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Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation

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Neuronal growth cones are guided to their targets by attractive and repulsive guidance cues[1]. In mammals, netrin-1 is a bifunctional cue, attracting some axons and repelling others[2–5]. Deleted in colorectal cancer (Dcc) is a receptor for netrin-1 that mediates its chemoattractive effect on commissural axons[6,7], but the signalling mechanisms that transduce this effect are poorly understood. Here we show that Dcc activates mitogen-activated protein kinase (MAPK) signalling, by means of extracellular signal-regulated kinase (ERK)-1 and -2, on netrin-1 binding in both transfected cells and commissural neurons. This activation is associated with recruitment of ERK-1/2 to a Dcc receptor complex. Inhibition of ERK-1/2 antagonizes netrin-dependent axon outgrowth and orientation. Thus, activation of MAPK signalling through Dcc contributes to netrin signalling in axon growth and guidance.

Signalling mechanisms triggered by netrin binding to Dcc are poorly understood. Dcc interacts with receptors of the Robo and UNC5H families, and adenosine A2b (refs 5, 8, 9), which are believed to modulate Dcc function. Beyond these cell-surface interactions, one aspect of Dcc signalling already characterized is its role as a dependence receptor, inducing apoptosis in the absence of netrin-1 through a mechanism involving caspase cleavage of its cytoplasmic domain[10]. However, signalling events downstream of

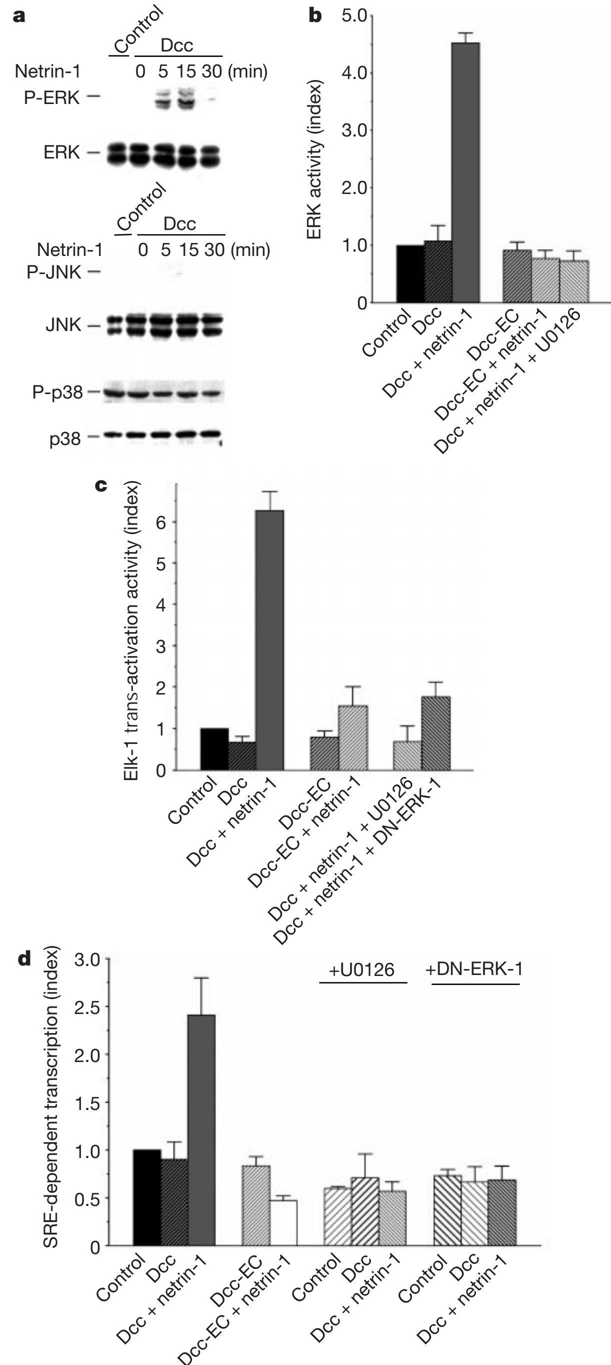

Figure 1 Dcc induces ERK-1/2 activation on netrin-1 binding. **a**, Western analysis of phosphorylated and total ERK-1/2, JNK and p38 in Dcc-transfected HEK 293 cells in response to 100 ng ml^{-1} netrin-1. **b**, ERK kinase activity after 15 min of 100 ng ml^{-1} netrin-1 in HEK 293 cells transiently expressing full-length or truncated Dcc (Dcc-EC; expression of the Dcc ectodomain). Control, 4 μM U0126. **c**, **d**, Elk-1 activation (**c**) or SRE-dependent gene expression (**d**) assessed by luciferase activity or SEAP expression, respectively, in HEK 293 cells transfected with full-length or truncated Dcc, with or without netrin-1 construct. Controls in **c** and **d**: co-transfection of dominant negative (DN) ERK-1, or addition of 4 μM U0126.

