Novel roles for Slits and netrins: axon guidance cues as anticancer targets?

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Abstract | Over the past few years, several genes, proteins and signalling pathways that are required for embryogenesis have been shown to regulate tumour development and progression by playing a major part in overriding antitumour safeguard mechanisms. These include axon guidance cues, such as Netrins and Slits. Netrin 1 and members of the Slit family are secreted extracellular matrix proteins that bind to deleted in colorectal cancer (DCC) and UNC5 receptors, and roundabout receptors (Robos), respectively. Their expression is deregulated in a large proportion of human cancers, suggesting that they could be tumour suppressor genes or oncogenes. Moreover, recent data suggest that these ligand–receptor pairs could be promising targets for personalized anticancer therapies.

Floor plate
A group of cells that occupy the ventral midline of the developing vertebrate nervous system, extending from the spinal cord to the diencephalon. They secrete morphogens and axon guidance molecules.

Commissural axons
Neurons that extend or project axons across the dorsal or ventral midline of the nervous system and have an important role in the coordination of sensory information received on both sides of the body.

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Netrin 1 — named from the sanscrit netr, 'the one who guides' — was purified by Tessier-Lavigne and colleagues as a soluble factor secreted by floor plate cells able to elicit the growth of commissural axons[1,2]. This discovery launched a scientific race to identify novel secreted or membrane-bound factors with repulsive or attractive activities for growing axons and migrating neurons. This led to the identification of the four canonical families of axon guidance cues and their receptors: netrin 1, which binds members of the UNC5 family and deleted in colorectal cancer (DCC); semaphorins, which bind plexins and neuropilins; Ephrins–Ephrin receptors (Eph); and Slits–roundabout receptors (Robos)[3,4]. Far from being confined to the developing brain, these ligand–receptor pairs have recently emerged as pivotal factors in tumour progression, especially during the late phases of tumour growth and dissemination[5]. As the importance of ephrins[6] and semaphorins[7] in cancer has been recently reviewed elsewhere, we focus on netrin 1, Slits and their receptors.

Netrins, Slits and Robos

Netrin 1 activates intracellular signal transduction pathways that are downstream of multiple receptors, including DCC and UNC5 homologue family members (UNC5A, UNC5B, UNC5C and UNC5D in humans, and UNC5H1, UNC5H2, UNC5H3 and UNC5H4 in rodents) (BOX 1; FIG. 1). Netrin 1 belongs to a larger family of laminin-related factors, which also includes two other secreted netrins (netrin 3 and netrin 4), and two glycosylphosphatidylinositol (GPI)-anchored membrane proteins, netrin G1 and netrin G2 (REF. 8). The different roles of netrin 1 and its receptors have been extensively described, but little is known about the function of these other netrins. Netrin 4, which shares little homology with netrin 1 (netrin 4, unlike other netrins, which display homology to the short arm of laminin-γ chains, is more related to laminin-β chains) has recently received more attention as a component of the basement membrane in the vasculature, kidneys and ovaries[9,10]. Slits and Robos (BOX 1; FIG. 1) were initially identified through their repulsive function at the midline of the central nervous system, but were also shown to modulate netrin 1–DCC signalling in commissural axons[11]. They function during many phases of brain development[12]. Three Slits (SLIT1, SLIT2 and SLIT3) and four Robo receptors (ROBO1–ROBO4) have been characterized in mammals[12].

From axon guidance to cancer progression

DCC is a prototypical receptor that functions in neuronal guidance, and its loss is implicated in the progression of colorectal cancer. Genetic studies in mice and *Caenorhabditis elegans* showed that DCC is a receptor that mediates netrin 1 attraction of commissural axons[13,14]. The gene encoding DCC was described in 1990 as a potential tumour suppressor involved in advanced stages of colorectal carcinogenesis[15]. Whether *DCC* is a tumour suppressor gene remains controversial mainly because of the absence of tumour predisposition in *Dcc*-mutant mice[13,16] (BOX 2), but *in vivo* evidence for such a role has been obtained for UNC5C, one of the other netrin 1 receptors[17,18]. *UNC5C* expression is downregulated in colorectal cancers, and in several other cancers, and this is also the case for

At a glance

- The axon guidance cues netrin 1 and Slits are causally implicated in human cancer. They are deregulated in a large proportion of human cancer, and the analysis of various mouse models has revealed that this deregulation is associated with tumour progression.
- Netrin 1, Slits and their respective receptors are implicated in tumorigenesis via the regulation of tumour cell migration, tumour cell survival and tumour angiogenesis.
- The netrin 1 receptors deleted in colorectal cancer (DCC) and UNC5 (UNC5A, UNC5B, UNC5C and UNC5D) are dependence receptors. They actively trigger apoptosis in the absence of netrin 1. This activity can function as a safeguard mechanism against tumour development.
- Slits–roundabout receptors (Robos) have a dual role in regulating angiogenesis. SLIT2–ROBO1 inhibits angiogenesis while SLIT2–ROBO4 promotes the stability of established vessels.
- Netrin 1 is upregulated in a large proportion of cancers, and an appealing therapeutic strategy could be to inhibit the interaction of netrin 1 with its receptors.

UNC5A and *UNC5B*, mainly through promoter methylation[17,19,20]. Tumour predisposition has been analysed in *Un5c*-deficient mice[17], and tumour frequency is not increased in these animals, suggesting that loss of UNC5C function is not sufficient to initiate tumorigenesis in mice. However, tumour aggressiveness is substantially increased in adenomatous polyposis coli-mutant ($Apc^{+/1638N}$);$Unc5c^{-/-}$ mice. Indeed, most $Apc^{+/1638N}$ mice develop mainly low-grade adenomas, whereas $Apc^{+/1638N}$;$Unc5c^{-/-}$ mice develop adenocarcinomas[17]. These data support a link between UNC5C inactivation and tumour progression, as well as a role for UNC5C as a tumour suppressor.

