Netrin-1 and its dependence receptors as original targets for cancer therapy

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Purpose of review

The dependence receptor notion has recently seen an interesting development. From a basic cell biology concept, which proposes that some transmembrane receptors can be active in the absence of their ligand and induce in the setting apoptosis, recent observations have provided new hope for the development of alternative targeted therapies. The purpose of this review is to show, with the example of netrin-1 dependence receptors, the path from cell biology to promising anticancer-targeted therapy.

Recent findings

The dependence receptors Deleted in Colorectal Cancer and Unc-5 homolog that bind netrin-1 had been implicated in nervous system development as they participate in neuronal navigation. They were also implicated beyond the developing brain with roles in angiogenesis regulation and homeostasis of various tissues. However, these receptors were shown to trigger apoptosis in the absence of netrin-1 and, as such, act as tumor suppressors. Recent data support the view that Deleted in Colorectal Cancer/Unc-5 homolog proapoptotic signals are indeed a safeguard mechanism regulating tumor growth and metastasis.

Summary

In this review, we will develop the different data supporting the view that a selective advantage for a tumor is to inactivate this dependence receptor's proapoptotic signal and will describe a putative therapeutic approach that is to reactivate this death signaling in tumor cells.

Keywords

apoptosis, cancer, dependence receptor, netrin-1, targeted therapy

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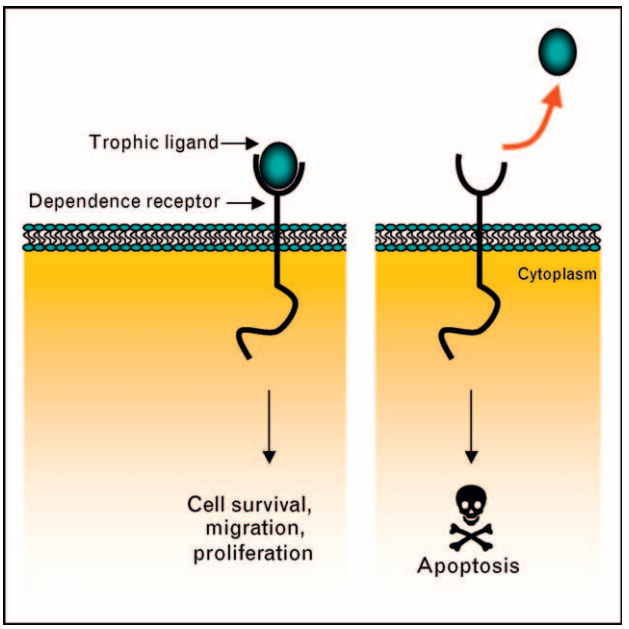
Introduction

It has been known for over half a century that cells depend on stimulation for their survival. Stimulation is mediated by various receptors and sensors. For example, cells may require specific soluble trophic factors, cytokines, hormones, extracellular matrix interactions, cell–cell interactions, or electrical activity for survival. For any given required stimulus, withdrawal leads to programmed cell death or apoptosis. It has generally been assumed that cell death induced by withdrawal of supporting factors is due to the loss of the associated positive survival signals such as Akt phosphorylation. Although such survival signals are clearly very important, data obtained over the past 10 years argue for a complementary and novel form of signal transduction that actively induces cell death following stimulus withdrawal. This 'negative signal transduction' is mediated by specific 'dependence receptors' that induce apoptosis in the absence of the required stimulus (e.g., when unbound by a trophic ligand), but block cell death in the presence of the required stimulus (e.g., when bound by a trophic ligand) (Fig. 1). Thus, the expression of various dependence receptors creates a state of dependence to their respective ligands. To date, more than a dozen of such receptors have been identified: the nerve growth factor receptor $p75^{NTR}$, RET (rearranged during transfection), TrkC, ALK, EPHA4, neogenin, some integrins, the Sonic Hedgehog receptor Patched (Ptc), and the netrin-1 receptors DCC (Deleted in Colorectal Cancer) and UNC5H1–4 (Unc-5 homolog 1–4). As detailed in different reviews on dependence receptors, the proapoptotic activity of these receptors has been implicated in both developmental processes and in cancer regulation [1–3]. We will concentrate this short review on the prototypical netrin-1 receptors (Fig. 2), as they represent the most studied example of dependence receptors and on their implication in cancer regulation.