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Dcc that mediate axon growth and guidance remain to be identified. We therefore examined whether classic signalling pathways mediate netrin actions.

The MAPK proteins, an evolutionarily conserved family of sequence- and structurally related enzymes, are interesting candidates for the downstream mediators of axon growth and guidance as they connect cell-surface receptors to critical regulatory targets[11]. To test their involvement, HEK 293 or NIH3T3 cells were transiently transfected with a full-length Dcc expression construct, and phosphorylation of major MAPK proteins (ERK-1/2, Jun N-terminal kinase (JNK)-1/2 and p38) was analysed. Netrin-1 caused increased phosphorylation of ERK-1/2, but not JNK-1/2 or p38, in Dcc-transfected cells (Fig. 1a and not shown). This phosphorylation was rapid (appearing in less than 5 min) but transient, disappearing within 30 min (Fig. 1a). Increased phosphorylation was accompanied by increased ERK-1/2 activity, assessed by the ability of immunoprecipitated ERK-1/2 to phosphorylate myelin basic protein (MBP) *in vitro* (Fig. 1b and data not shown). This activation was not observed with a truncated form of Dcc that lacked its cytoplasmic domain. Activation was completely blocked by U0126, a specific inhibitor of MAP-kinase kinase (MEK)-1 and MEK-2 (Fig. 1b). Only slight inhibition, however, was observed with PD098059 (not shown), an inhibitor specific for MEK-1 over MEK-2 (ref. 12), suggesting that in these cell lines either MEK-2 mediates most of the netrin-induced ERK activation, or that either MEK-1 or -2 alone are redundant and must both be inhibited to block activation.

Some of the effects of ERK-1/2 are mediated through regulation of specific transcription factors[13]. We found that netrin activation of full-length, but not truncated, Dcc activated the transcription factor Elk-1 (Fig. 1c). We also examined ERK-dependent transcription from a reporter gene controlled by a serum response element (SRE) enhancer element. Whereas Dcc alone had no effect, co-expression of Dcc and netrin-1 enhanced SRE-dependent transcription, an effect requiring the Dcc intracellular domain (Fig. 1d). Both Elk-1 activation and SRE-dependent transcription were blocked by U0126 or by expression of dominant negative ERK-1 (Fig. 1c, d). Thus, netrin-induced ERK-1/2 activation can cause transcriptional activation.

