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Review

The brain within the tumor: new roles for axon guidance molecules in cancers

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Abstract

Slits, semaphorins and netrins are three families of proteins that can attract or repel growing axons and migrating neurons in the developing nervous system of vertebrates and invertebrates. Recent studies have shown that they are widely expressed outside the nervous system and that they may play important roles in cancers. Several of the genes encoding these proteins are localized on chromosomal region associated with frequent loss-of-heterozygosity in tumors and cancer cell lines and there is also significant hypermethylation of their promoter suggesting that they may act as tumor suppressors. In addition, proteins in all these families and their receptors appear to control the vascularization of the tumors. Last, many axon guidance molecules also regulate cell migration and apoptosis in normal and tumorigenic tissues. Overall, this suggests that molecules that could mimick or block the activity of axon guidance molecules may be used as therapeutic agents for the treatment of malignancy.
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Abbreviations: Abl, abelson; AHV, alcelaphine herpes virus; ALPS, agrin/laminin/perlecan/slit; CSPG, chondroitin sulfate proteoglycan; DCC, deleted in colorectal cancer; DNA, desoxyribonucleic acid; Dpp, decapentaplegic; Dutt1, deleted in U2020 cells; FNIII, fibronectin type III; GEF, guanine exchange factor; GPI, glycosyl phosphate inositol ; GTP, guanine tri-phosphate; HSP, heparan sulfate proteoglycan; HUVEC, human umbilical vein endothelial cell; Ig, immunoglobulin; L(2)gl, *lethal giant larvae*; LG, laminin G; LOH, loss of heterozygosity; LRR, leucine rich repeat domains; MAPK, microtubule associated protein kinase; MDR, multidrug resistance; mRNA, messenger ribonucleic acid; MRS, Met related sequence; NRP1, neuropilin-1; PAE, porcin aortic endothelial; PDZ, PSD-95/Dlg/Z0-1; PI3K, phosphatidyl inositol 3 kinase; PPARs, peroxisome proliferator activated receptors; RGM, Repulsive Guidance Molecule; RNA, ribonucleic acid; Robo, roundabout; SDF-1, stromal-derived factor-1; Sema, semaphorin; TGF, tumor growth factor; TIM, T-cell, immunoglobulin and mucin domain proteins; VEGF, vascular endothelial growth factor

Introduction

Nervous systems are composed of excitable cells, the neurons, connected by long cellular processes, the axons, that form synaptic networks of increasing complexity from simple invertebrates to primates. However, many cells in the nervous system (up to 90% in human[1]) are not neurons but glia: astrocytes, microglial cells and oligodendrocytes. Astrocytes and microglial cells exert several functions such as the maintenance of brain homeostasis, while oligodendrocytes (that do not exist in invertebrates) produce the myelin that enwraps axons thereby allowing the rapid saltatory conduction of action potentials. During development, all these cells differentiate from multipotent progenitors that proliferate in restricted locations of the embryo. In most animal species, postmitotic neurons and glial cells migrate, sometimes over long distances, to reach their final destination. Concomitantly, neurons extend axons, tipped at their leading edge by a growth cone, toward their target cells, grow dendrites and establish synaptic contacts. All these precisely orchestrated cellular events are regulated by a plethora of secreted and membrane-bound proteins, most of which were identified in the last 10–15 years using genetic and biochemical approaches.[2] Interestingly, the mechanisms and the molecules that control neural development are highly conserved in evolution.[3] Recently, it was discovered that many so-called 'axon guidance' molecules also control neuronal migration and neuronal survival. In addition, they are not confined to the nervous system but are widely expressed in many developing and mature organs in the body.[4] Their normal function in the adult CNS and other adult tissues is essentially unknown. However, an increasing number of studies suggest that they are involved in many pathological processes and in particular cancers.

We will review here recent studies on the function in cancer of three families of axon guidance molecules and their receptors, the netrins, the slits and the semaphorins that have many common properties: most are secreted and all can be repulsive or attractive for growing axons and migrating neurons. Particular attention will be paid to recent data giving new insights on the diversity/complexity of ligand–receptor interactions. The implication of the ephrins and their Eph receptors, another family of axon guidance molecules, in tumorigenesis has been also demonstrated and recently reviewed and will not be discussed here.[5]

Semaphorins and their Receptors

The Semaphorins were simultaneously discovered in insects and chick embryos as very potent axon repellents able to induce the collapse of axonal growth cones.[6,7] In less than 10 years, more than 30 semaphorins have been cloned in mammals and some orthologs have been found in virtually all animal phyla and in certain DNA viruses.[8] Eight semaphorin subclasses were distinguished based on sequence similarity and distinctive structural features.[9] The semaphorin subclasses, 1 and 2, contain semaphorins identified in invertebrate species. Subclasses 3–4–6 and 7 contain vertebrate semaphorins (Figures 1 and 2). Class 5 semaphorins exist both in vertebrate and invertebrate species (Figure 2). All semaphorins share a highly conserved 500 amino-acid motif, the semaphorin domain that is also present in other proteins (Winberg *et al*[10] and Gherardi *et al*[11]; see below).

Class 3 secreted semaphorins have been by far the most studied and all were shown to be chemorepulsive for many classes of axons (Figure 1).[8] However, a few semaphorins can also attract some axons and dendrites.[12–14] Pioneering studies showed that their receptors are multimolecular complexes with neuropilins as binding moieties and plexins as signaling moieties. Among the six class 3 semaphorins identified to date, Sema3A, Sema3C, Sema3D, Sema3E were initially found to bind to neuropilin-1 with similar affinities.[15–18] A second neuropilin family member, neuropilin-2, which has at least six spliced variants,[16,17] can bind Sema3C and Sema3F, but not Sema3A with high-affinity.[17] The cytoplasmic tail of neuropilins is very short but can interact with a cytoplasmic protein containing a central PSD-95/Dlg/ZO-1 (PDZ) domain that might work as a molecular adapter coupling neuropilin-1 to membrane trafficking machinery.[19] Many studies have shown that secreted semaphorin function requires plexins as signaling subunits. Plexins are large membrane spanning proteins with a highly conserved cytoplasmic domain devoid of any obvious enzymatic activity. Their extracellular domain also contains a divergent semaphorin domain and two to three MET-related sequences (MRS, Figure 1). Although plexins do not have kinase activity, they share sequence homology in the extracellular domain with the receptor tyrosine kinases MET (the receptor for scatter factor-1/hepatocyte growth factor) and Ron (the receptor for macrophage-stimulating protein[20]). Nine plexins were identified and regrouped into four subclasses (plexin-A–plexin-D). Several studies showed that plexin-A interact with