There is also evidence that loss of ROBO1 in mice is tumorigenic. Most *Robo1*-knockout mice exhibit postnatal morbidity, but surviving mice suffer from bronchial hyperplasia and focal dysplasia[21]. This correlates with the observation that *ROBO1* and *ROBO3* are inactivated or lost in different human cancers, such as invasive cervical, lung, breast and kidney cancer[22]. *ROBO1* is located in 3p12.3, a locus frequently affected by homozygous deletion and loss of heterozygosity (LOH) in lung cancer. However, associating variations in the expression levels of Robo receptors with cancer is apparently more complex than it is for DCC and UNC5, as some studies suggest that Robo expression is increased in some cancers, such as colorectal carcinomas[23]. However, as discussed below, this might be explained by the expression of Robo receptors in endothelial cells[23]. Not much is known about Slits and cancer development, although it is generally assumed that *SLIT2* and *SLIT3* could be tumour suppressor genes as they are frequently inactivated in various cancers through hypermethylation of their promoter regions and allelic loss[22,24,25]. Similarly, inactivation of *Slit2* and *Slit3* in mice is associated with the formation of hyperplastic disorganized lesions in the mammary epithelium[26].

Despite the fact that it was first reported that netrin 1 expression is decreased in some cancers, such as prostate cancer[27], netrin 1 functions as an oncogene[5], and is upregulated in different cancers, including two-thirds of metastatic breast cancers, 50% of non-small-cell lung cancers, and a large proportion of neuroblastomas and pancreatic adenocarcinomas[28–31]. Intriguingly, although netrin 1 expression is generally low in sporadic colorectal cancers, it is upregulated in colorectal cancers that have developed in patients with inflammatory bowel disease (IBD)[32]. Thus, inflammation may be an important regulator for netrin 1 function (BOX 3). The mechanisms behind netrin 1 upregulation in cancer are currently unknown. Upregulation does not seem to be related to gene amplification, but rather occurs at the level of gene expression, as suggested by nuclear factor-κB (NF-κB)-dependent expression of netrin 1 in IBD-associated cancers[33]. Inhibition of netrin 1 activity using a recombinant decoy netrin 1 receptor suppressed colorectal tumour progression in this mouse model[32]. Netrin 1 upregulation might also be involved in cancer initiation and progression, as demonstrated by the tumour predisposition of netrin 1-transgenic mice[34]. Conditional expression of netrin 1 in the gastrointestinal tract of $Apc^{+/1638N}$ mice is associated with tumour initiation (focal hyperplasia and low-grade adenoma), but more importantly with tumour progression (adenocarcinoma development)[34]. Netrin 4 has also been reported to be deregulated in human cancers[27,35]; however, its implication in cancer progression is still confusing at this stage[36–38].

Together, these data suggest that downregulation of Robos, Slits, DCC and UNC5 family members, and upregulation of netrin 1, are causal factors in tumour progression (TABLE 1). How these changes might affect tumour development is thought to be based on three main mechanisms.

Box 1 | **Netrins and Slits during development**

Netrins constitute a family of extracellular proteins that share sequence homology with laminin and control axon guidance and cell migration[1]. In addition, netrins are also involved in other functional roles, such as tissue morphogenesis, vascular development, cancer and cell survival. In vertebrates, netrins consist of three secreted proteins, netrin 1, netrin 3 and netrin 4 and two glycosylphosphatidylinositol (GPI)-anchored membrane proteins, netrin G1 and netrin G2 (REF. 39). Secreted netrins can function either as chemoattractants or as chemorepellents. This dual activity is dependent on the presence of distinct receptors, cell types and cellular context. Netrin 1 attraction requires receptors of the deleted in colorectal cancer (DCC) family[97], which includes the vertebrate-associated receptors, DCC and neogenin, the *Caenorhabditis elegans* receptor UNC-40 and the *Drosophila melanogaster* Frazzled protein. The adenosine receptor A2b was shown to be a functional receptor for netrin 1, but the role of A2b in netrin 1-mediated axon guidance is controversial[98,99]. Recently, the Down's syndrome cell adhesion molecule (DSCAM) has been identified as a netrin 1 receptor[100]. Netrin 1 and its receptors are also expressed in non-neural tissues, such as the pancreas, mammary gland or lung, suggesting a role in the morphogenesis of branched organs[87]. Recent studies also argue that netrin 1 has a role in autoimmune diseases[20].

Slits are secreted glycoproteins and the main ligands for Roundabout receptors (Robos)[101]. However, heparan sulphate proteoglycans (HSPGs) have been identified as co-receptors for Slits. Slit was originally discovered in *D. melanogaster*[102,103]. In mammals, there are three Slit genes, all of which are expressed in many developing and adult tissues. Robo receptors belong to the immunoglobulin superfamily of cell adhesion molecules (ICAMs). The archetypical Robo receptor contains five immunoglobulin motifs, three fibronectin type III domains and four conserved cytoplasmic domains, which are expressed in different combinations in the Robo receptor family[12]. All Robo receptors can be alternatively spliced to generate various isoforms with distinct functions[104]. In zebrafish and mammals, a fourth Robo receptor, ROBO4 (also known as Magic Roundabout) is expressed by endothelial cells and is involved in angiogenesis[82,105], but its extracellular domain is quite distinct and its ability to bind Slits is controversial[81,84,106]. As with other ICAMs, Robo receptors are capable of homophilic and heterophilic interactions, suggesting that they have Slit-independent functions[107].