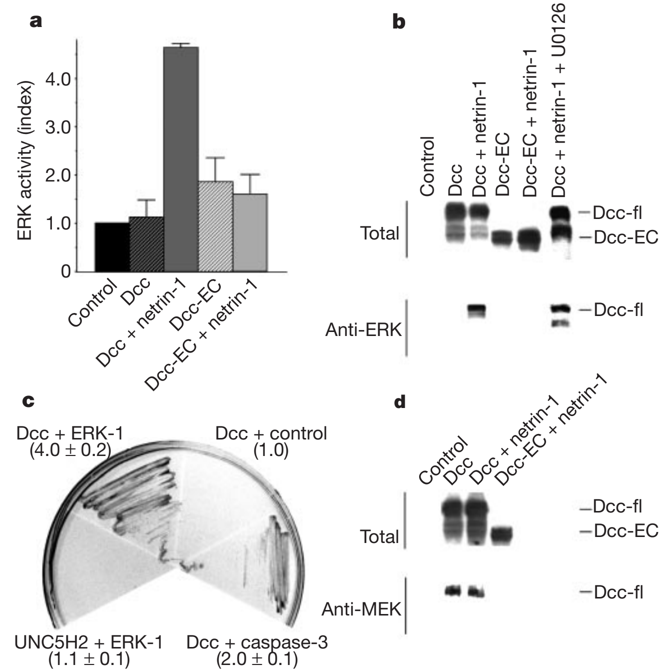

Figure 2 Interaction of Dcc with ERK-1/2 and MEK-1/2. HEK 293 cells transfected with mock, full-length (fl) or truncated Dcc-EC constructs were treated for 15 min with netrin-1 (**a**, **b**, **d**). **a**, ERK kinase assay performed on a Dcc immunoprecipitate. **b**, Immunoprecipitation using an anti-ERK-1/2 antibody followed by Dcc immunoblot. **c**, Interaction of the Dcc intracellular domain and ERK-1 assessed using the yeast two-hybrid system. Quantitative strength of the interaction is indicated in parentheses. **d**, Immunoprecipitation using a MEK-1/2 antibody followed by Dcc immunoblot. One Dcc molecule is immunoprecipitated by approximately nine ERK molecules or four MEK molecules (not shown).

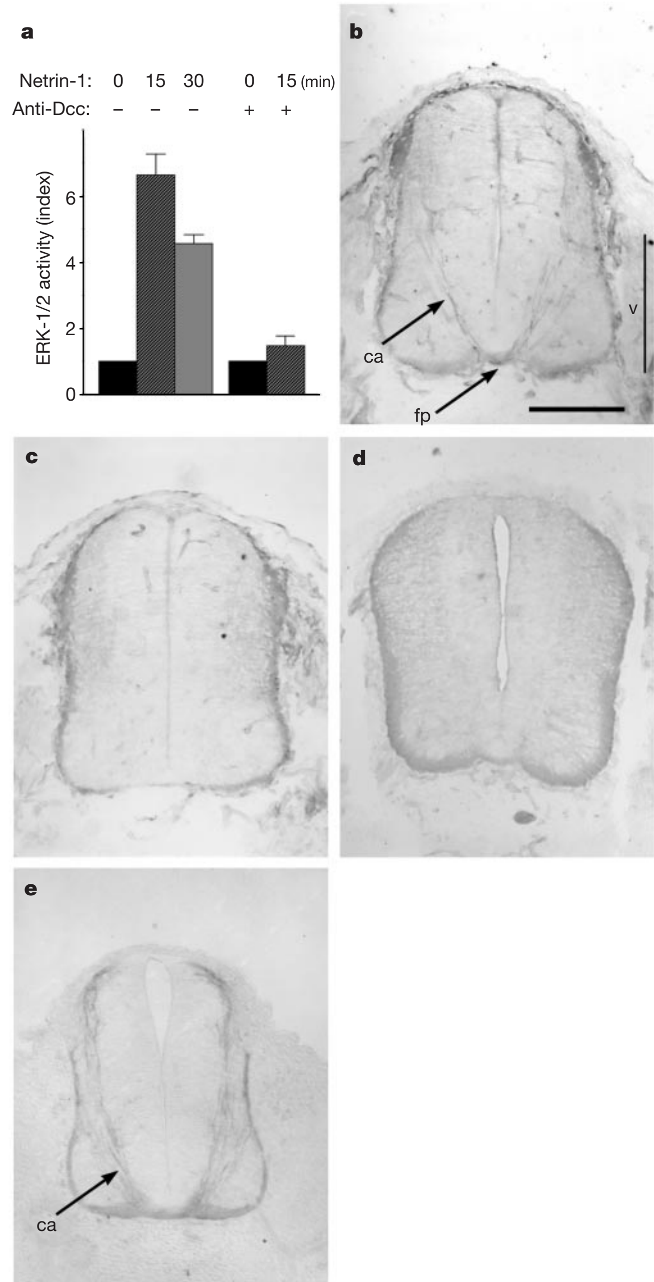

Figure 3 Netrin-1-induced ERK-1/2 activation occurs in commissural neurons. **a**, Rat E13 spinal cords were dissected and cultured in L15 medium[4]. A total of 100 ng ml^{-1} netrin-1 was added for 0, 15 or 30 min in the presence or absence of 10 μg ml^{-1} anti-Dcc (AF5) antibody, and ERK-1/2 activity was measured using an ERK kinase assay. **b**–**d**, ERK-1/2 activation occurs in commissural axons during spinal cord development. Phospho-ERK staining on transverse sections of spinal cords from E13 wild-type (**b**), *netrin-1* $^{-/-}$ (**c**) or *Dcc* $^{-/-}$ (**d**) embryos. **e**, Dcc staining. ca, commissural axons; fp, floor plate; v, ventral spinal cord. Scale bar, 200 μM.

