

# Netrin-1, a missing link between chronic inflammation and tumor progression

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**N**etrin-1, discovered as a neuronal navigation cue, has been recently proposed to play a crucial role during colorectal tumorigenesis by regulating apoptosis. This survival activity is mediated via the inhibition of the so-called netrin-1 dependence receptors. The netrin-1 receptors, DCC (for Deleted in Colorectal Cancer) and UNC5H (UNC5 homologues), indeed belong to the functional family of dependence receptors that share the ability to induce apoptosis in the absence of their ligands and such a trait has been hypothesized to confer these receptors a tumor suppressor activity as their presence render cell survival dependent on ligand availability. As a consequence, human tumors show either a loss of dependence receptors or a gain of netrin-1, allowing tumors to escape this safeguard mechanism. We recently found that netrin-1 is a direct transcriptional target of the transcription factor NF $\kappa$ B, and that a fraction of colorectal tumors show a netrin-1 gain parallel to NF $\kappa$ B activation. Moreover, colorectal cancers from patients affected by inflammatory bowel diseases (IBD) show upregulation of netrin-1. Several evidences suggest a tight link between chronic inflammation and tumorigenesis, mainly through NF $\kappa$ B activation. We propose that induction of netrin-1 expression via NF $\kappa$ B in IBD patients could affect colorectal tumor promotion and progression and that inhibition of netrin-1 could be an innovative target for drug therapy in inflammation-driven colorectal cancers.

## Introduction

Netrin-1 is a laminin-related molecule initially discovered as a diffusible molecule produced by a ventral structure in the developing spinal cord, the floor plate, which attracts commissural axons.<sup>1</sup> Netrin-1 was thus shown to act as a chemoattractive or chemorepulsive cue for many migrating axons and neurons during the development of nervous system. This effect is regulated by the interaction with its main receptors, DCC (for Deleted in Colorectal Cancer)<sup>2-4</sup> and UNC5H (UNC5 homologue).<sup>5,6</sup> DCC is a type I transmembrane glycoprotein protein of roughly 175–190 kDa with a single membrane spanning domain. The sequences present in DCC's large extracellular domain of roughly 1,100 amino acids bear strong similarity to those found in neural cell adhesion molecule (NCAM) protein family members, and include four immunoglobulin-like domains and six fibronectin type III-like motifs (Fig. 1). The DCC cytoplasmic domain of roughly 325 amino acids shows little similarity to proteins with well-established functions. UNC5H receptors regroup actually a family of 4 receptors: UNC5H1, UNC5H2, UNC5H3 and UNC5H4 also called UNC5A-B-C-D in human. These receptors are type I transmembrane receptors composed of two immunoglobulin-like (Ig-like) domains, two thrombospondin type II domain, a transmembrane domain, a Zu-5 domain (homologous to Zo-1 protein) and a Death Domain (Fig. 1). Both DCC and UNC5H have been shown to transduce navigation signals to neurons or axons in response to netrin-1.