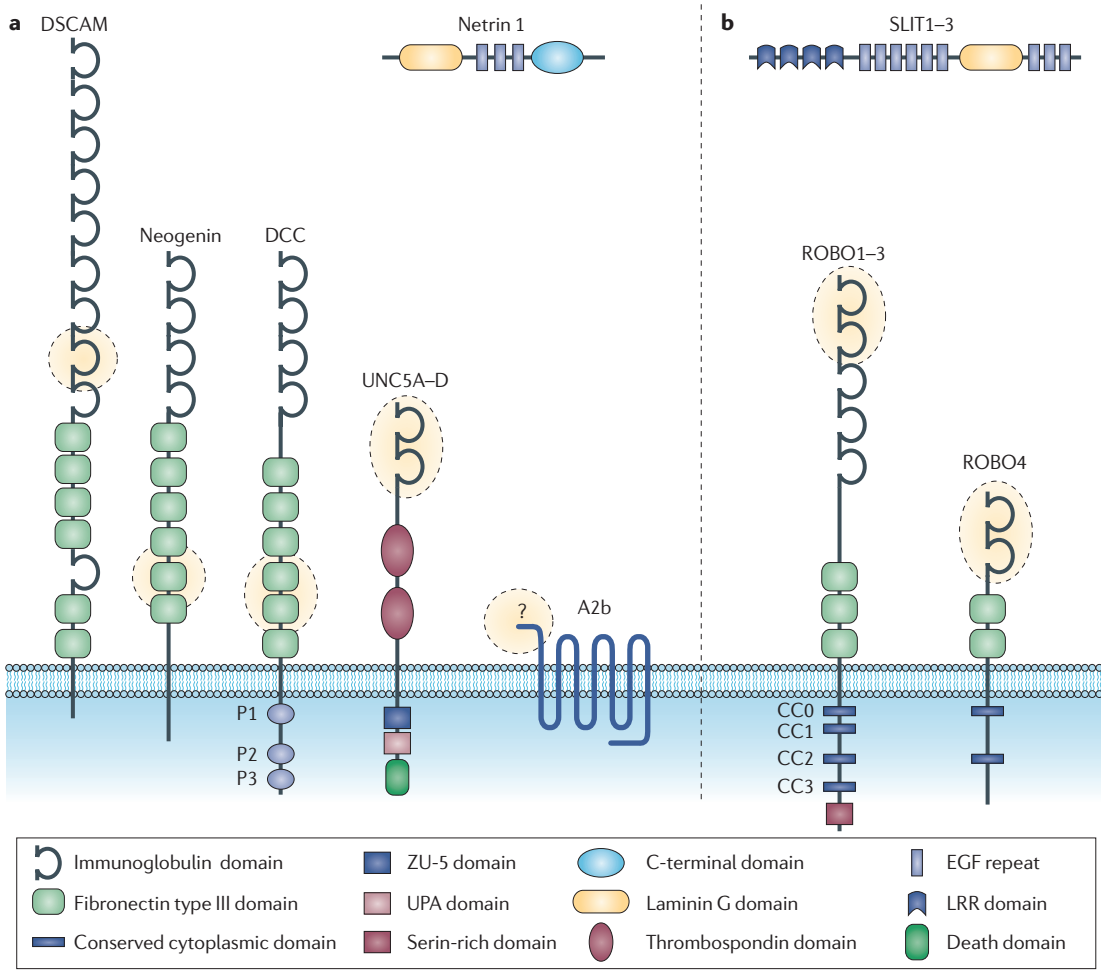

Figure 1 | **Slits and netrin 1 and their receptors. a** | Schematic representation of netrin 1 and its receptors Down's syndrome cell adhesion molecule (DSCAM), neogenin, deleted in colorectal cancer (DCC), UNC5A–D and A2b. Netrin 1 interacts with its receptors though its laminin G domain; the interaction site on each receptor is indicated in yellow and the question mark indicates a controversial interaction site. **b** | Schematic representation of Slits and their receptors ROBO1–3 and ROBO4. P1, P2 and P3 represent regions that are specifically conserved among DCC orthologues, and they are thought to have functional roles in DCC activity[11]. CC0, CC1, CC2 and CC3 are domains conserved among Robo receptors. Please note that ROBO3 does not have a CC1 domain. LRR, leucin-rich repeat; UPA, UNC5, PIDD and Ankyrin domain; ZU, zona occludens.

Tumour cell migration. In the developing central nervous system, Slits and netrin 1 and their receptors control axon outgrowth and neuronal migration through the activation of GTPases and the modulation of cytoskeleton dynamics[39,40]. These cytoplasmic changes are often observed during cancer cell migration, which led researchers to hypothesize that these ligand–receptor pairs could also affect tumour cell migration, although this hypothesis has so far only received limited direct experimental support. There is no strong evidence for a netrin 1–DCC or netrin 1–UNC5 role in cancer cell migration, although it was shown that netrin 1 could stimulate the migration of human melanoma[41], glioblastoma and pancreatic adenocarcinoma cells[31]. Binding of netrin 1 to DCC is thought to activate CDC42 or RAC1 (REF. 42). However, in most assays, expression of netrin 1 fails to affect tumour cell proliferation, differentiation or migration. These contrasting results could be explained by technical issues, as functionally active netrin 1 is difficult to purify, and by the fact that netrin 1 might not be a potent, diffusible chemoattractive factor for cancer cells. Although it is secreted, netrin 1 is a highly charged protein that is known to bind to many extracellular matrix components, such as dystroglycans and heparan sulphates, suggesting that it may not diffuse much in adult tissues[43,44]. Netrin 1 is more likely to function as a short-range cue, as previously shown in the *Drosophila melanogaster* nervous system[45]. Therefore, testing netrin 1 activity on cancer cells could require the development of more appropriate migration assays and the use of immobilized netrin 1.

Although there is more evidence in favour of a role for Slit–Robo in tumour cell migration, the exact cellular and molecular mechanism by which this might occur is still unclear. The downregulation of *SLIT2* and *SLIT3* expression in many human cancers suggests that SLIT2 and SLIT3 could inhibit tumour cell migration. Accordingly, in glioma or medulloblastoma cells, ectopic

Box 2 | Is DCC a tumour suppressor?