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To begin to investigate how Dcc activates ERK-1/2, we examined whether immunoprecipitates of Dcc and its interacting proteins contain ERK-1/2 activity. A 15 min netrin-1 treatment resulted in the presence of MBP phosphorylating activity and ERK-1/2 protein in a Dcc immunoprecipitate (Fig. 2a, b), but only from cells expressing full-length, not truncated, Dcc (Fig. 2a, b). In a control experiment, ERK-1/2 was activated by glial cell line derived neurotrophic factor (GNDF) binding to the Ret receptor tyrosine kinase expressed in HEK 293 cells, but did not immunoprecipitate Ret (not shown). In a yeast Gal4 two-hybrid assay, we found evidence for direct interaction of the Dcc intracellular domain with full-length ERK-1 (Fig. 2c), suggesting that Dcc may directly recruit ERK-1 in response to netrin-1. This interaction seems to be required for netrin-induced ERK activation as it was abolished by deletion of a domain in Dcc required for the interaction (C.F., F.L., E.S., M.T.-L. and P.M., manuscript in preparation). As ERK activation usually occurs through formation of a module composed of a MAPK, a MAPKK and a MAPKKK protein[11], we tested whether the MAPKK MEK can interact with Dcc. Full-length, but not truncated, Dcc immunoprecipitated with endogenous MEK-1/2 (Fig. 2d); how-