Netrin-1 is a laminin-related molecule initially discovered as a diffusible molecule produced by a ventral structure in the developing spinal cord, that is, floor plate, that attracts commissural axons [4]. Netrin-1 is a member

Figure 1 The dependence receptor notion



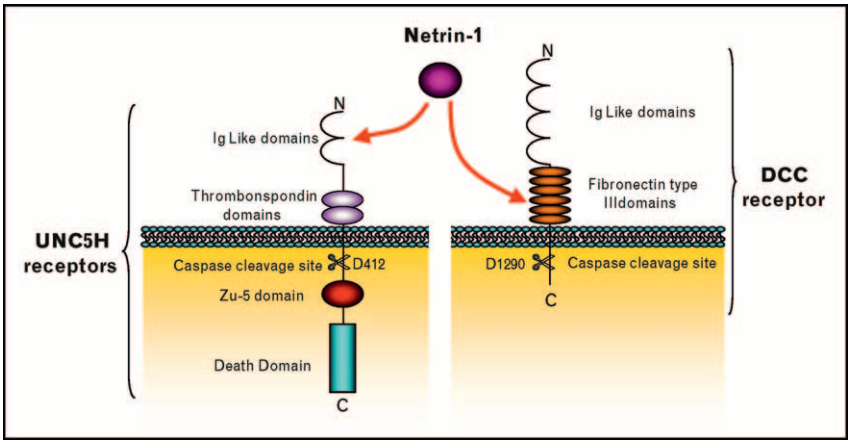
Although the classic view of transmembrane receptors supports that they are inactive in the absence of ligand, dependence receptors are active when unbound and induce apoptosis.

of a family of homologous molecules, which include netrin-3, netrin-G1, netrin-G2, and netrin-4/b-netrin but the main attention has been focused on netrin-1 rather than on its other homologs. One reason for this interest is the dramatic phenotype of the netrin-1 mutant mice, which show major developmental defects in the nervous system [5,6]. Netrin-1 was shown to act as a chemoattractive or chemorepulsive cue for many migrating axons and neurons. This effect is believed to occur through the binding to two main families of type 1 transmembrane receptors: DCC (for deleted in colorectal cancer) and its homolog neogenin and the UNC5H, that is, UNC5 homolog receptors (UNC5H1, UNC5H2, UNC5H3, and UNC5H4, also called UNC5A, UNC5B, UNC5C, and UNC5D) (Fig. 2). However, recent data support the implication of netrin-1 and its main receptors beyond the brain.

Along this line, netrin-1 and its receptors are also expressed in nonneural tissues such as pancreas [7,8], mammary gland [9], or lung [10] that has suggested their role in the morphogenesis of 'branched' organs. Furthermore, the interaction of netrin-1/receptors and, more specifically, netrin-1/UNC5H2, was recently shown to be involved in the morphogenesis of endothelial vessels, but the precise nature of this involvement is not yet clearly established. Indeed, although it is clearly shown that UNC5H2 expressed by the tip cells is a key player in developmental angiogenesis [11], the present data published either suggest an antiangiogenic role or a proangiogenic role for netrin-1 [11–13].

In adults, the functions of the netrin-1/receptors are difficult to study, especially because of the perinatal lethality of transgenic animals deficient for the genes encoding netrin-1 and its receptors. It appears, however, that the expression of netrin-1 and its receptors is relatively ubiquitous. Specifically because of their expression in many cell types of the adult nervous system, netrin-1 and its receptors could be implicated in cell–cell interaction and axonal regeneration [14]. A recent study [15] also argues for a role of netrin-1 in autoimmune diseases and in the inflammatory reactions associated to these diseases.

Figure 2 Netrin-1 and its receptors



DCC and UNC5H are type I transmembrane receptors that interact with netrin-1. The different domains in DCC and UNC5H are indicated. These receptors bind netrin-1 on the two immunoglobulin-like domains (UNC5H) and on the 4–5th fibronectin type III domains (DCC). Their intracellular domain is essential for cell death induction and particularly the caspases cleavage site located at D412 (UNC5H) and D1290 (DCC). DCC, Deleted in Colorectal Cancer; Ig, immunoglobulin; UNC5H, Unc-5 homolog.

Netrin-1 receptors as prototypical dependence receptors

Although netrin-1 and its receptors were clearly identified as key mediators of nervous system development, the netrin-1 receptor DCC, as illustrated by its name Deleted in Colorectal Cancer, was, on the contrary, first identified as a candidate tumor suppressor rather than as a mediator of axon guidance. The *DCC* gene is deleted through allelic loss in the majority of colorectal cancers [16]. The observation that *DCC* expression is lost or reduced in colorectal cancers suggests that DCC expression somehow represents a constraint for tumor development. Such receptor bifunctionality – mediation of neuron/axon migration during development, and putative tumor suppression in various cancers – has proven to be a common theme for dependence receptors. Along this line, it was shown that DCC expression in various cell lines lacking endogenous DCC expression leads to cell death induction [17,18]. Moreover, addition of the DCC ligand, netrin-1, was shown to be sufficient to inhibit apoptosis [17,19,20]. This led to the designation of DCC as a dependence receptor.