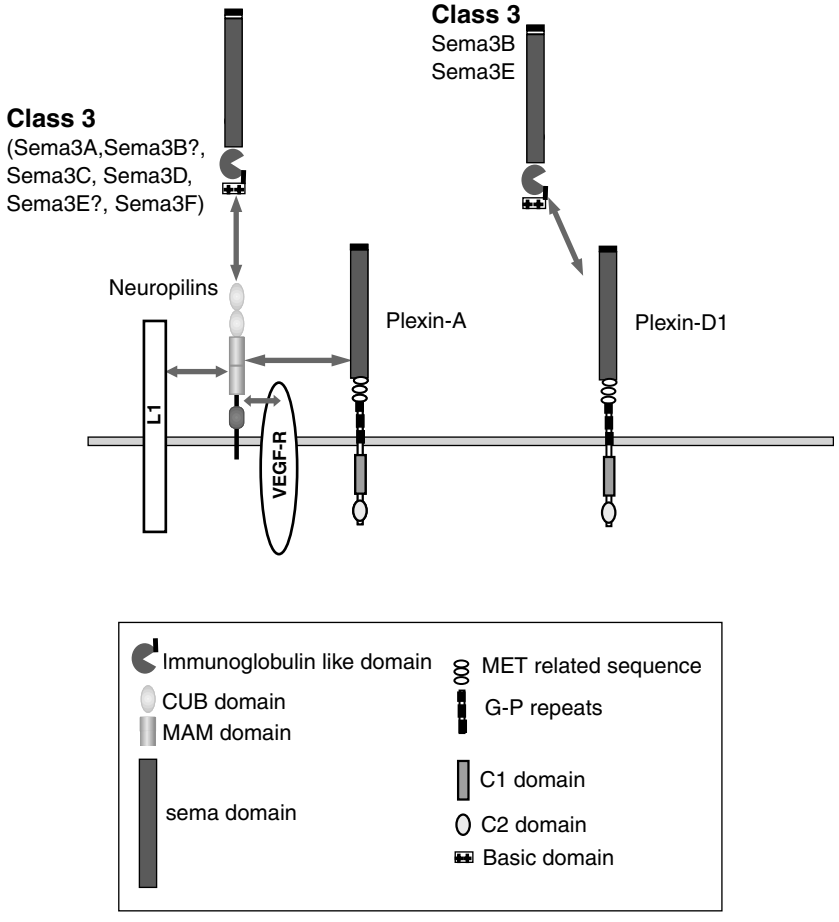

Figure 1 Secreted semaphorins and their receptors. All class 3 semaphorins identified to date were initially found to bind to neuropilins and use plexin-As as signaling subunits. The cell adhesion molecule L1-CAM is also part of the receptor complex for Sema3A. Recent data suggest that, at least in endothelial cells, Sema3E and Sema3B are unable to bind to neuropilins and direct signal through plexin-D1. Neuropilin-1 can also bind VEGF

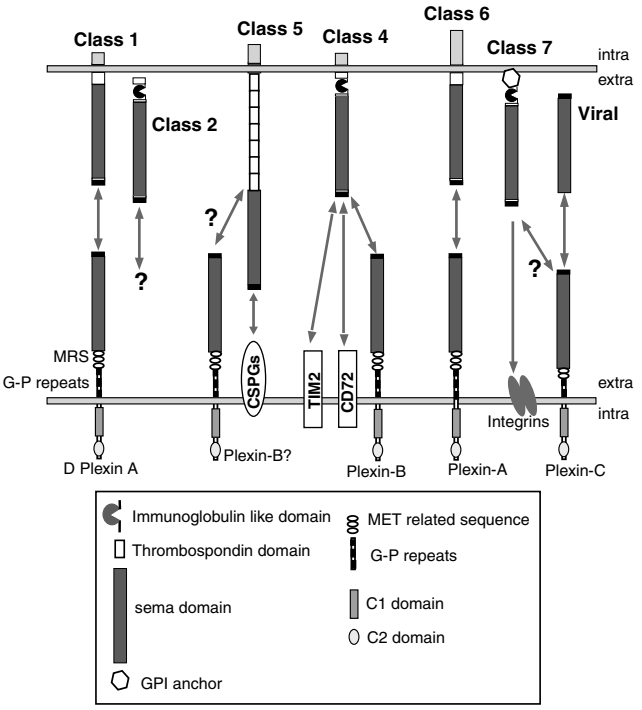

Figure 2 Membrane bound semaphorins and their receptors. Class 4 semaphorins have several known receptors. Sema4A receptor is a member of the T cell, immunoglobulin and mucin domain proteins (TIM) family, Tim-2.[64] Sema4D can bind plexin-B1 but its major receptor in the immune system is CD72. Other class 4 receptors are unknown. Class 5 semaphorins contains thrombospondin repeats in their extracellular domain.[68] Sema5A binds to plexin-B3 but also chondroitin sulfate proteoglycans and heparan sulfate proteoglycans. Sema6D has recently been shown to bind plexin-A1. Sema7A is bound to the plasma membrane by a GPI anchor and could bind to plexin-C1 and integrins

neuropilins and are the signaling moiety of the receptor complex for secreted semaphorins.[8] A cell adhesion molecule of the immunoglobulin (Ig) superfamily, L1-CAM is also part of the receptor complex for Sema3A.[21] L1 binds to neuropilin-1 and upon Sema3A binding, L1 and NP-1 are cointernalized through a clathrin-dependent mechanism mediated by L1.[22] Interfering with this endocytosis blocks Sema3A inhibitory activity on axons. It remains to determine if additional cell adhesion molecules are involved in the signaling of other secreted semaphorins.

However, more recent data challenge this classical model.[23] During development, Sema3E was found to repel migrating endothelial cells and control vascular patterning *in vivo*. Surprisingly, in this system Sema3E function is mediated by plexin-D1 and does not require neuropilin-1. Moreover, and in contradiction with earlier studies, Sema3E, but also Sema3B were found unable to bind to neuropilins. This result is hard to reconcile with previous findings that showed that Sema3B can act as antagonist for Sema3A on receptors containing neuropilin-1.[24] It also suggests that Sema3B and other secreted semaphorins could signal through different receptors in different systems. Additional studies will be required to explain these discrepancies. Last, at least some Plexin-A are also receptors for class 6 transmembrane semaphorins (Toyofuku *et al*,[25] see below).