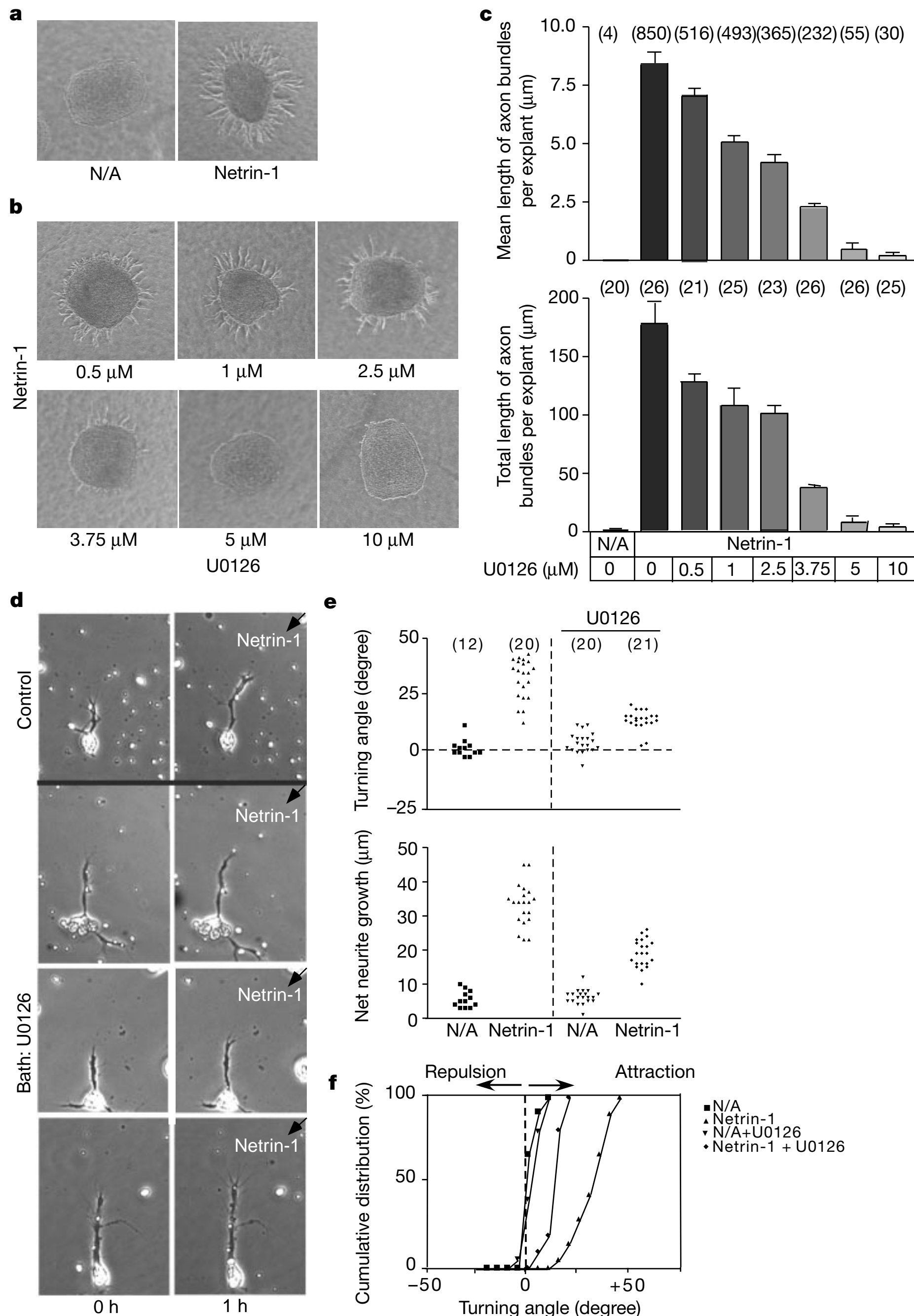

Figure 4 ERK-1/2 inhibition blocks netrin-mediated outgrowth and turning. **a**, E13 rat dorsal spinal cord explants cultured alone (N/A) or with netrin-1 (65 ng ml^{-1}). **b**, Explants cultured with netrin-1 (65 ng ml^{-1}) and increasing concentrations of U0126. **c**, Quantitative analysis. Numbers tested are in parentheses. **d**, Images of representative *Xenopus* spinal growth cones before and after a 1 h exposure to a netrin-1 gradient (indicated by an arrow), with and without U0126 (10 μM). **e**, Scatter diagram of turning angles (top) and net neurite extension during a 1-h period (bottom). Numbers of growth cones tested are in parentheses (symbols as in **f**). Cumulative distribution plot of turning angles (proportion with angles less than a given angle). Magnification: **a**, **b**, ×15; **d**, ×300.

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ever, unlike ERK-1/2, MEK-1/2 interacted with Dcc equally in the presence or absence of netrin-1. These results suggest that MEK-1/2 may be latently bound to Dcc before recruitment and activation of ERK-1/2 in response to netrin-1; the recruitment, however, seems independent of MEK-1/2 activity, as it was not blocked by U0126 (Fig. 2b). Alternatively, we cannot exclude the possibility that MEK-1/2 bound nonspecifically to the intracellular domain of over-expressed Dcc. Future studies will determine whether this apparent docking mechanism triggers ERK-1/2 activation or instead simply amplifies activation stimulated by other factors.

We next examined whether ERK-1/2 contributes to the effects of Dcc on axon growth and guidance. We first tested whether ERK-1/2 activation occurs in a population of netrin-responsive neurons (commissural neurons). These neurons have cell bodies in the dorsal spinal cord that extend axons to the ventral spinal cord in response to netrin-1 (refs 2, 4). Netrin-1 stimulates outgrowth of commissural axons from explants of E13 (embryonic day 13) rat dorsal spinal cord by activating Dcc on these axons[4,6]. Application of netrin-1 to isolated E13 rat spinal cord induced ERK-1/2 activation, with kinetics similar to those observed in Dcc-expressing HEK 293 cells (Fig. 3a). Dcc is required for this activation, as it was blocked by pre-treatment with a function-blocking anti-Dcc antibody[6] (Fig. 3a). We next examined whether ERK-1/2 activation can occur specifically in commissural axons, using immunohistochemistry on tissue sections with an antibody to phosphorylated ERK-1/2. High levels of phospho-ERK-1/2 were detected in commissural axons between E10.5 and E13 (Fig. 3b and data not shown), ages at which the axons are growing to the floor plate[2]. In netrin-1 and Dcc knockout animals we observed diminished phospho-ERK-1/2 staining in the ventral spinal cord (Fig. 3c, d); this finding is consistent with activation of ERK-1/2 by netrin-1, but its interpretation is complicated by the fact that commissural axon extension requires netrin-1 and Dcc, and is therefore impaired in these animals[4,7]. Nonetheless, these results suggest that Dcc-dependent ERK activation occurs in commissural axons in response to netrin-1.