In addition to DCC, netrin-1 also binds another family of receptors, the UNC5/UNC5H family [21]. Although DCC is proposed to mediate the axonal guidance-related chemoattractive effect of netrin-1, it is believed that UNC5H/UNC5 is more related to the chemorepulsive effect of netrin-1 [22]. However, the predominant work describing the role of UNC5H in axon repulsion was performed in a system in which UNC5H2 was truncated of its C-terminal domain, which contains a death domain, usually found in proteins implicated in cell death regulation. The presence of this death domain and the fact that UNC5H were described as netrin-1 receptors led us and others to assess whether UNC5H are also netrin-1 dependence receptors. Similarly to DCC, the expression of the different UNC5H receptors induces apoptosis in various cell lines, and the presence of netrin-1 inhibits this effect [23,24]. The proapoptotic activity of unbound DCC or UNC5H was not only demonstrated *in vitro* in immortalized cell lines but was also more recently shown *ex vivo* using primary neurons [25,26•] or *in vivo* using animal models such as mice, chicken, or zebrafish upon inactivation of netrin-1, its receptors, or both [23,25,27••].

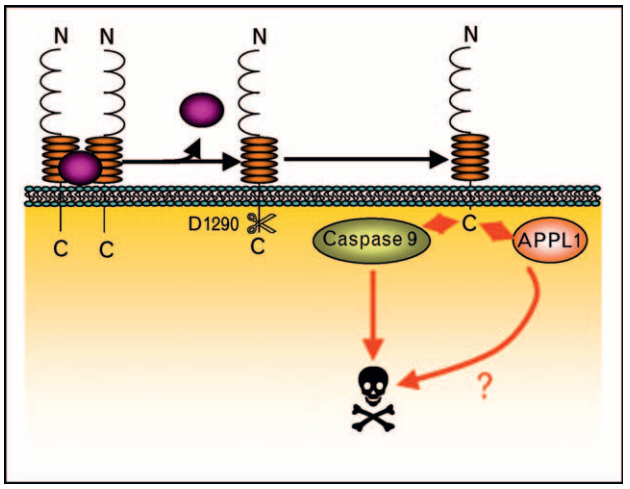
Death and antideath signaling of netrin-1 receptors

The duality of functions of these receptors is associated with a duality in signaling. In presence of netrin-1, DCC and UNC5H transduce classic signals and, in most cases, the activation of these pathways, which we are not going to describe in detail, have been shown to be important for the guidance activity of these receptors. The current view is that in the presence of netrin-1, DCC and UNC5H oligomerize and are then able to recruit different adapter proteins, different kinases such as focal adhesion kinase or extracellular signal-regulated kinases 1 and 2, and, in some case, are phosphorylated [28–30]. These signaling pathways initiated upon ligand binding not only affect migratory and differentiation properties of the cell but also actively inhibit the negative/proapoptotic signaling. Tang *et al.* [26•] and Franke [31] have recently shown that in response to netrin-1, Fyn is activated and phosphorylates phosphatidylinositol 3(P 13)-kinase enhancer, a small GTPase, that in turn is able to interact with UNC5H2 and to inhibit UNC5H2-induced apoptosis.

Although the positive signaling has been studied in depth, how unbound DCC or UNC5H trigger apoptosis is not fully understood. As a common feature of dependence receptors, both DCC and UNC5H receptors are cleaved by caspase in their intracellular domain, and this cleavage is a prerequisite for apoptosis induction by the receptors. The current view is that this cleavage allows the exposure or the release of a proapoptotic domain called addiction/dependence domain (ADD) [23,32,33]. In the case of DCC, the caspase cleavage allows the exposure of a domain located upstream to the caspase site [17]. It has then been described that the DCC–ADD recruits a caspase-activating complex that differs from those implicated in the death receptors and intrinsic-mitochondrial classical apoptotic pathways. In absence of netrin-1, DCC recruits and activates caspase 9, thus allowing caspase 3 activation, but this process does not require cytochrome *c* release and subsequent formation of an apoptosome (cytochrome *c*–apaf-1–caspase 9) complex as is the case in the classical mitochondrial pathway [19]. DCC does not interact directly with caspase 9, so it might recruit one or more adaptor proteins (Fig. 3). One of them may be DCC interacting protein 13 alpha (DIP13α), a protein identified as an interactor of the DCC–ADD and shown to be important for DCC-induced cell death [20] (Fig. 3). However, the precise role of DIP13α in DCC-triggered apoptosis remains quite obscure, as it does not seem to mediate interaction of DCC with caspase 9 and further studies performed on DIP13α, also known as adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1, has not provided clear evidence for a role of this protein in apoptosis induction.