Deleted in colorectal cancer (*DCC*) is located at chromosome 18q, a locus deleted in 70% of colorectal cancers and many other tumour types[15]. Several studies have linked chromosome 18q loss of heterozygosity (LOH) to reduced DCC expression at the mRNA[108] or protein[109] levels. Furthermore, deletions in the 18q chromosome region and/or loss of DCC expression were associated with a poor prognosis in patients with colorectal tumours. However, in the mid-1990s, the idea that *DCC* was a tumour suppressor gene was challenged: only a few mutations were detected in *DCC* in human tumours. Another tumour suppressor gene *SMAD4* was mapped at 18q, close to *DCC*[110], and initial studies on *DCC* inactivation in mouse models failed to demonstrate any link between DCC loss and tumour predisposition[13]. However, reintroduction of *DCC* clearly suppresses the tumorigenic properties of tumour cells[111]. Moreover, a recent analysis of *DCC* LOH in human colorectal cancers, using more restrictive markers, supports the view that *DCC* is a tumour suppressor[70,108]. However, there is still no causal evidence linking DCC inactivation to cancer predisposition in any animal model. Therefore, a DCC tumour suppressor function still needs to be validated.

SLIT2 expression or addition of recombinant SLIT2 inhibits tumour cell migration[46,47]. Similarly, ectopic SLIT2 expression in breast cancer cells inhibits tumour cell migration (and tumour growth in engrafted mice models) through a mechanism implicating β-catenin modulation[48]. Conversely, lowering the level of SLIT2–ROBO1 signalling inactivates the AKT–GSK3 pathway, leading to increased β-catenin stabilization and nuclear export, decreased β-catenin–E-cadherin association and E-cadherin expression at the cell surface[49] (FIG. 2). This in turn reduces cell adhesion and increases lung cancer cell migration and motility. In addition, stimulation of the SLIT2–ROBO1 pathway has been associated with silencing of the pro-metastatic partners CXCL12–CXCR4, leading to inhibition of chemoinvasion *in vitro* and the inhibition of tumour growth in an engrafted mouse model[26,50]. Interestingly, in breast cancer cells, SLIT2 also inhibits tumour cell migration by affecting the direction of migration. This effect was shown to be dependent on the deubiquitylating enzyme USP33 (also known as VDU1), which directly interacts with ROBO1 (REF. 51). Thus, Slit–Robo pathways might regulate cancer cell migration through direct Robo-mediated intracellular signalling. However, in some cases, it is possible that Slit–Robo crosstalks with the hepatocyte growth factor (HGF)–MET signalling pathway[52]. Silencing of *SLIT2* or ectopic expression of a soluble decoy Robo increases HGF-induced migration, matrix invasion and tubulogenesis, concomitantly with upregulation of CDC42 and downmodulation of RAC1 activity[52].

There is no general consensus about whether SLIT2–ROBO1 induces a repulsive signal in tumour cell migration, as described for migrating neurons[53,54]. Indeed, several studies have suggested that SLIT2 stimulates rather than inhibits glioma or breast cancer cell migration, at least *in vitro*[55,56]. Along this line, an interesting study links microRNA (miRNA)-dependent ROBO1 expression and gastric cancer metastasis. Tie *et al.*[57] have shown, using both *in vitro* and *in vivo* approaches, that gastric cancer metastasis is associated with the downregulation of a specific set of miRNAs, including miR-218-1, an miRNA hosted in an intron of the *SLIT3* gene, which directly inhibits *ROBO1* expression. They present a model in which the acquisition of metastatic propensity occurs as a result of the downregulation of miR-218 and an upregulation of ROBO1 (REF. 57). Interestingly, miR-218 may also regulate ROBO1 function during angiogenesis (see below)[58].

These conflicting data regarding pro-migratory or anti-migratory activity highlight the danger of generalizing findings obtained from a limited number of cancer cell lines collected at various stages of tumour progression. From the currently available data mostly obtained *in vitro*, one could propose the following model: at the primary site, the migration of cancer cells in response to attractants is limited by the SLIT2–ROBO1 signalling pathway. However, in tumour cells with metastatic properties, the Slit–Robo system might contribute to increased motility.

Tumour cell death and survival. As elegantly described by Hanahan and Weinberg[59], not only do tumour cells need to acquire increased invasive and migratory properties, but they also have to survive. Over the past decade, we and others have proposed that a category of transmembrane receptors, known as dependence receptors, could have a role in inhibiting tumour progression by killing tumour cells (FIG. 3). DCC and UNC5 family members represent prototypic dependence receptors that have two different functions depending on whether they are bound to netrin 1. The binding of netrin 1 activates MAPK-, focal adhesion kinase (FAK)- or AKT-dependent pathways, whereas in the absence of netrin 1 both DCC and the four UNC5 receptors trigger apoptosis[60–63] both *in vitro* and *in vivo*[64–66]. The mechanism through which the unbound receptors trigger apoptosis is currently unclear, but is thought to occur through an interaction with pro-apoptotic effectors, such as death-associated protein kinase (DAPK)[67] or caspase-9 (REF. 68). Based on the crystal structure of the UNC5B intracellular domain, Wang *et al.*[69] proposed that in the presence of ligand, the pro-apoptotic domain of UNC5B is masked by a structural supramodule that is in a closed conformation. In the absence of ligand, this domain is unmasked through the opening of this supramodule.

Box 3 | Netrins, Slits and inflammation?