SEMA3C was the first secreted semaphorin proposed to be involved in tumorigenesis.[26] A screen for genes responsible for non-MDR (multidrug resistance) drug resistance in human ovarian cancer cell line (TyKnuR) and lung cancer cell line (Lu65/CDDP and MS-1/CDDP) identified SEMA3C as a gene overexpressed in these cells. Several glioma cell lines also express SEMA3C, neuropilins and plexin-As.[27] The role of SEMA3C in these cancer cell lines is unclear. Sema3C binds neuropilins. As neuropilin-1 is implicated in angiogenesis (see below), SEMA3C could control directly or indirectly the vascularization of the tumors. Sema3C was also shown to promote the survival of cultured cerebellar granule cells[28] and to attract cortical axons.[13] Sema3C could thus have an autocrine/paracrine protective action on tumor cells or stimulate their migration. It will be important to determine if these cancer cell lines express SEMA3C receptors, such as neuropilin-2.

SEMA3E expression was also correlated positively with tumor progression in mouse mammary carcinoma[29] and its mRNA (messenger ribonucleic acid) is overexpressed in metastatic human lung adenocarcinoma cell lines (HAL-8Luc).[30] However, sema3E function in tumorigenesis is also unclear. On dissociated neurons, Sema3E has either a repulsive action or a growth promoting one that is dose-dependent.[31,32] As mentioned before, this protein and its receptor plexin-D1 were recently shown to control angiogenesis during development[23,33,34] and both could exert a similar function in tumors.

To date, most studies on semaphorins and cancer have focused on sema3B and sema3F. It is known that there is a frequent allele loss in chromosome region 3p in many cancers (ovarian, breast gastric, renal, lung, etc.) and putative tumor suppressor genes were mapped to the 3p21.3 locus. Interestingly, both SEMA3B and SEMA3F were mapped to this region and thus suggested to play a direct role in tumorigenesis.[35] Moreover, SEMA3B has been found at reduced levels or not expressed in lung cancer cells[36,37] and is also often mutated, suggesting that SEMA3B may play a suppressive role in tumorigenesis. Last, SEMA3B promoter is hypermethylated in non-small-cell lung cancer cell lines or in tumor samples, and there is a significant loss of heterozygosity (LOH) in some tumors.[36,38] SEMA3B transfection in lung cancer cell lines or application of exogenous soluble Sema3B ectodomain decrease colony formation and induces apoptosis.[36] An antiproliferative activity of SEMA3B has been shown for breast cancer cell lines.[39] In addition, in lung and breast cancer cell lines, SEMA3B effects are antagonized by the angiogenic factor vascular endothelial growth factor (VEGF) 165,[39] suggesting that SEMA3B tumor suppressor activity involves VEGF signaling. Ovarian adenocarcinoma cells (HEY cells) also express 25-fold less SEMA3B than in normal human ovary and have decreased tumorigenic properties in xenograft model.[40] In addition, after stable transfection with SEMA3B expression constructs, their proliferation rate is decreased. Last, SEMA3B may also act as a mediator of p53-suppressor activity in glioblastoma cell lines.[41] The emerging model[39] suggests that in premalignant cells, the activation of the p53 pathway leads to a decrease of SEMA3B expression and/or an overexpression of its antagonist VEGF, therefore allowing cancer cells to survive and

proliferate. As HEY cells and lung cancer cells express neuropilins, it was proposed that in tumor cells, SEMA3B signals through these receptors and competes with neuropilin-mediated VEGF signaling. However, recent data suggest that this is not likely to occur as, at least in transfected cells, sema3B does not bind to neuropilins but to plexins (see above). The expression of plexins in most cancer cell lines will have to be carefully investigated.

SEMA3F expression is also downregulated in several cancer cell lines and tumors (see Roche *et al*[35] for a review) but it is overexpressed in migrating lung cancer cells.[42] SEMA3F inhibits the attachment and spreading of breast cancer cells (MCF7) apparently through interaction with neuropilin-1 and not neuropilin-2.[43] SEMA3F is also able to antagonize VEGF action on these cells. Like Sema3A, Sema3F can inhibit angiogenesis and endothelial cell migration but through neuropilin-2 binding.[44,45] SEMA3F overexpression in mouse fibrosarcoma or ovarian cancer cells block their proliferation.[36,46] More recently it was shown that SEMA3F overexpression in highly metastatic melanoma cells (that only express neuropilin-2 and not neuropilin-1, VEGF-R1 or VEGFR-2) inhibits adhesion and migration but not proliferation.[44] These SEMA3F-transfected melanoma cells injected into nude mice do not become metastatic. All these results suggest that SEMA3F could inhibit endothelial cell invasion and/or tumor cell migration.

Many recent studies have focused on the semaphorin receptors neuropilins that were found to play a pivotal role in angiogenesis. Binding experiments revealed that in addition to binding most class 3 semaphorins, neuropilin-1 is a receptor for VEGF-A (the VEGF165 but not the VEGF121 isoform), VEGF-B, VEGF-E and placental-derived growth factor-2 (see Bielenberg *et al* [44] for references). Neuropilin-1 is expressed by tumor cells and endothelial cells, where it is a coreceptor for VEGFR-2 mediating VEGF function in angiogenesis.[47,48] Interestingly, Sema3A binding to neuropilin-1 blocks the migration of endothelial cells.[49] However, several class 3 semaphorins, including Sema3A are also expressed by endothelial cells[50] and could have an autocrine action. Accordingly, Sema3A seems to exert a permissive role on angiogenesis by inhibiting integrins-mediated adhesion of endothelial cells allowing their deadhesion.[50] The analysis of neuropilin-1 knockout mice has confirmed that neuropilin-1/VEGF interaction is required for normal development of the vasculature.[51] A soluble neuropilin-1 isoform was identified and found to have robust antitumor activity.[52] Recently, two other soluble forms of neuropilin-1, sIIINRP1 and sIVNRP1, generated by alternative splicing, were discovered and both are expressed in human cancerous tissue.[53] These soluble neuropilins also bind VEGF165 and sema3A. Likewise, neuropilin-2 is a receptor for VEGF165, VEGF145 and placental-derived growth factor-2.[54]