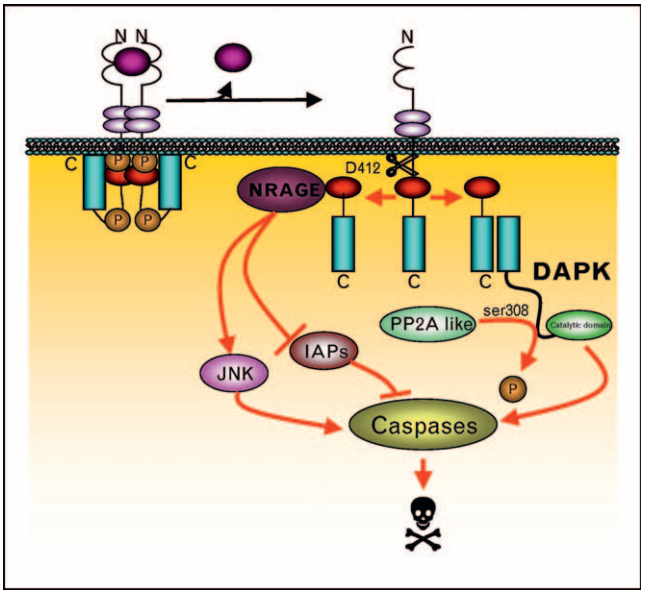
In the case of UNC5H receptors, the caspase cleavage that occurs in the absence of ligand releases a proapoptotic fragment that basically encompasses the whole intracellular domain, which contains the ZU-5 and death domains. Indeed, it was shown that this fragment was sufficient to induce apoptosis when it is myristoylated and overexpressed in immortalized cells [23]. This suggests that a submembrane complex including

Figure 3 Cell death signal through unbound Deleted in Colorectal Cancer



In the absence of netrin-1, DCC monomerizes and reveals a caspase cleavage site at D1290. This cleavage allows the exposure of the DCC–ADD that is responsible for caspase 9 and APPL1 recruitment and induction of apoptosis. ADD, addiction/dependence domain; APPL, adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1; DCC, Deleted in Colorectal Cancer.

Figure 4 Cell death signal through unbound Unc-5 homolog



In the absence of netrin-1, UNC5H receptors adopt an opened conformation, which allow their cleavage at D412 by protease. The cleavage fragment recruits DAPK, which is activated by a PP2A-like phosphatase. An alternative pathway for cell death includes NRAGE interaction. NRAGE may activate the JNK pathway and inhibits IAP to induce apoptosis. DAPK, death-associated protein kinase; IAPs, inhibitor of apoptosis proteins; JNK, c-Jun N-terminal kinase; NRAGE, neurotrophin receptor p75 interaction MAGE homolog; PP2A, protein phosphatase 2A.

UNC5H–ADD could be responsible for apoptosis induction. The ZU-5 domain and the death domain were shown to interact with proapoptotic proteins such as neurotrophin receptor p75 interaction MAGE homolog (NRAGE) and the serine–threonine death-associated protein kinase (DAPK), respectively (Fig. 4). The initial view of NRAGE implication in UNC5H1-induced cell death was that, in the absence of netrin-1, NRAGE is recruited by UNC5H1 through its ZU-5 domain. This interaction would block the inhibitor of apoptosis protein and, as a consequence, would activate caspases. Another possible role of NRAGE could be the activation of the c-Jun N-terminal kinase pathway, which also participates in apoptosis induction [34,35]. However, the implication of the ZU-5 domain in cell death was recently challenged with the determination of the three-dimensional structure of the intracellular domain of UNC5H2 [36••]. Indeed, Wang *et al.* [36••] elegantly demonstrated that UNC5H intracellular domain displays two conformations: a closed conformation in which ZU-5 and death domain interact together, the ZU-5 serving as zip preventing the exposure of the proapoptotic death domain and an opened conformation in which the ZU-5 is not interacting with the death domain, allowing induction of apoptosis through this domain. The working hypothesis is then that upon netrin-1 absence, the death domain of UNC5H is released, interacts with DAPK, and allows its activation with DAPK, which occurs via a loss of its autophosphorylation [37]. One possible explanation for this loss of DAPK autophosphorylation could be the activation of a protein phosphatase and, along this line, it has recently been shown that DAPK is dephosphorylated by a protein phosphatase 2A (PP2A)-like phosphatase [38]. Future work will show whether UNC5H interacts with PP2A and triggers PP2A activation upon netrin-1 withdrawal.