Inflammation and cancer are intimately connected, and it is intriguing to note that recent reports support the implication of netrins and Slits in inflammatory processes, including tumour development[32]. Indeed, it was shown that SLIT2 inhibits leukocyte chemotaxis[112]. Through a variety of *in vitro* approaches and animal models, SLIT2 was shown to inhibit migration of neutrophils, lymphocytes and macrophages in response to inflammatory signals[113–115]. This seems to occur through the suppression of Rho family GTPase activity[115]. However, recent reports suggest that the situation is probably more complicated, as SLIT3 was shown to promote monocyte migration[116], and a gradient of SLIT2 seems to support the migration of eosinophils while repressing the migration of neutrophils[117]. Interestingly, netrin 1 was also shown to inhibit leukocyte migration through an UNC5B-dependent mechanism[118]. Thus, the deregulation of netrins and Slits in the tumour might also control tumour-associated inflammation.

Structural supramodule
In UNC5B, several domains form a larger, structural module that inhibits the pro-apoptotic activity of the death domain and might also confer other activities.

Table 1 | **Expression of Slits and netrin 1, and their receptors in cancer**

Gene	Locus	Expression in cancer	Refs
DCC	18q21	• LOH and homozygous deletion in colorectal and pancreatic cancers • Downregulation of expression in prostate cancer • Loss of protein function in many cancers	15,27,70, 107,108,119
UNC5A	5q35.3	• LOH in colorectal cancers • Downregulation in breast, ovary, uterus, prostate, stomach, rectum, colon, thyroid, lung and kidney cancers	19
UNC5B	10q21-22	• LOH in colorectal cancers • Downregulation in breast, ovary, uterus, prostate, stomach, rectum, colon, thyroid, lung and kidney cancers	19
UNC5C	4q21-23	• LOH in colorectal cancers • Downregulation in breast, ovary, uterus, prostate, stomach, rectum, colon, thyroid, lung and kidney cancers	19,70
ROBO1	3p12	• Homozygous deletion in lung and breast cancers • Downregulation and LOH in prostate, breast, kidney and lung cancers • Upregulation in colorectal and prostate cancer	22,23,27 120,121
ROBO3	11q24.2	• Downregulation in cervical cancer, lung, breast and kidney cancers • Upregulation in prostate cancer	22,27
SLIT1	10q23.3-q24	• Downregulation in gliomas, breast and lung cancer • Upregulation in prostate tumours	25,27
SLIT2	4p15.2	• LOH in lung, colorectal, cervical, head and neck, and bladder cancers • Downregulation in gliomas, acute lymphocytic leukaemia, breast, colorectal and lung cancers • Upregulation in prostate tumours	22,24,27, 122,123,124
SLIT3	5q35	• Downregulation in gliomas, breast and lung cancer • Upregulation in prostate tumours	22,25,27
NTN1	17p12-13	• Downregulation in prostate tumours • Upregulation in metastatic breast cancer, non-small-cell lung cancer, aggressive neuroblastoma, pancreatic adenocarcinoma and IBD-derived colorectal cancers	27–32

IBD, inflammatory bowel disease; LOH, loss of heterozygosity. Table is modified, with permission, from REF. 5 © (2004) Macmillan Publishers Ltd. All rights reserved.

The ability of these receptors to trigger apoptosis in the absence of their ligand has been proposed as a mechanism for tumour suppression. The idea is that tumour cells expressing such dependence receptors undergo apoptosis as soon as tumour cells become too numerous for the quantity of available secreted netrin 1 in the surrounding tissue, or as soon as they metastasize in secondary tissues where netrin 1 is not expressed or is only weakly expressed. This hypothesis is supported by *in vivo* studies using either transgenic mice overexpressing netrin 1 or *Unc5c*-knockout mice. Such mice have fewer apoptotic cells in tissues, such as the intestinal villi where the netrin 1/receptor ratio is altered, and these mice are more likely to develop aggressive intestinal cancers[17,34]. The existence of dependence receptors suggests that only cancer cells with alterations in this pathway will be able to grow and spread. There are three mechanisms by which this could occur: downregulation of receptor expression, as extensively shown for DCC and UNC5 in human colorectal cancers[15,17,19,70]; inactivation of the dependence receptor-mediated death pathway, as shown by the downregulation of DAPK in human cancers[29,71]; and autocrine secretion of the ligand, which has been detected in a large proportion of human cancers[28–31]. Indeed, silencing of netrin 1 or interference with the netrin 1–UNC5 interaction increases tumour cell death *in vitro* and inhibits primary tumour growth and metastasis in different animal models[28–31]. Interestingly, selection against the dependence receptor death pathway seems to be a pre-requisite in colorectal cancers. All of the colorectal tumour samples analysed in one study at mRNA level showed either a gain in the expression of netrin 1 and no receptor loss, or a loss of receptor expression but no gain of netrin 1 (REF. 33).

The mechanisms leading to the loss of DCC or UNC5 receptors or the gain of netrin 1 are largely unknown. *UNC5B* and more recently *UNC5D* were described as transcriptional targets of p53 (REFS 5,62). Loss of *UNC5B* can suppress p53-mediated apoptosis[5,62], and silencing of *UNC5D* results in resistance to adriamycin-mediated, p53-dependent apoptosis[63]. Despite these findings, no correlation between p53 status and UNC5 levels in tumour samples has been reported.

Whether Slit–Robo has any role in tumour cell death or survival remains unknown, but warrants investigation, as ROBO1 can interact with DCC in a Slit-dependent manner to silence netrin 1 chemoattraction in commissural axons[11]. It will be interesting to know whether Slit–Robo expression has an effect on DCC-induced apoptosis and regulates tumour cell survival. Interestingly, silencing of *SLIT2* in lung cancer cells is associated with increased Akt activation, a well-known marker of cell survival[49], and forced SLIT2 expression in squamous cell carcinomas increases tumour cell apoptosis both *in vitro* and when these cells are engrafted in nude mice[72].