To determine whether ERK activation is required for netrin function, dorsal spinal cord explants from E13 rat embryos were grown for 16–18 h in collagen gels with or without netrin-1 (ref. 2). U0126 blocked netrin-1-induced axon extension in a dose-dependent manner ($P < 0.0001$ at all doses) (Fig. 4a–c). This effect does not represent a general inhibition of outgrowth but is, rather, specific for netrin-1 signalling, as the netrin-independent commissural axon outgrowth that can be observed when explants are grown for 40 h (ref. 6) was not affected by U0126 (total axon length per explant with and without U0126 (mean ± s.e.m.): $223 \pm 60\,\mu m$ ($n = 18$) and $279 \pm 57\,\mu m$ ($n = 17$), respectively ($P = 0.586$)). These observations support a requirement for ERK-1/2 in netrin-mediated outgrowth of commissural axons. In contrast to U0126, the p38 inhibitor SB-203580 actually increased the number of axon bundles emanating from explants in response to netrin-1 (not shown), an effect perhaps explained by a known side effect of SB-203580, that is, activation of c-Raf/ERK signalling[14].

We next tested whether MAPK signalling is required for netrin-1 to re-orient axons, using an assay in which *Xenopus* spinal neurons turn towards a source of netrin-1; an effect mediated by Dcc[5,15,16]. Whereas ERK-1/2 inhibition did not appear to affect the basal growth of axons, both netrin-1-induced turning and the netrin-1-stimulated increase in extension rate were antagonized by U0126 (Fig. 4d–f). Of note, the effects of netrin-1 were not completely blocked even though U0126 blocked ERK-1/2 activation by netrin-1 in *Xenopus* spinal neurons (not shown), suggesting an additional involvement of a MEK-1/2-independent pathway in the acute turning response.

Our results support a role for the MAPK pathway in responses to the chemoattractant netrin-1. This pathway has previously been implicated in neurite extension stimulated by neurotrophic factors activating Trk receptors and by cell adhesion molecules like N-cadherin, laminin and L1 (refs 17–21). Conversely, activation of some repulsive receptors of the Eph family can repress MAPK signalling[22,23], although a causal role in repulsion has not been established. How does ERK activation affect axonal growth and guidance? Some effects probably result from phosphorylation of cytoskeletal ERK targets such as microtubule-associated proteins and neurofilaments[24]. Interestingly, a MAPK-dependent mechanism drives internalization of the cell adhesion molecules ApCAM and L1 at the rear of the growth cone, which may allow protein cycling from the rear to the leading edge necessary for growth cone advance[24–26]. We also show that Dcc-stimulated MAPK activation leads to activation of the transcription factor Elk-1 and SRE-regulated gene expression, providing a mechanism for transcriptional control by netrin-1. Elk-1 is present not just in neuronal cell bodies but also in axon terminals[27], but whether axonal Elk-1 participates in axon guidance is unknown. Other targets of ERK-1/2 involved in axon guidance may be translation regulators. New protein translation is stimulated by netrin-1 and required for netrin-mediated attraction of *Xenopus* retinal growth cones *in vitro*[28]. Principal factors for translation initiation like eIF4E and eIF4E-BP1 are phosphorylated by an ERK-1/2-dependent pathway[29], providing a potential mechanism linking netrin-1 to protein synthesis for axon growth and guidance. □

Methods

Cell transfection, immunoblotting and netrin-1 production

Transient transfections of HEK 293 or NIH3T3 cells were performed using Lipofectamine following the manufacturer's suggested procedure (Life Technology). Immunoblots using different commercially available antibodies (phospho-ERK-1/2, Cell Signaling Technology; ERK-1/2, Sigma; phospho-p38, Cell Signaling Technology; p38, Cell Signaling Technology; phospho-JNK, Cell Signaling Technology; JNK, Cell Signaling Technology) were performed as described previously[10]. Netrin-1 was purified from netrin-1-producing HEK 293-EBNA cells[2].

Site-directed mutagenesis and plasmid constructs

The full-length Dcc-expressing construct pDcc-CMV-S and the netrin-1-expressing construct pGNET1-Myc were as described[2,10]. The construct pDcc-EC-CMV, allowing the expression of the ectodomain of Dcc, was generated by a Quikchange strategy (Stratagene) replacing amino acid 1,121 by a stop codon. This was done using pDcc-CMV-S as template and the following primers: 5′-GCTGTGATTTGCACCTGACTCGAGCGACGCTCT TCAGCC-3′ and 5′-GGCTGAAGAGCGTCGCTCGAGTCAGGTGCAAATCACAGC-3′. The construct pRc/CMV-ERK1 T192A, coding for a dominant negative form of ERK-1, was provided by V. Fafeur. Haemagglutinin (HA)-tagged P44 (ERK-1) was provided by J. Pouyssegur. The two-hybrid construct pACT2-ERK-1 coding for the Gal4-activating domain fused to ERK-1 was generated by introducing, in the *Bam*H1–*Eco*R1 restriction sites of pACT2, the polymerase chain reaction product obtained from HA-P44 template and 5′-TATGGATCCGAGGCGGGGAGCCCCGG-3′ and 5′-TATGAATTCTTAGGGGG CCTCTGG -3′ primers.