Netrin-1 dependence receptors as homeostasis regulators

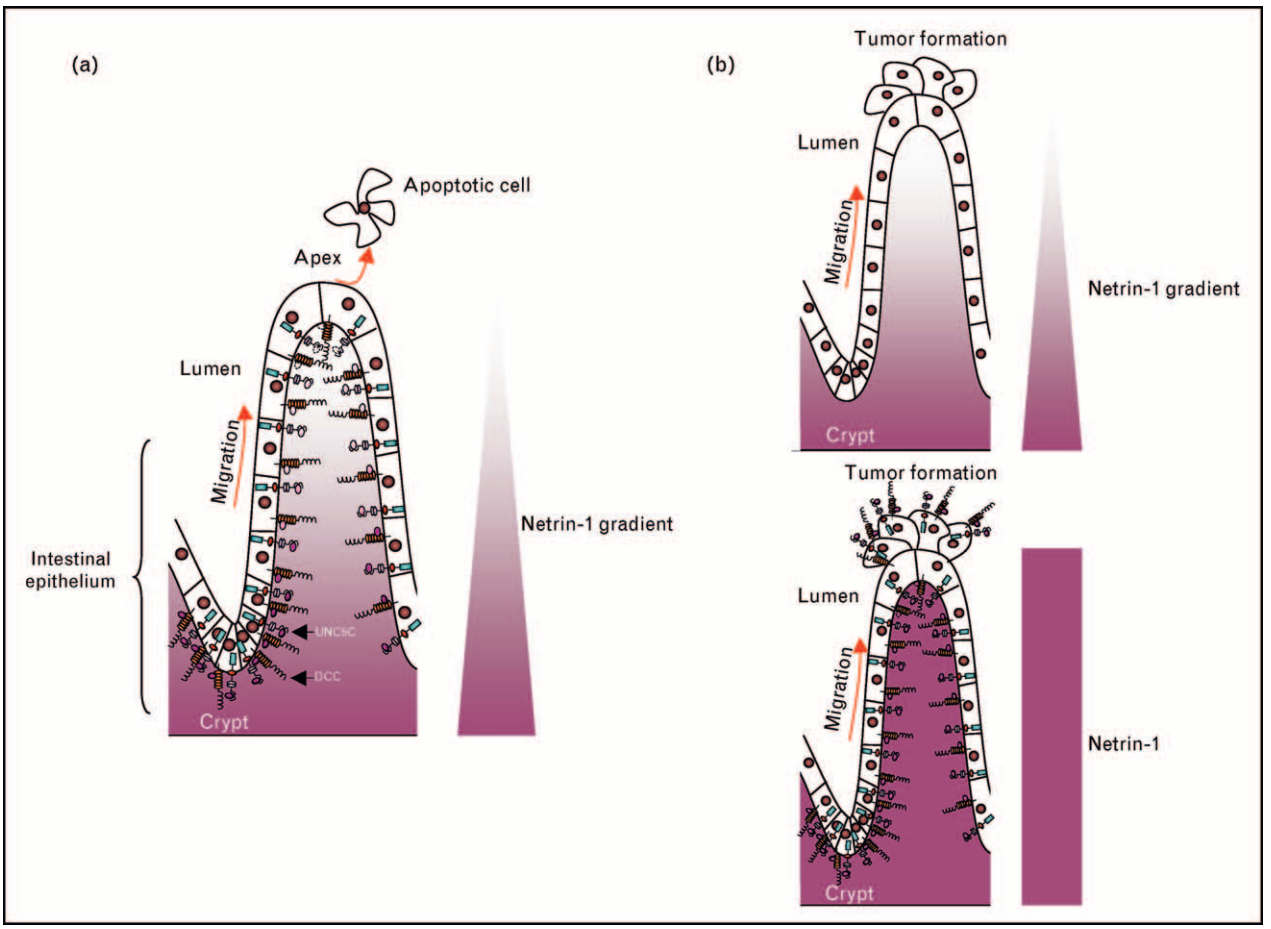
The duality inherent to the dependence receptor function is speculated to play an important role during development by allowing both the migration/differentiation through the positive signal triggered by these receptors in the presence of netrin-1 and the regulation of cell positioning through elimination of cells that would differentiate/migrate in tissues/regions devoid of netrin-1. Along this latter line, the role of netrin-1 as a survival cue during development has been described both during nervous system development [25,26•] and, more recently, during developmental angiogenesis [27••]. Yet the role of the proapoptotic activity of these netrin-1 receptors in adults also appears to be important as a regulator of cell fate and, more generally, homeostasis. In adults, the expression profile of netrin-1 and its receptors suggests that such a mechanism of 'dual control' may exist in other cell types, particularly in tissues with intense cell renewal such as the