Angiogenesis. Recent studies have shown that many axon guidance receptors are shared between endothelial and neuronal cells, and that axon guidance molecules have a key role in angiogenesis[73,74]. However, it is fair to say that the literature is currently quite confusing and rich in contradictory observations, which raises scepticism among many researchers in the field of angiogenesis.

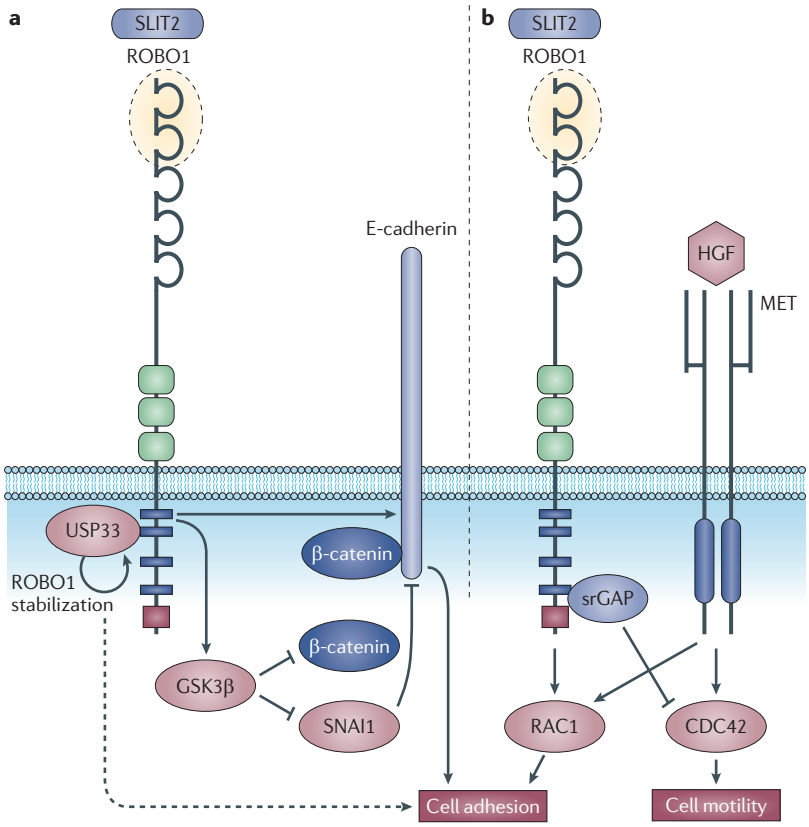

Figure 2 | **Implication of SLIT2–ROBO1 in the regulation of tumour cell migration.** **a** | SLIT2–ROBO1 interaction promotes the activation of glycogen synthase kinase 3β (GSK3β), which phosphorylates both β-catenin and SNAI1, which is a potent repressor of E-cadherin expression. This phosphorylation step creates a recognition motif for β-transducin repeat-containing protein (βTrCP), a ubiquitin ligase, and thus targets these two proteins for degradation through the ubiquitin–proteasome pathway. Thus, SLIT2–ROBO1 promotes E-cadherin expression and downmodulates β-catenin. Paradoxically, SLIT2–ROBO1 increases the stabilization of the E-cadherin–β-catenin complex at the plasma membrane, which facilitates cell adhesion. The SLIT2–ROBO1 effect on cell adhesion is supposed to be enhanced by the deubiquitylating enzyme USP33 that interacts with ROBO1 and promotes its stabilization[51]. **b** | SLIT2–ROBO1 inhibits hepatocyte growth factor (HGF)–MET-induced tumour cell migration, matrix invasion and tubulogenesis. The interaction between SLIT2 and ROBO1 induces the recruitment of Slit–Robo-specific GTPase-activating proteins (srGAP), which inhibits CDC42 and thus antagonizes HGF–MET-induced cell motility. Concomitantly, SLIT2–ROBO1 potentiates RAC1 activity, thus promoting cell adhesion.

There are some strong genetic indications that netrin 1 and its receptors, as well as Robos and Slits, are important for angiogenesis. *Unc5b* inactivation in mice induces angiogenic defects during development[75], and silencing of *netrin1* or *robo4* in zebrafish is associated with major vessel defects[76,77]. Indeed, morpholino-mediated knock down of *robo4* leads to asynchronous intersomitic vessel sprouting, resulting in a reduction and misdirection of intersomitic vessels[76]. Thus, the initial view was that Slit–Robo had a pro-angiogenic effect during development and probably had a similar role during tumour angiogenesis. Unbiased analysis of the transcriptional network governing the angiogenic switch in human pancreatic cancer identified *ROBO1* and *SLIT1* as putative pro-angiogenic genes[78]. SLIT3 has been recently shown to be a potent angiogenic factor both *in vitro* and *in vivo*[79]. Moreover, Wang and colleagues have shown that neutralization of ROBO1 using a ROBO1 blocking antibody reduces microvessel density and tumour mass of human melanoma cells grown as xenografts in mice[80].

Different studies have shown that disruption of ROBO4 activity is associated with reduced angiogenesis *in vivo*[81–83]. However, several discrepancies have arisen regarding the intrinsic mechanism and the nature of the ligand. Although several groups consider SLIT2 as the active ROBO4 ligand in angiogenesis, others doubt SLIT2 binds ROBO4 either *in vitro* or in endothelial cells[73,81]. In this context it is important to note that the ROBO4 extracellular domain is structurally different from other Robo receptors (BOX 1). Similarly, it is unclear whether binding of SLIT2 to ROBO4 in endothelial cells inhibits cell migration[84], or whether this interaction promotes endothelial cell migration[76,81]. Expression of SLIT2 has been detected in a number of cancers[80], and a recent study has shown a direct correlation between the level of SLIT2 immunoreactivity and microvessel density, and the recurrence of ovarian endometriomas[85]. This is not in agreement with reduced SLIT2 expression in cancers (discussed above)[40], suggesting that SLIT2 is not simply a pro-angiogenic or anti-angiogenic factor. Similarly, it was recently shown that SLIT2–ROBO4 promotes vascular stability through the inactivation of the GTPase ARF6 (REFS 82,83) and antagonizes pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) (FIG. 2). How this affects tumour angiogenesis remains to be determined. Indeed, if we hypothesize that SLIT2–ROBO4 supports vascular stability in tumours, it could improve primary tumour growth, but could inhibit tumour progression, as tumour cell extravasion and enhanced metastatic disease are thought to require leaky vessels. It remains possible that SLIT2 binding to ROBO4 supports tumour vascular stability, thereby promoting primary tumour growth, whereas SLIT2 binding to ROBO1 stimulates endothelial cell migration and metastasis[86].