The analysis of neuropilin expression in tumors and tumor cell lines showed that there is a differential expression of neuropilin-1 in two rat prostate carcinoma cell lines (AT2.1 and AT3.1), which have a differential motility in Boyden chambers. AT3.1 cells are more motile and express higher level of neuropilin-1 than AT2.1 cells. Upon transfection with neuropilin-1, AT2.1 cells increase their level of migration in Boyden chamber. They also form larger tumors when grafted in rats, possibly through an enhancement of angiogenesis involving VEGF signaling. Likewise, in glioblastoma, neuropilin-1 expression is increased in endothelial cells and neoplastic astrocytes.[55] A possible role for Sema3A, in neuropilin-1 tumor activity has been recently investigated.[56] This showed that VEGF binding to Neuropilin-1 is required for the survival of breast carcinoma cells. Those cells also express SEMA3A (and plexin-A1, a neuropilin-1 coreceptor) and lowering SEMA3A expression stimulates their migration. Likewise, SEMA3A expression is decreased in mesothelioma.[57] In these cells, SEMA3A expression is transcriptionally induced by VEGF, through a p38 MAPK (microtubule associated protein kinase)-dependent pathway. As for SEMA3B, it is thought that a deregulation of the VEGF : SEMA3A ratio occurs in tumor cells, increasing their invasive potential. Overall, these experiments suggest that neuropilin-1 antagonists could be used to block tumor growth. These antagonists could be soluble neuropilin-1 recombinant proteins that could sequester VEGF, or Sema3A protein or peptides that could block tumor progression and endothelial cell migration. Some peptides able to mimick sema3A proapoptotic activity on cultured neurons have been identified,[58] but their activity on cancer cell lines or tumors has not been tested so far. Other possible therapeutic agents are sIIINRP1 and sIVNRP1 that can block breast cancer cell migration.[53]

Although the majority of semaphorins are membrane-bound, their function in normal and pathological conditions remains largely unknown. Their receptors have also started to be identified and these studies suggest that they might be different in the nervous system and in other tissues, in particular in the immune system.

Class 4 semaphorins, the largest subclass with at least seven members, were shown to bind to type B plexins. In transfected cells, Sema4D binds to plexin-B1 leading to the small GTPases Rac1 and RhoA signaling pathway.[59,60] Plexin-B1, -B2 and -B3 can also form receptor complexes with Met and Ron.[61,62] It was shown that SEMA4D fixation on plexin-B1 can trigger invasive response of NIH3T3 cells *in vitro* by activating MET and Ron.[61,62] Moreover, plexin-B1 is overexpressed, constitutively phosphorylated and associated with MET in liver, colon, gastric and pancreas carcinoma cell lines.[62] Interestingly, plexin-B1 overexpression is sufficient to activate MET. Type B plexins could thus have an important role in the regulation of tumor invasion through their interaction with tyrosine kinase receptors such as MET and Ron. Accordingly, in the nervous system, plexin-B1 and plexin-B2 are highly expressed in regions of intense cell proliferation and migration (see Worzfeld *et al*[63] and AC unpublished data).

Several class 4 semaphorins, such as Sema4A and Sema4D/CD100, are also involved in immune response.[64] In the immune system, it was proposed that plexin-B1 is also a functional receptor for Sema4D,[65] but most data show that Sema4D major receptor is CD72, a member of the C-type lectin family.[64] In the immune system, Sema4A receptor is a member of the T cell, Ig and mucin domain proteins (TIM) family, Tim-2,[64] and Tim-2 cytoplasmic region contains a consensus tyrosine phosphorylation site that is phosphorylated upon Sema4A binding. The receptors for other class 4 semaphorins are unknown. Despite their number and wide

distribution, the expression and function of class 4 semaphorins in cancer is largely unknown. SEMA4D/CD100 is expressed on activated B and T lymphocytes and at high level in lymphoid and myeloid leukemia cells lines.[66] SEMA4D is also expressed in T-cell non-Hodgkin's lymphoma and a subset of B-cell non-Hodgkin's lymphoma. B-cell chronic lymphocytic leukemia is characterized by the expansion of CD5+ B lymphocytes and it was suggested that this could involve a signal delivered by T cells to the malignant B cells. Normal and malignant B cells express CD100/SEMA4D while its receptor plexin-B1 is expressed by activated T lymphocytes. Thus, CD100/plexin-B1 interaction could activate a survival or proliferation signal in CD5+ B cells that may favor the expansion of leukemic clones.[65]

In addition, it was shown that Sema4D promotes angiogenesis through Plexin-B1. Plexin-B1 is expressed in human umbilical vein endothelial cell (HUVEC) and porcin aortic endothelial (PAE) endothelial cells. Sema4D potently induced chemotaxis and tubulogenesis in PAE endothelial cells and enhanced blood vessel formation in mice. Interestingly, the angiogenic responses provoked by Sema4D do not seem to require MET activation.[67] It is still unknown if Sema4D is implicated in tumor angiogenesis.

Class 5 semaphorins have four known members that all contains thrombospondin repeats in their extracellular domain.[68] In the nervous system, Sema5A is expressed by oligodendrocytes and inhibits axonal growth.[69] During development Sema5A is bifunctional, both attractive and repulsive, for some axons.[70] It was shown that in transfected cells, Sema5A binds to plexin-B3[71] but the physiological relevance of this interaction is unclear. However, more recently, Sema5A activity on neurons was shown to require chondroitin sulfate proteoglycans and heparan sulfate proteoglycans.[70]

In *Drosophila*, neoplastic growth of the brain can be induced by inactivation of the *lethal giant larvae l(2)gl* gene. Moreover, upon transplantation, l(2)gl tumor cells invade and metastasize to distant organs.[68] A genetic screen for suppressors of the l(2)gl phenotype lead to the identification of Sema5C (tumor growth is blocked in the absence of sema5C). There are at least three class 5 semaphorins in human SEMA5A, SEMA5B and SEMA5D.[68] Antibody staining showed that SEMA5A and SEMA5D are expressed in human melanoma cells (A2058) and SEMA5D in ovarian cancer cells. Likewise, SEMA5A is overexpressed in uterine leiomyomata[72] and SEMA5B in human renal cell carcinoma.[73]

Class 6 semaphorins are closely related to insect class 1 transmembrane semaphorins. Recent studies in chick embryos have shown that Sema6D is a ligand for plexin-A1 and that their interaction is important for mediating the expansion of the cardiac primordium.[74] However, at later stages of heart development plexin-A1 acts as a ligand and Sema6D as its receptor.[25] Upon plexin-A1 binding Abl kinase is recruited to the cytoplasmic tail of Sema6D and activated, resulting in phosphorylation of enabled and dissociation from Sema6D.[25] This is the first direct evidence of bi-directional signaling in this system and of a role for semaphorins as receptors. Other transmembrane semaphorins were also suggested to act as receptors for instance Sema1a and Sema4D, in *Drosophila* embryo and in immune cells respectively.[75,76] Other class 6 semaphorins are able to inhibit axonal growth in dissociated neuronal culture[77–79] but their receptors are still unknown. There are several isoforms of SEMA6A and SEMA6B generated by alternative splicing.[80] One isoform of SEMA6B is downregulated in two human glioblastoma cell lines upon treatment with retinoids. This effect appears to require peroxisome proliferator activated receptors (PPARs[81]) that are transcription factors belonging to the nuclear hormone receptor superfamily and can associate with retinoic acid receptors. These data suggest that SEMA6B may play a role in tumor progression. Last, SEMA6A was mapped to 5q21–22, which is known to be deleted in certain forms of lung cancer.[82] SEMA7A, the only known class 7 semaphorin, is bound to the plasma membrane by a glycosyl phosphate inositol (GPI) anchor.[83] SEMA7a (also known as CD108 in the immune system) is a close homologue of the alcelaphine herpes virus (AHV) semaphorin or AHVsema. Both were found to bind to plexin-C1.[20,84] However, recent data suggest that in the nervous system integrins and in particular those containing the β1 subunit, are functional receptors for Sema7A.[85] A possible function of SEMA7A in cancer is unknown.

