimulation. DCC knockout mice (kindly provided by S. Ackerman; The Jackson Laboratory) were maintained in the B6 background. Embryos derived from $Dcc^{+/-}$ matings were used for cortical neuronal culture. Genotyping was carried out by the polymerase chain reaction (PCR) as described previously[49].

Yeast two-hybrid studies. The C-terminal domain of chicken FAK (amino acid residues 800–1052) was generated by PCR, was subcloned downstream of the Gal4 DNA-binding domain in pGBT10 and was used as bait to screen a mouse oligo(dT)-primed cDNA library in pACT2 (ref. 39). Clones that grew on plates lacking leucine, tryptophan and histidine with 25 mM 3-aminotriazole were selected and assayed for β-galactosidase activity. The original clone encoded the C-terminal region of neogenin, containing amino acids 1158–1493.

Expression vectors. The cDNAs encoding neogenin, DCC and DCC mutants were amplified by PCR and were subcloned into mammalian expression vectors downstream of a Flag or a Myc epitope tag with a signal peptide and under the control of the CMV promoter. The cDNAs of FAK and PYK2 were described previously[39]. Point mutations in DCC and FAK were generated using the Quick Change kit (Stratagene). The authenticity of all mutants was verified by DNA sequencing. cDNA sequences corresponding to the cytoplasmic domains of DCC (amino acids 1108–1445), DCCΔP3 (amino acids 1108–1425) and neogenin (amino acids 1158–1527) were cloned into pGEX-2T (Pharmacia) to generate GST fusion proteins.

Protein-protein interaction assays. Immunoprecipitation was carried out as previously described[39]. Briefly, cell lysates (1 mg of protein) were incubated with indicated antibodies (1–2 μg) at 4 °C for 1 h in a final volume of 1 ml modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM sodium chloride; 1% NP40; 0.25% sodium-deoxycholate and proteinase inhibitors)[39] with constant rocking. After the addition of protein A/G–agarose beads, the reactions were incubated at 4 °C for 1 h. Immune complexes were resolved by SDS-PAGE and

were subjected to immunoblotting. The GST pull-down assay was carried out as described previously[39].

Culture and immunostaining of neurons. Primary cortical neurons were cultured as described previously[50]. For immunostaining, E17 rat cortical neurons (DIV3) were used for immunocytochemistry as previously described[50]. Briefly, they were fixed with 4% paraformaldehyde for 20 min, blocked with 5% donkey serum and incubated with antibodies against DCC (A20 goat polyclonal) and FAK (PY397 rabbit polyclonal). Double-labeled immunostaining was done with appropriate fluorochrome-conjugated secondary antibodies. Images were taken on a Zeiss fluorescence microscope at 63× with Axiocam CCD (charge-coupled device) camera and were assembled in PhotoShop (Adobe).

Neurite outgrowth and growth cone turning in *X. laevis* spinal neurons. Blastomere injection of mRNAs and culture of *X. laevis* spinal neurons were done as previously described[22]. Briefly, fertilized embryos at the two-cell stage were injected unilaterally using the Eppendorf microinjector 5242 (Eppendorf). A 1:1 mixture of FAK RNA (2–3 μg/μl) and GFP RNA (2–3 μg/μl) was injected into embryos. Neurons derived from the RNA-injected blastomere were identified by GFP fluorescence.

For assaying neurite outgrowth, neurons derived from injected embryos at stage 20–22 were plated on coverslips and were cultured in medium with or without purified recombinant netrin-1 (10 ng/ml) for 24 h. Total neurite length of each individual GFP^+ and GFP^- neuron was measured and analyzed for different conditions. The growth cone turning assay was carried out as previously described[19,22]. Briefly, a microscopic gradient of netrin-1 was produced by a pipette (5 μg/ml netrin-1 and 50 μg/ml BDNF in the pipette), which was placed 100 μm away from the center of the growth cone at an angle of 45° with respect to the initial direction of neurite extension. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the center of the growth cone at the onset and at the end of the assay[22]. Net neurite extension rate was determined by measuring the total trajectory of the neurite at the end of the experiment with imaging software. Only those growth cones with net growth of more than 10 μm were included for analysis.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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