The situation is more complicated for netrin 1 and its receptors, as *in vitro* and *in vivo* studies indicate that netrin 1 can be either pro-angiogenic or anti-angiogenic[39,75,77,87]. Eichmann and colleagues showed that genetic inactivation of *Unc5b* in mice was associated with increased angiogenesis, thereby suggesting an anti-angiogenic activity for netrin 1. However, Li and colleagues showed that the inactivation of *netrin1a* was associated with a loss of vessels during zebrafish development[75,77]. Kroll and colleagues recently confirmed that *netrin1a* inactivation is associated with vessel loss, and they elegantly demonstrated the importance for zebrafish vessel development of a signalling cascade that seems to involve netrin 1, the receptor Unc5b and the ELMO1–DOCK180 complex, which regulates RAC1 (REF. 88). However, the situation is more puzzling as divergent results were obtained using other *Unc5b*-deficient mice or other netrin 1 morpholinos in zebrafish[75,89]. Therefore, it is possible that netrin 1 has a more subtle role in angiogenesis than simple pro-angiogenic or anti-angiogenic regulation and might be dependent on which receptors are expressed and

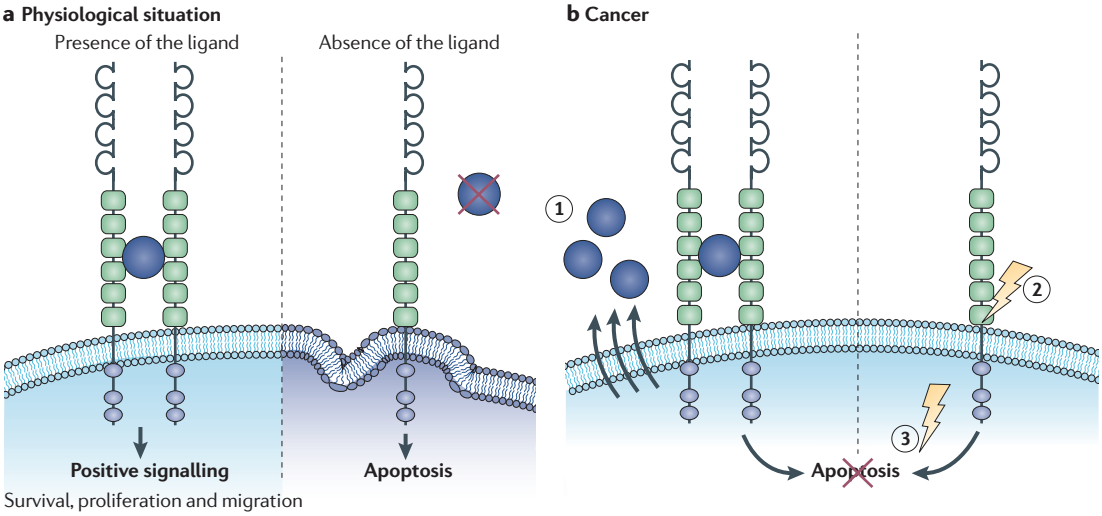

Figure 3 | **The dual signalling of dependence receptors. a** | In a physiological situation, dependence receptors such as deleted in colorectal cancer (DCC) and UNC5A–D share the property of inducing two types of signalling according to the presence of their ligand. In the presence of the ligand, dependence receptors are dimeric or multimeric and induce a positive signal known to promote cell survival, migration and/or proliferation. When disengaged from their ligand, dependence receptors are monomeric and initiate an apoptotic cell death. **b** | In a tumour cell, three main selective advantages could be acquired that involve the dependence receptor signalling pathway. An autocrine production of the ligand (**1**); loss of function (through loss of heterozygosity or epigenetic silencing) of the receptor (**2**); and loss of pro-apoptotic partners (**3**) all prevent the apoptotic pathway.

bound by netrin 1, similar to the situation with SLIT2 and ROBO1 and ROBO4. Indeed, some have argued that netrin 1 acts through an unknown receptor, which is distinct from DCC and UNC5 receptors[77]; however, UNC5B is so far the only netrin 1 receptor known to be expressed in endothelial cells[75]. It has been suggested that netrin 1 regulates endothelial cell migration, even though this has led to the conclusion that netrin 1 either inhibits migration[75] or promotes it[77]. For many investigators, however, it has been impossible to detect any effect of recombinant netrin 1 on either the proliferation or the migration of endothelial cells[88]. The relatively low activity of recombinant netrin 1 might partly explain this absence of effect, as adenovirus-mediated expression of netrin 1 has been shown to be associated with increased migration of HUVEC cells *in vitro* and focal neovascularization *in vivo*[90]. However, we have recently proposed an alternative model that could reconcile most of the current models, in which netrin 1 could function as a survival factor for endothelial cells by blocking UNC5B pro-apoptotic activity[74]. Indeed, we have shown that netrin 1 acts as a survival factor for endothelial cells by blocking UNC5B-induced apoptosis in zebrafish[66]. The regulation of endothelial cell survival is an alternative mechanism to fine-tune angiogenesis that has been overlooked[91]. However, induction of apoptosis by anti-angiogenic factors leads to vessel quiescence or regression, whereas promotion of endothelial cell survival favours sprouting of new capillaries[92]. Such a mechanism could explain the increase in the number of vessels in *Unc5b*-mutant mice, as there are fewer unbound UNC5B dependence receptors, and the loss of vessels in *netrin1*-silenced zebrafish, where there will be more unbound UNC5B dependence receptors. However, it is fair to say that this hypothesis does not solve all the discrepancies reported so far, suggesting that other, more subtle, mechanisms may be important. Therefore, netrin 1 could exert a general positive action on blood vessel development through a combination of a survival effect on endothelial cells, together with an as yet unknown effect that is associated with increased or decreased endothelial cell migratory capacity. Only two published papers have directly looked at the function of netrin 1–UNC5B in tumour angiogenesis, and these are contradictory. Mice with cancer cell line xenografts that express ectopic netrin 1 have reduced angiogenesis[93], whereas Dumartin and colleagues[31] report a pro-angiogenic effect of netrin 1 on human pancreatic cancer cell lines grown in an avian model.