The Slits and Robos

The SLITS is the most recently discovered family of chemotropic factors.[86] Slit (d-Slit) was first identified in *Drosophila* embryo. In fly, Slit is synthesized in the central nervous system by midline glia cells and in the absence of *slit*, longitudinal and commissural axons all converge and coalesce at the midline.[86–89] More recent work has demonstrated that Slit is a chemorepulsive factor and a key regulator of midline crossing and axonal fasciculation.[90,91] Slit homologues have since been found in virtually all vertebrate species. In mammals, three *slit* genes (*slit1–slit3*) have been cloned.[86] All encode large ECM glycoproteins of about 200 kDa (Figure 3), comprising, from their N terminus to their C terminus, a long stretch of four-leucine rich repeats, seven to nine EGF repeats, and a domain, named ALPS (agrin/laminin/perlecan/slit),[86,92] LNS[93] or laminin G (LG) module.[94] Slits are proteolytically processed into a large N-terminal and shorter C-terminal fragments in cell culture and *in vivo.*[86] Slit cleavage fragments have different cell association characteristics, with the smaller C-terminal fragment being more diffusible and the larger N-terminal and full-length fragments being more tightly cell associated. Vertebrate Slits have also been shown to repel developing axons and migrating neurons.[95] Slit proteins also repel migrating muscle precursors in fly embryos[87] and mesodermal cells in zebrafish embryos.[96] However, in rodents Slit2 can stimulate axonal elongation and branch formation of sensory axons from the dorsal root ganglia[97] and attract migrating cells *in Drosophila* embryo.[98]

Roundabout (robo) proteins are the Slit receptors.[87,99,100] Robo is an evolutionary conserved family of transmembrane receptors.[89,90,101,102] Robo proteins define a small subgroup within the Ig superfamily (Figure 3) characterized by the presence of five Ig-like followed by three fibronectin type III (FNIII) repeats, a transmembrane portion and a long cytoplasmic tail containing robo-specific motifs.[103] So far, three *robo* genes have been found in flies[89,90,103] and

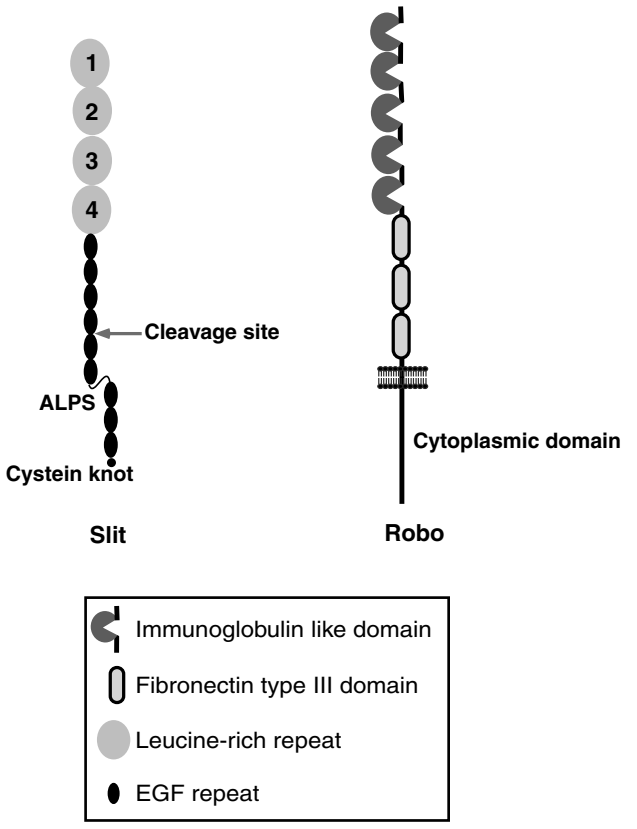

Figure 3 Slits and their receptors. Slit are large ECM glycoproteins comprising, from their N terminus to their C terminus, a long stretch of four leucine rich repeats, seven to nine EGF repeats, and an LG module. Slits are proteolytically processed into a large N-terminal and shorter C-terminal fragments. Roundabout (robo) are slit receptors and define a small subgroup within the immunoglobulin superfamily characterized by the presence of five Ig-like followed by three fibronectin type III (FNIII) repeats, a transmembrane portion and a long cytoplasmic tail containing robo-specific motifs

mammals.[99,100] In mammals, CDO is another protein with 5Ig-3FNIII,[104] but its sequence is overall rather divergent from those of the three Robo receptors, suggesting that CDO probably does not belong to this family. Genetic and biochemical evidence shows that Slits are ligands of the robo1–robo3 receptors.[89,90,103] A fourth putative *robo* gene, called *magic roundabout* or *robo4* was recently cloned and is only expressed by endothelial cells.[105] However, it lacks some of the Ig domains and FNIII domains found in other robo proteins and its capacity to bind slits is still debated.[106,107]

The N terminal region of slits contains a stretch of four leucine-rich repeat domains (LRR) connected by disulfide bonds. These and the Ig domains of robo are important for signaling. Structure–function analysis in vertebrate and *Drosophila* revealed that the LRRs of slits are required and sufficient to mediate its repulsive activities in neurons.[108–111] More recent studies have shown that in *Drosophila* all three Robo receptors compete for a single active binding site in the second LRR of Slit.[108] Neither the FN3 domains nor Robo dimerization are required for slit binding. The major robo1–3 binding site of slit is in the second of the four LRRs, is evolutionary conserved and has a similar affinity for all robos. However, slit affinity is higher when all LRRs are present, probably due to its dimerization. On the receptor side, several results suggest that the first two Ig domains of robos are required for slit binding. First, the genetic deletion of Ig1 and Ig2 results in abnormal lung development.[112] Second, antibodies against robo Ig1 inhibit tumor growth in mice[113] and neurite outgrowth *in vitro*.[114] Third, robo1 Ig1–2 are important for slit binding and function *in vitro*.[115]