intestine and colon. It was indeed shown that netrin-1 is produced at the bottom of crypts formed by the intestinal villi, whereas DCC is detected throughout the villi [39], with the view being that there is a gradient of netrin-1 concentration along the intestinal villus. The working model is then that, at the bottom of the crypts, the proliferating cells expressing DCC are subject to a high concentration of netrin-1 and are thus protected from death by apoptosis. In contrast, cells that stop proliferating and instead differentiate and migrate towards the apical part of the intestinal villus and are subjected to a decreasing concentration of ligand, which gradually leads to cell death by apoptosis (Fig. 5). This model is supported by the fact that, in mice, forced expression of netrin-1 throughout the intestinal epithelium causes a reduction of half the rate of intestinal apoptosis [39]. As such, the gradient of netrin-1 would represent a safeguard mechanism regulating the 'time of life' of the intestinal cells by eliminating cells that have undergone many cycles of proliferation in the crypts or that have been submitted to mechanical and chemical-associated injuries. As a consequence, cells that could escape apoptosis through this mechanism are potentially dangerous cells. Along this same line, mice that are forced to express netrin-1 or that are inactivated by one of its dependence receptors show increased intestinal tumor progression [39–41], thus supporting the view that netrin-1 and its receptors DCC or UNC5H are key regulators of tumor development.

Dependence receptors as tumor suppressors

The ability of DCC and UNC5H to trigger apoptosis in settings of netrin-1 limitations is thus speculated to be a safeguard mechanism preventing primary tumor proliferation within a tissue with limited expression of netrin-1 or tumor metastasis in niches devoid of netrin-1. As such, it would be expected that aggressive tumor cells should

Figure 5 Regulation of colon homeostasis by the pairs Unc-5 homolog/netrin-1 and/or Deleted in Colorectal Cancer/netrin-1



(a) In normal condition, a netrin-1 gradient is formed from the bottom of the crypt to the apex, whereas DCC and UNC5H receptors are expressed all over the epithelium. In this setting, epithelial cells that are highly proliferative and located at the bottom of the crypt avoid apoptosis, whereas cells that are migrating toward the apex find less and less netrin-1 available and die. (b) When UNC5C/DCC are mutated and inactivated (upper panel) or if netrin-1 is abnormally expressed (below panel), the normal homeostasis of the intestine is modulated, cells are not eliminated through apoptosis and this provides a selective advantage for tumor development. DCC, Deleted in Colorectal Cancer; UNC5H, Unc-5 homolog.

block this dependence receptor pathway to survive. The primary way to do so is to inactivate the dependence receptor by itself. Along this line, the receptor DCC was described in 1990 as a potential tumor suppressor gene involved in the advanced stages of colorectal carcinogenesis because its expression is strongly reduced in this disorder. Indeed, the *DCC* gene is located at chromosome 18q, a chromosome region not only deleted in 70% of colorectal cancers but also in many other tumor types [16,42]. Several studies have linked loss of heterozygosity of chromosome 18q and a reduced expression of DCC at the RNA [43] or protein [44] level. Furthermore, deletion of the chromosomal region 18q or loss of DCC expression was associated with bad prognosis in colorectal tumors. The presence of the tumor suppressor gene *SMAD4* locus at 18q close to DCC [45] and the low number of mutations found within the *DCC* gene are data that have raised doubt on the tumor suppressor role of DCC. However, a whole series of studies show that the reintroduction of *DCC* gene suppresses the tumorigenic properties of cells, which have lost the expression of DCC [46,47]. Recent data obtained through the use of more restrictive markers also confirm the loss of DCC in 70% of colorectal cancers [48]. Furthermore, it was observed that not only in over 90% of colorectal tumors but also in many other tumors [49], UNC5H expression is significantly reduced, particularly, through epigenetic processes such as promoter methylation [15,40].

To demonstrate that netrin-1 receptors loss of expression observed in the human disorder is a causal event for cancer progression, animal models have been used. Although initial data on DCC inactivation in mouse fails to demonstrate any link between DCC loss and tumor predisposition [50], a more recent study [51] strengthens the view that netrin-1 dependence receptors are key negative regulators of cancer progression. First, forced expression of netrin-1 throughout the intestinal epithelium in transgenic mice, which is supposed to inhibit both DCC and UNC5H-induced apoptosis in these tissues, was associated with the development of focal hyperplasia and adenoma in the intestine [39]. Moreover, in the mouse model for intestinal adenoma predisposition (carrying a mutation in the adenomatosis polyposis coli or *APC* gene), netrin-1 overexpression favored adenocarcinoma formation [39]. Thus, netrin-1 overexpression is associated not only with the initiation of tumorigenesis but also with tumor progression. To more specifically mimic the human colorectal cancer disorder in which there is generally not a gain of netrin-1 but rather a loss of netrin-1 receptors, intestinal tumor progression was analyzed in UNC5H3 mutant mice [40]. Mutation of *UNC5H3* gene in mice is also associated with intestinal tumor progression [40], thus demonstrating that, *per se*, UNC5H3 (and therefore by analogy probably DCC and other UNC5H receptors) is a tumor suppressor.