Even though netrin 4 is quite different from netrin 1, and even though there is no consensus on the nature of the netrin 4 receptor, the function of netrin 4 in tumour angiogenesis seems to be as complex as for netrin 1. Although several groups showed that netrin 4 inhibits angiogenesis and more specifically tumour angiogenesis[36,38], Li and colleagues convincingly demonstrated that netrin 4 induces lymphangiogenesis *in vivo*, which may contribute to tumour dissemination[37]. Thus, it is fair to say that the function of netrin 1 and netrin 4 in tumour angiogenesis has yet to be understood. However, netrin 1 seems to be upregulated in a large proportion of different cancer types, suggesting that netrin 1 may not only promote tumour progression by inhibiting epithelial cell death, as shown so far in different animal models, but may also do so by enhancing the development and the survival of new vessels within the tumour[8].

Targets for anticancer strategies

As discussed in this Review, Slits, netrins and their respective receptors have been causally implicated in tumour progression and limitation. This involves various (and sometimes undefined) roles in tumour cell migration, tumour cell survival and angiogenesis. As such, these soluble factors and transmembrane receptors are attractive therapeutic targets in cancer: they are extracellular proteins, a fact that allows the development of biological agents such as antibodies and recombinant proteins, and are, at first glance, only weakly expressed in adult tissues, suggesting possible low target-related toxicity and a therapeutic window.

There are initial proof-of-concept studies in different animal models that show that interfering with Robos using either a ROBO1-specific antibody or a recombinant ROBO4 ectodomain protein might inhibit angiogenesis and/or tumour growth[80]. The role of SLIT2–ROBO1 in tumour progression described above suggests that specific inhibition of SLIT2–ROBO1 could lead to metastasis inhibition, but this will be difficult to establish in humans. Moreover, given that SLIT2–ROBO4 might promote vascular stability[82,83], inhibition of SLIT2–ROBO4 could be associated with tumour cell spreading, with obvious unwanted effects. Future work is required to more accurately assess the crosstalk between ROBO1 and ROBO4, and drug development will benefit from this assessment.

At first glance, the situation seems to be more clear-cut for netrin 1. Different teams have provided proof of concept in mice and chicken models of cancer that silencing of netrin 1 by netrin 1 small interfering RNA (siRNA) or interference with netrin 1–receptor interaction is associated with inhibition of tumour growth and metastases[28–32]. These studies proposed that disrupting netrin 1 binding to its receptors could represent an efficient anticancer strategy in a large proportion of cancers in which netrin 1 is expressed in an autocrine or a paracrine manner, such as pancreatic adenocarcinoma, metastatic breast cancer, non-small-cell lung cancer and stage IV neuroblastoma. Initial drug development has focused on biological agents that mimic DCC interaction with netrin 1. One of these biologicals, DCC-5fbn, contains the fifth fibronectin domain of DCC, which is known to interact with netrin 1. It does not seem to block the binding of netrin 1 to its receptors but instead seems to block receptor multimerization in response to netrin 1, a pre-requisite for netrin 1 anti-apoptotic activity[94] and probably also for other netrin 1-induced signalling pathways[11]. One important question remains as to the toxicity of drugs targeting netrin 1 and its receptors, as little is known about the possible roles of these proteins in adult tissues.

Additional strategies include targeting the signalling pathways downstream of netrin 1 and its receptors or preventing secretase activities. Both DCC and Robos were recently shown to be cleaved in their extracellular domain by a disintegrin and metalloproteinase domain 10 (ADAM10), which might regulate their activities[95,96]. Clinical studies are currently underway for ADAM-selective inhibitors, and future work could assess whether the mode of action of these candidate drugs could also include inhibition of DCC or Robos and their effect on tumour cell migration, survival and angiogenesis.

Conclusion

Over the past few years, axon guidance molecules have steadily gathered more attention in the field of oncology, but there is still a long way to go before one understands and reconciles the rather contradictory results obtained so far. From our point of view, this is to a large extent due to the fact that most researchers have tried to simply transfer or apply developmental neurobiology models to cancer. However, how axon guidance molecules participate in tumorigenesis is obviously much more complex. A second explanation for the conflicting results is probably related to the incomplete or erroneous biochemical characterization of most ligand–receptor pairs and the absence of genetic validation of the interactions. Most of the studies, including our own, primarily rely on basic immunoprecipitation of overexpressed proteins. The biological reality is probably different and one should take into account the local concentration of the diverse receptors and ligands, their dissociation constants and the extensive crosstalk between these signalling pathways. Despite more basic research being required to better understand how these proteins work, the proof of concept obtained by interfering with netrin 1, Slits and their respective receptors is exciting, and in the near future, these molecules may well turn out to be efficient targets for anticancer therapies.

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Competing interests statement

The authors declare competing financial interests. See Web version for details.

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