Although slits and robos were only recently discovered there is mounting evidence suggesting that they are also involved in cancers. As mentioned previously, deletions and heterozygous loss on the short arm of chromosome 3 occur frequently in lung cancer. In lung tumor cell lines, homozygous deletions have been characterized in regions 3p12, 3p14 and 3p21, for instance in cell line U2020.[116] Interestingly, ROBO1/Dutt1 was mapped within the deletion and its promoter region is hypermethylated in primary lung, renal and breast tumors. So far, no somatic point mutation of ROBO1 (or of its ligands slits) was reported in tumors. These data suggest that ROBO1 may be a tumor suppressor gene.[112] A targeted mutation of mouse *robo1* was generated by deletion of exon 2, mimicking a deletion that naturally occurs in human small cell lung cancer cell line NIH-H219X, and resulted in the removal of Robo1 Ig1. In total, 63% of Robo1$-/-$ homozygous die in the first 24 h because of respiratory failure due to abnormal lung development. A few homozygous survive up to 1 year and show bronchial hyperplasia, but no spontaneous tumor formation was detected. Recently, the tumor susceptibility of Robo1 heterozygous mice was analyzed.[113] During their second year of life, Robo1 heterozygotes develop lymphoma and carcinomas, such as invasive lung carcinomas. In malignant tumor samples from Robo1$+/-$ mice, the expression of Robo1 is undetectable. Moreover, the study of the remaining allele showed that its promoter is hypermethylated. Overall, these studies support a role for Robo1 as a tumor suppressor gene, at least in the mouse.

Slit1, slit2 and slit3, the three known Robo ligands may also be involved in tumorigenesis. First, SLIT2 is expressed in many tumor cell lines[117] such as human melanoma (A375), bladder squamous carcinoma (SCaBER), neuroblastoma (SK-N-SH), small cell lung cancer (NCI-H446), carcinoma of urinary bladder (T24), colon adenocarcinoma (LoVo), breast cancer (ZR-75-30), nasopharyngeal carcinoma (CNE), hepatocellular carcinoma (SMMC-7721), salivary gland carcinoma (Acc), rhabdomyosarcoma (A673) and primary tumors (melanoma, invasive breast carcinoma, colorectal carcinoma, etc.). Moreover, there appears to be a gradient of slit2 expression in primary tumors with highest concentration at the center.

SLIT1–3 expression is upregulated in prostate tumors,[118] but decreased in breast and lung cancer cell lines and tumors and in gliomas.[119,120] SLIT2 is mapped to 4p15.2, a region associated with frequent LOH in many tumors. Accordingly, the inactivation of SLIT2 in tumors was shown to be epigenetic and caused by the hypermethylation of the promoter region.[119,120] Very recently, a similar methylation of the promoter region of SLIT1 (1q23.3–q24) was detected in glioma tumor cell lines.[121] This study also showed that SLIT3 (5q35–34) promoter is frequently hypermethylated in breast, lung, colorectal and glioma tumor cell lines and in primary

breast tumors and gliomas. The observation that exogenous slit2 suppresses colony growth in breast cancer cell lines[119] supports a possible tumor suppressor function of SLIT2. However, in tumors that express high level of SLIT2, its function is likely to be different. Many studies suggest that slit2 and its receptors have a potent angiogenic activity. Robo1 is expressed on HUVECs and their migration is increased by slit2 in Boyden Chamber assay.[117] This chemotactic activity of slit2 is dose dependent, requires phosphatidylinositol-3 kinase (PI-3K) activation and can be inhibited by recombinant ectodomain (RoboN). *In vivo*, both RoboN or antibodies against the first Ig domain of Robo1 reduce tumor microvessel densities and tumor size while exogenous slit2 has a opposite proangiogenic activity.[117] However, the expression of Robo proteins by endothelial cells in normal or metastatic tissue has not been reported yet and slit1/slit2 knockouts have an apparent normal vasculature.[122] Therefore, the physiological relevance of these results is still unclear. Slits might also have other function in metastatic cells. Tumor cells often migrate to distant organs leading to secondary tumor formation and chemokines play a role in this process. Recently, slit2 was shown to be a potent inhibitor of stromal-derived factor (SDF)-1 induced leukocyte chemotaxis.[123] This effect requires the interaction of CXCR4 with Robo1 that are both expressed by leukocytes. Breast cancer cells and human melanoma also express CXCR4, ROBO1 and ROBO2 and chemokines such as CXCL12 stimulate the migration of cancer cells.[124] It has been shown that slit inhibits CXCL12/CXCR4-induced breast cancer cell (DU4475) chemotaxis, chemoinvasion and adhesion. Slit inhibits CXCL12-induced phosphorylation of the focal adhesion component FAK and RAFTK/Pyk2 and paxillin. It also inhibits CXCL12-induced Src kinase and PI3-kinase activities, p44/42 MAP kinase and activity of the matrix metalloproteinase MMP-2 and MMP-9 two proteolytic enzymes that play a role in tumor invasion through degradation of the extracellular matrix.[124–126]

Netrins and their Receptors

Although the existence of chemoattractive factors for growing axons was long suspected,[127] the first direct experimental evidence for their existence was only obtained in 1986.[128] Soon after, it was shown that in vertebrates, the ventral midline of the developing CNS, also called the floor plate, secretes some attractants for spinal cord commissural axons.[129] The biochemical purification of this attractant led to the identification of netrin-1, a laminin related protein,[130] whose function in regulating axon guidance at the midline of the nervous system is conserved in evolution.[3] There are at least three netrin genes in mammals (netrin-1, netrin-3/NTN2L and netrin-4[131–134]). Netrin-1 can attract several classes of axons throughout the developing nervous system but also acts as a repulsive factor for some axons.[135] In addition, netrin-1 controls neuronal migration in the developing and adult brain[136] and the migration of pancreatic progenitors,[137] neural crest cells,[138] oligodendrocyte progenitors[139,140] and endothelial cells (see below). In the adult mouse brain, netrin-1 is expressed by some neurons and myelinating oligodendrocytes.[141] In neurons, netrin-1 has several known receptors (Figure 4), Deleted in colorectal cancer (DCC), UNC5A, UNC5B, UNC5C and UNC5D[142,143] and the adenosine receptor A2b.17,[144,145] (see Patel and Van Vactor[146] for a review). In neurons, DCC was shown to mediate the attractive activity of netrin-1, in association with A2b.[144,145] UNC5s seem required for netrin-1 repulsive activity, at least *in vitro*[147–149] and this might also require interaction with DCC.[140,150] However, in *C. elegans and Drosophila* embryo[150] UNC5 could signal independently of DCC. The function of UNC5/netrin-1 interaction in vertebrate development *in vivo* is still unknown.