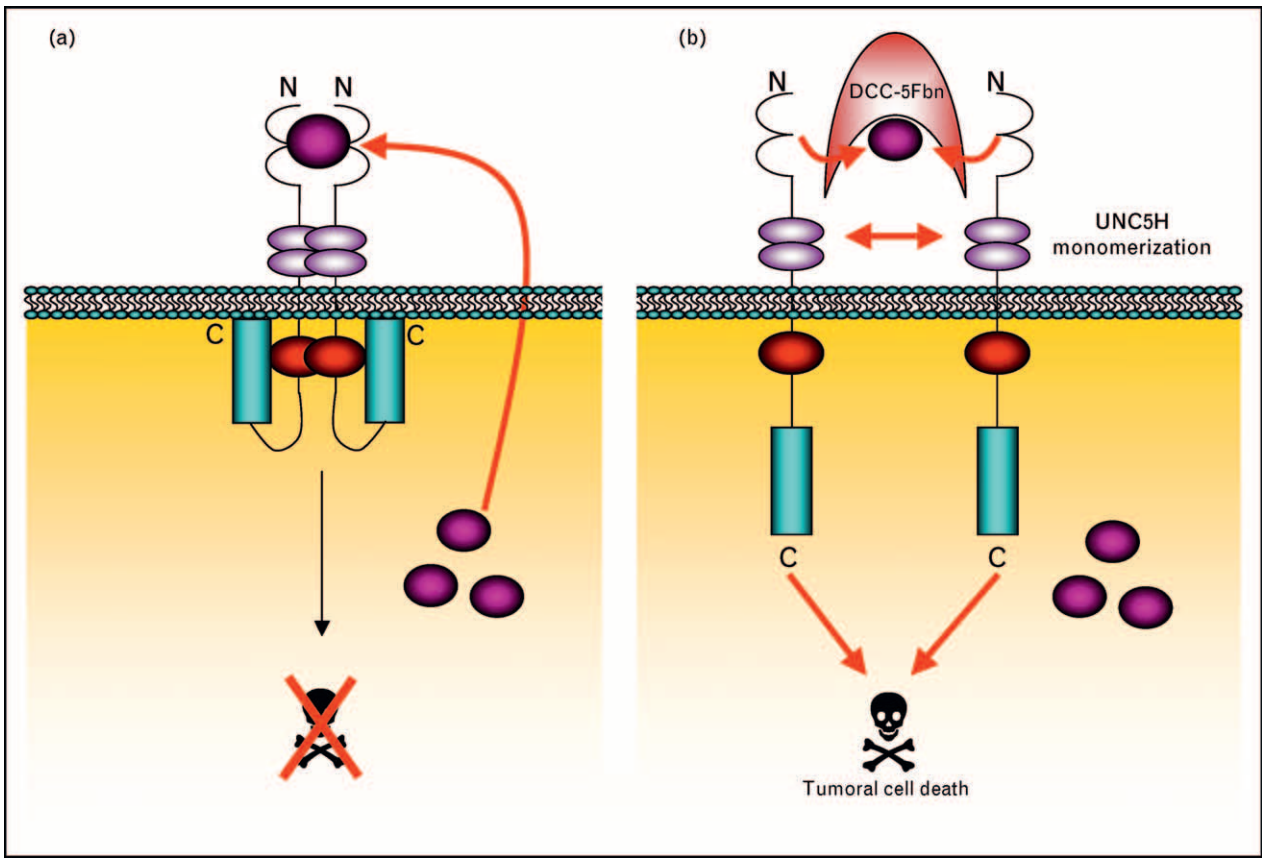
Dependence receptors as original targets for cancer therapy

If the demonstration that netrin-1 dependence receptors are tumor suppressors is interesting in terms of academic research, the loss of expression of these receptors observed in many cancers such as colorectal cancer fails, at a first glance, to make them appealing for therapy. However, the model of dependence receptors predicted that instead of losing netrin-1 receptors, a second potential selective advantage for tumor cell survival could be an autocrine expression of the ligand. This should, theoretically, lead to constitutive blocking of apoptosis induced by these netrin-1 dependence receptors.