MAPK activity assay

The MAPK immunoprecipitation kinase assay was performed using agarose-coupled ERK-1/2 antibody following Euromedex's instructions.

Elk-1 *trans*-reporting assay

HEK 293 or NIH3T3 cells were transiently transfected with the Dcc-expressing constructs together with pFA2-Elk-1 and pFR-Luc vectors (Stratagene). Elk-1 activation was measured using an Elk-1 *trans*-reporting assay (Stratagene) according to manufacturer's instructions.

Reporter gene assay

HEK 293 or NIH3T3 cells were transiently transfected with the Dcc- and/or netrin-1-expressing constructs together with pSRE-SEAP vector (Clonetech). Twenty-four hours after transfection, SEAP was measured using Great EscAPe SEAP Detection System (Clontech).

Two-hybrid analysis

The two-hybrid system matchmaker II (Clontech) was performed as described previously[8] using pAS2.1-Dcc-IC and pACT2-ERK-1. As a positive control, yeast cells were co-transformed with Dcc-IC and caspase-3 (ref. 8); as a negative control, yeast cells were co-transformed with Dcc and empty Gal4AD vector, or with UNC5H2 and ERK-1. The strength of the interaction was also measured following the manufacturer's instructions and is presented as the ratio between the level of β-galactosidase substrate degraded after 20 min for each co-transformed yeast and the one observed in the Dcc/Gal4AD control yeast.

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Immunoprecipitation and immunohistochemistry

Immunoprecipitations were carried out on HEK 293 cells transfected with pDcc-CMV-S or pDcc-EC-CMV using either anti-ERK-1/2 or anti-MEK-1/2 to immunoprecipitate endogenous ERK-1/2 or MEK-1/2, respectively. All immunoprecipitations were performed using 36 mg of lysates. The Dcc interaction with ERK-1/2 or MEK-1/2 was detected by immunoblot using an anti-Dcc antibody[10].

We carried out production, breeding and genotyping of the netrin-1- and Dcc-deficient mice as described previously[4,7]. Timed pregnant (plug date, E0) netrin-1 and Dcc mutant mice were killed, and individual embryos were fixed overnight at 4 °C using 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Cryostat sections were stained with an anti-phospho ERK antibody (Cell Signaling Technology) or an anti-Dcc antibody. Immunostainings were visualized with a diaminobenzidine reaction.

Commissural axon outgrowth and *Xenopus* spinal turning assay

We performed both assays exactly as described[16]. The outgrowth from E13 explants was quantified by measuring the total length of all axon fascicles emerging from explants (total length of axon) or the mean of the length of these axon fascicles (mean length of axon bundles).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Molecular identification of a renal urate–anion exchanger that regulates blood urate levels

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Urate, a naturally occurring product of purine metabolism, is a scavenger of biological oxidants implicated in numerous disease processes[1–3], as demonstrated by its capacity of neuroprotection[4,5]. It is present at higher levels in human blood (200–500 µM) than in other mammals[6], because humans have an effective renal urate reabsorption system, despite their evolutionary loss of hepatic uricase by mutational silencing[6–8]. The molecular basis for urate handling in the human kidney remains unclear because of difficulties in understanding diverse urate transport systems and species differences[6,9,10]. Here we identify the long-hypothesized[9–11] urate transporter in the human kidney (URAT1, encoded by *SLC22A12*), a urate–anion exchanger regulating blood urate levels and targeted by uricosuric and anti-uricosuric agents (which affect excretion of uric acid). Moreover, we provide evidence that patients with idiopathic renal hypouricaemia (lack of blood uric acid) have defects in *SLC22A12*. Identification of URAT1 should provide insights into the nature of urate homeostasis, as well as lead to the development of better agents against hyperuricaemia, a disadvantage concomitant with human evolution.

Urate exists primarily as a weak acid at physiological pH with a pK_a value of 5.75 (ref. 10); therefore, the structure of the urate transporter should be similar to that of members of the organic

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