Netrin also binds to slit2[132] and to neogenin, a DCC-related receptor.[151] (However, recent studies suggest that, at least in the nervous system, neogenin is a receptor for a GPI-linked protein named RGM (repulsive guidance protein[152,153]). Last, in fetal pancreatic epithelial cells, netrin function appears to be mediated by $\alpha 6\beta 4$ integrins.[137] All these receptors are

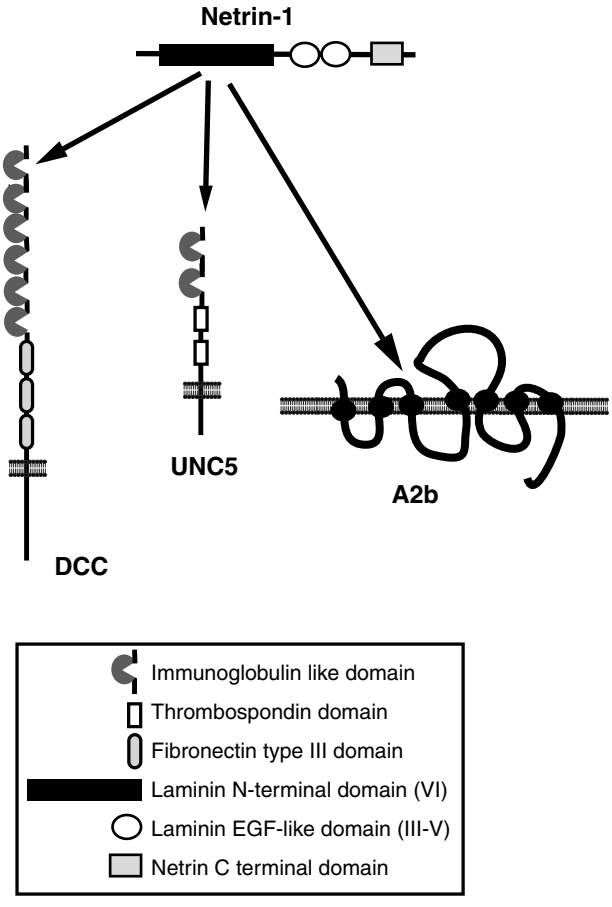

Figure 4 Netrin-1 and its receptors. Netrin-1 is a laminin related protein, containing a laminin N-terminal domain, two laminin EGF-like domains and a netrin C terminal domain. Several transmembrane netrin-1 receptors are known. Deleted in Colorectal Cancer (DCC) contains six Ig-like and three fibronectin type III (FNIII) repeats. UNC5A–UNC5D are composed of two Ig-like and two thrombospondin domains. Netrin also binds the adenosine receptor A2b, a seven membrane domain receptor

expressed in many developing and adult tissues in normal condition (see Hinck[4] for a review).

DCC was first characterized as a gene frequently deleted in colorectal cancers.[154] DCC expression is also downregulated in prostate tumors,[118] human gastric carcinoma[155] endometrial cancer cell lines[156] among others (for a recent review, see Arakawa[157]). DCC is at 18q21.2, a locus of chromosome arm 18q associated with frequent LOH in gastrointestinal cancers, suggesting that DCC is a tumor suppressor gene. Likewise, the expression of UNC5 genes is frequently downregulated in many primary tumors[158] such as colorectal tumors, kidney tumors and lung tumors in association with significant LOH.

One promising model suggests that UNC5 and DCC function in tumorigenesis is related to apoptosis. All these receptors were demonstrated to be dependence receptors (see Bredesen this issue): In the absence of their ligand netrin-1, their cytoplamic domain is cleaved by caspases and massive cell death occurs when they are overexpressed in cultured cells. Moreover, there is a death domain at the C-terminus end of UNC5 proteins. The exact mechanism by which DCC and UNC5 receptors trigger apoptosis is still largely unknown, but in the case of UNC5, a p53-dependent pathway may be involved (see Arakawa, this issue).

It was proposed that DCC and UNC5s act as tumor suppressors only when their ligand netrin-1 is not present. According to this model, the normal function of DCC and UNC5s could be to induce the death of tumor cells that have migrated away from their normal location, in territories where the ligand netrin-1 is absent. Therefore, in tumor cells, the lack of functional DCC or/and UNC5 would make the tumor cells resistant to apoptosis.[159] Likewise, an excess or abnormal expression of netrin-1 would protect tumor cells still expressing DCC and UNC5 from death. This also suggests that netrin-1 function in normal tissues would be to interfere with DCC and UNC5s-dependent apoptosis. In support of this model, it has recently been shown that transgenic mice that overexpress netrin-1 in the intestine develop spontaneous intestinal tumors.[160] However, an overexpression of netrin in or around human tumors has not been reported yet. As for semaphorins and slits, another possible function of netrins in cancer could be to regulate angiogenesis. It has just been shown that endothelial cells express UNC5B and A2b receptors and respond to netrin-1[161,162] and vascular defects were detected in UNC5B knockouts. The exact mechanism of action of Netrin-1 in endothelial cells is still debated as it was shown to inhibit[162] or stimulate HUVECs migration.[161]

Conclusions

Overall these data suggest that axon guidance molecules of the semaphorin, slit and netrin families and their receptors play important roles in tumorigenesis in many tissues and several possible functions and common properties are emerging (Table 1).

The development and growth of tumors require the simultaneous formation and sprouting of new blood vessels from pre-existing capillaries and veins.[163] Surprisingly, many of these novel axon guidance molecules are angiogenic factors. Blood vessels that irrigate tumors were shown to express Robo1, neuropilin-1, plexins-B1 and -D1 and UNC5B. In the tumors, some axon guidance proteins such as netrin-1 and slit2 are upregulated and may directly increase angiogenesis upon binding their receptors on endothelial cells. Other axon guidance molecules, such as Sema3E, Sema3A and Sema3C, may act as inhibitors of angiogenesis in normal condition for instance by interfering with VEGF function. Their downregulation in some tumors may result in a stimulation of blood vessel development.

Many secreted axon guidance proteins such as netrin-1, Sema3B and Sema3A also play a role in apoptosis. In this case, their normal function could be to kill premalignant cells to block their migration and proliferation. The downregulation of their expression in tumors and/or the upregulation of the expression of their receptors could allow some malignant cells to survive, proliferate and migrate (Table 1).