In this sense, it was recently shown that netrin-1 is upregulated and autocrinally produced in more than 60% of metastatic breast cancer [52••], in 47% of lung cancer [53••], in 38% of neuroblastoma [54••], and in a large fraction of pancreatic cancer [55]. The mechanism associated with this gain of netrin-1 is, at this stage, unclear. It does not appear to be due to gene amplification but rather to a change in promoter activity [54••]. Along this line, it was recently demonstrated that netrin-1 is a direct transcriptional target of the nuclear factor kappa B (NFκB) transcription factor, and that NFκB activation in tumor cell is associated with netrin-1 upregulation [56]. As a direct consequence in sporadic colorectal cancer, netrin-1 receptors expression is decreased, whereas netrin-1 is rarely upregulated; in colorectal cancers associated with chronic inflammatory diseases such as ulcerative colitis and Crohn's disease, netrin-1 is massively upregulated [57•]. This suggests that such a gain of netrin-1 expression allows tumor cells to survive independently of the limited presence of netrin-1 in the extracellular environment (Fig. 6). This can confer to the tumor cell both a selective advantage to survive while growing in the primary site but also to migrate freely away from the 'physiological' accessibility of the ligand netrin-1, and thus to colonize other tissues and metastasize. Along this line, a cohort's study [55] shows that a high-rate netrin-1 expression significantly influences the time of relapse of patients with pancreatic adenocarcinoma and is associated with a poor prognosis. Similarly, a recent study [54••] demonstrated a high expression of netrin-1 in metastatic neuroblastoma with poor prognosis.

This gain of netrin-1 has been demonstrated to be a selective advantage for tumor cells to survive. Indeed, in tumor cells with autocrine expression of netrin-1, downregulation of this ligand by small interfering RNA strategies or interference with netrin-1/receptors interaction by a decoy recombinant protein have been shown to be associated with tumor cell death, both *in vitro* and in different animal models related to metastatic breast cancer, lung cancer, neuroblastoma, or inflammatory

Figure 6 Netrin-1/receptors interference as a promising therapeutic strategy



(a) In a large fraction of breast, lung, and pancreas cancers, and in neuroblastomas, a specific growth selective advantage is occurring, that is, netrin-1 is autocrinally produced. This mechanism triggers inhibition of DCC/UNC5H-induced apoptosis and is associated with tumor growth and dissemination. (b) Netrin-1 titrating compounds have been developed and restore cell death in these specific netrin-1 high tumors. DCC, Deleted in Colorectal Cancer; UNC5H, Unc-5 homolog.

bowel diseases-associated colorectal cancer (Fig. 6). Thus, a tempting alternative therapeutic approach could be to develop compounds that either sequester netrin-1, netrin-1 receptors, or that inhibit/interfere with netrin-1/receptors interaction. Some biologics, which target netrin-1/receptors interaction, are currently under preclinical evaluation. Such compounds are potentially of great interest because in the first case, it would concern a wide fraction of patients with different types of cancers (lung, breast, etc.). Second, as the target is outside the cell, this leads to the possible development of biologics and to a possible reduced high-dose-associated toxicity. Third, such dependence receptors-targeted-based therapy could be associated with other conventional anticancer treatments such as chemotherapy. Indeed, the deregulation of classical apoptotic pathways is frequently associated with resistance of tumor cells to conventional anticancer treatment, whereas targeting dependence receptors should trigger apoptosis independently of the classic intrinsic and extrinsic pathways of cell death [19,58]. Fourth, a recent report suggests that netrin-1 upregulation promotes not only the survival of epithelial cells but also that of endothelial cells. Along this line, it was shown during zebrafish development that netrin-1 promotes angiogenesis by allowing the survival of endothelial cells via inhibition of UNC5H2-induced apoptosis [27••]. Thus, even though it has to be shown that this prosurvival effect of netrin-1 on endothelial cells also occurs in adult and in higher organisms, it could be tempting to speculate that titrating netrin-1 in tumors with high netrin-1 level could not only be associated with the death of tumor epithelial cells but also of endothelial cells, and consequently of vessels breeding the tumor.

Conclusion

Altogether the in-vivo assays with such interfering drugs are really promising so far [52••–54••] and this suggests that a large effort has to be made to develop drugs targeting netrin-1. However, a major uncertainty is related to the effect of these compounds in normal cells and, as a consequence, to the side effects of such treatment. Although

netrin-1 and its receptors are known to play an important role during the development, very little is known about the effect in adult organisms. What effect will netrin-1 perturbation have in gut homeostasis or in branching organs? Toxicology studies should soon tell whether these drugs represent a definitive hope for cancer treatment. One should also keep in mind that, to date, more than 15 dependence receptors have been identified, and the list is probably far from being completed. If it is assumed that each of these receptors is a tumor suppressor and that, consequently, a loop of autocrine production of ligands for these receptors should have been selected in a fraction of cancer, it could be tempting to speculate that this paradox of dependence receptors opens a large field of original targets in cancer therapy.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 75).

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