Therefore, for therapeutic use it will be important to develop reagents that can either mimick the activity of secreted semaphorins such as Sema3A, or interfere with slit or netrin binding to their receptors. Such molecules have already been successfully used on developing neurons[164,165] but still have to be tested on tumors. Moreover, the 3D-structure of several of these axon guidance proteins, in particular semaphorins and neuropilins, have been recently solved, which should help designing new molecules.[11] Likewise, the signaling cascades activated by many axon guidance proteins have started to be identified in neurons[2,8] and they may be similar in tumors. Other promising studies have shown that it is possible to switch the activity of netrin-1, slits and sema3A from attractive to repulsive, or *vice versa*, simply by modulating the intracytoplasmic concentration of molecules such as cyclic nucleotides or calcium.[166] It will be important to determine if the same applies to tumor cells. As axon guidance molecules have multiple functions in different cells, it may be difficult to develop tumor specific therapeutic agents. Two examples are slit2 and Sema3A that in addition to their role in angiogenesis and neuronal development are very potent inhibitors of dendritic cell activation and of the immune response.[167,168] However, it is not because tumor cells express some axon guidance proteins that their function in these cells is identical and it would probably be a mistake to directly apply models based on neurons to tumor cell biology. For instance, the semaphorin receptors appear to be distinct in the immune system and nervous system. In the developing heart, at least one semaphorin acts as a receptor. Robo proteins, plexins and neuropilins may also have homophilic properties.[114,169] Last, many of these receptors, such as DCC and UNC5[149] DCC and Robo1,[170] plexin-A4 and Robo2,[171] have been shown to interact in growth cones.

At this time, this research field is at the beginning, and most studies on axon guidance proteins in tumors are still mostly descriptive and sometimes contradictory.

Knockout mice for slit1–slit3, robo1–robo3, neuropilins, plexin-A3, netrin-1, DCC, UNC5 are already available and many others in those families are being generated. Some important answers on the possible implication of axon guidance proteins in tumorigenesis will probably come from the analysis these knockout mice.

Table 1 Possible function and expression of axon guidance molecules in cancer

	Possible function in cancer	Expression in cancer	References
SEMA3A	May inhibit angiogenesis and migration of endothelial cells (through neuropilin-1 binding)	Downregulated in mesothelioma. Expressed in breast carcinoma cells, lowering its expression stimulates their migration.	15–18, 50, 56–57
SEMA3B	May play a suppressive role in tumorigenesis, through neuropilins binding to compete with neuropilin-mediated VEGF signaling	Mapped to the 3p21.3 locus where there is a frequent allele loss in many cancers. Downregulated or mutated in lung cancer cells and downregulated in ovarian adenocarcinoma cells	35–41
SEMA3C	Could control the vascularisation of the tumors through Npn-1 binding. Promotes the survival of cultured cerebellar granule cells	Overexpressed in human ovarian cancer cell lines, lung cancer cell lines and glioma cell lines	13, 17–18, 26–28
SEMA3E	Controls angiogenesis and vascular patterning during development through plexin-D1 binding	Overexpressed in mouse mammary carcinoma metastatic cell lines and metastatic human lung adenocarcinoma cell lines	18, 23, 29–34
SEMA3F	Inhibits angiogenesis, endothelial cell invasion and/or tumor cell migration through Npn-2 binding. Inhibits attachment and spreading of breast cancer cells (MCF7 cells) through interaction with Npn-1	Mapped to the 3p21.3 locus where there is a frequent allele loss in many cancers. Downregulated in several cancer cell lines and tumors. Overexpressed in migrating lung cancer cells	17, 35–36, 42–46
Sema4D	Trigger invasive response of NIH3T3 cells by activating MET and Ron. Promotes angiogenesis through plexin-B1	Highly expressed in lymphoid and myeloid leukemia cells lines. Expressed in non-Hodgkin's lymphoma and in malignant B cells	20, 61–62, 65–67
SEMA5A	Binds to plexin-B3, binds to CSPG and HSPG in neurons	Expressed in human melanoma cells (A2058), over expressed in uterine leiomyomata	68, 70–2
SEMA5B		Expressed in human renal cell carcinoma	68, 73
Sema5C	Blocks tumor growth in Drosophila		68
SEMA5D		Expressed in human melanoma cells and in ovarian cancer cells	68
SEMA6A		Mapped to 5q21–22 deleted in lung cancer	82
SEMA6B		Downregulated in human glioblastoma cell line upon treatment with retinoids	81
Sema6D	Binds to plexin-A1 to regulate cardiac cell proliferation and migration		25
Plexin-A1	Is a coreceptor for Neuropilin-1, binds to Sema6D	Expressed in breast carcinoma cells. Expressed in glioma cell lines	20, 25, 27, 56
Plexin-A2	Is a coreceptor of Neuropilins	Expressed in glioma cell lines	27
Plexin-B1-3	Form receptor complexes with MET and Ron		61–2
Plexin-B1	Plexin B1 overexpression activates MET. Binds to Sema4D.	Overexpressed, constitutively phosphorylated and associated with MET in liver, colon, gastric and pancreas carcinoma cells. Expressed in glioma cell lines	20, 27, 62
Neuropilin-1	Binds to class 3 semaphorins. Binds to VEGF-A (VEGF165 but not VEGF 161), VEGF-B, VEGF-E and PDGF-2 coreceptor with VEGFR-2 to mediate VEGF function in angiogenesis	Upregulated in endothelial cells and neoplastic astrocytes in glioblastoma. Expressed in breast carcinoma cells. Expressed (as soluble forms sIIINRP1 and sIVNRP1) in human cancerous tissue (in glioma cell lines and lung cancers)	44, 47–9, 51–53, 55–56
Neuropilin-2	Binds to class 3 semaphorins receptor for VEGF165, VEGF145 and PDGF2	Expressed in glioma cell lines	17, 27, 54
SLIT1-3	Binds to ROBO1-3	Upregulated in prostate tumors, downregulated in breast and lung cancer cell line and tumors and gliomas	118–120
SLIT1	Inhibits CXCL12/CXCR4-induced breast cancer cells (DU4475) chemotaxis		124
SLIT2	Suppresses colony growth in breast cancer cell lines. Possible angiogenic activity. Attracts HUVECs	Expressed in human melanoma, bladder squamous carcinoma, neuroblastoma, small-cell lung cancer, carcinoma of urinary bladder, colon adenocarcinoma (LoVo), breast cancer, nasopharyngeal, hepatocellular and salivary gland carcinoma, rhabdomyosarcoma and primary tumors	117, 119
ROBO1	Binds to Slit1-3. Reduces lymphoma and carcinoma succeptibility. Possible angiogenic activity	Mapped in a 3p region deleted in lung cancer cell line (U2020)	113, 117
Netrin-1	Binds to DCC, UNC5A-D, neogenin, slit2, A2b Overexpression induces intestinal cancer Implicated in neuronal migration		142–146, 160
DCC	Netrin-1 receptor, controls apoptosis	Downregulated in prostate tumors	118
UNC5	Netrin-1 receptor, controls apoptosis	Downregulated in many primary cancer (colorectal, kidney, and lung tumors)	158

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