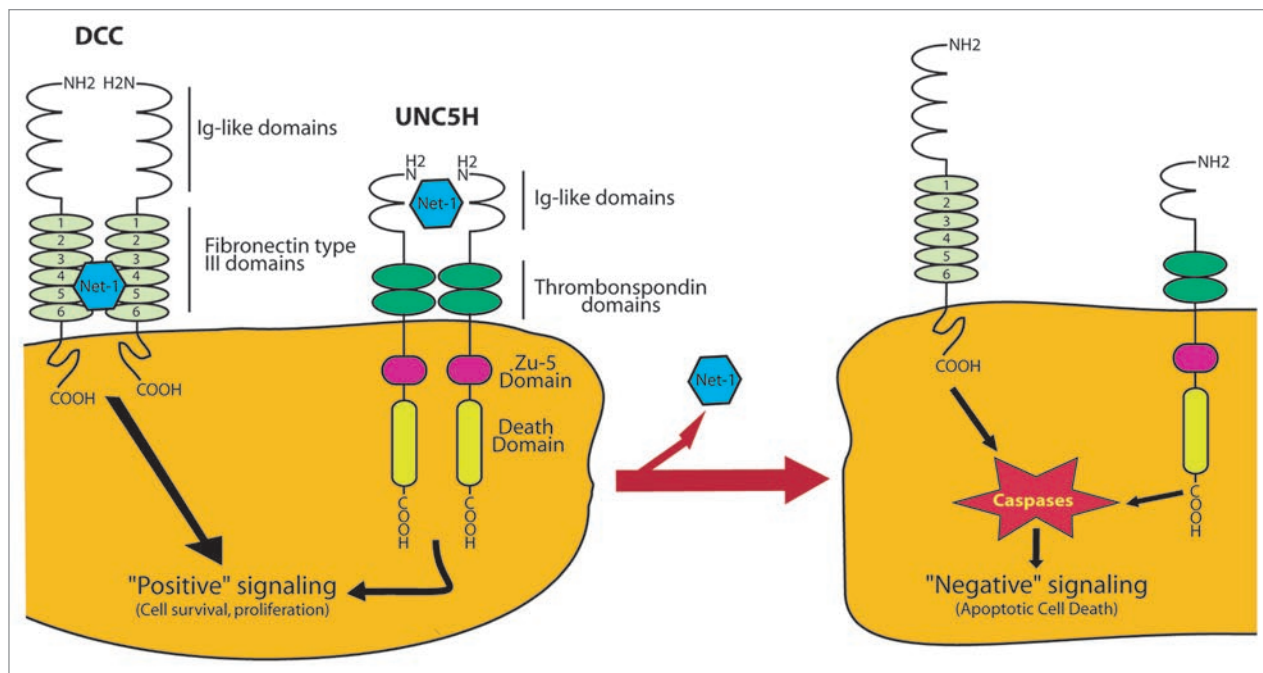
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**Figure 1.** Netrin-1 receptors and the dependence receptor notion. Netrin-1 transduces a “positive” signal when bound to its receptors DCC and UNC5H. The different receptor domains are indicated. Netrin-1 binds DCC on the 4<sup>th</sup> and 5<sup>th</sup> fibronectin-like domains, and UNC5H on the two Ig-like domains. In the presence of netrin-1, DCC and UNC5H dimerize and activate several pathways involved in cell survival, proliferation and axon guidance. In the absence of netrin-1, both receptors monomerize and induce “negative” signaling pathways that trigger caspase-dependent apoptosis. Ig, immunoglobulin; DCC, Deleted in Colorectal Cancer.

However, in the last past years netrin-1 has been proposed as a completely different molecule regulating cell survival and modulating tumorigenesis.<sup>7</sup> Indeed, netrin-1 receptors DCC and UNC5H belong to a new family of cellular receptor, the so-called “dependence receptors”.<sup>8,9</sup> Such receptors, which include also the low-affinity neurotrophin receptor p75NTR,<sup>10</sup> RET,<sup>11</sup> TrkC,<sup>12</sup> Patched<sup>13</sup> and neogenin<sup>14</sup> initiate two completely opposite signaling pathways: when ligand is available, these receptors transduce a positive signal leading to cellular proliferation, differentiation, migration or survival. However, in the absence of their ligand, these receptors are not inactive, like “classical” receptors, but rather induce a “negative signaling” that triggers caspase-dependent apoptotic cell death (Fig. 1).<sup>15</sup> Then, the expression of the dependence receptors within a cell renders this cell dependent on the presence of the ligand for its survival in the cellular environment (reviewed in refs. 15 and 16).

It is interesting to note that most of the known dependence receptors are candidate tumor suppressors. The overall hypothesis

is that the expression of such a receptor represents a protective mechanism that limits tumor development through apoptosis induction of tumor cells that would grow or migrate beyond the regions of ligand availability. Consequently, cell transformation toward malignant/metastatic phenotype is associated with the constitutive inhibition of the death signal induced by these receptors and this can be achieved by at least three mechanisms: either the loss of dependence receptor expression, the loss/mutation of the death signaling above these receptors or the autocrine expression of the ligand. This has been demonstrated per se for netrin-1 dependence receptors. Indeed, DCC expression is lost in a large fraction of human cancers,<sup>17,18</sup> as well as UNC5H genes, hence suggesting that the loss of these receptors represents a selective advantage for tumor development.<sup>19-21</sup> Along this line, UNC5H3 mutant mice show increased intestinal tumor progression.<sup>20</sup> Moreover, in mice the targeted overexpression of netrin-1 throughout the digestive tract increases initiation and promotion of intestinal tumorigenesis, as

a result of netrin-1-dependent cell death inhibition in the intestinal epithelium.<sup>7</sup> In the same line, a netrin-1 gain has recently been described in several human cancers, such as colorectal cancer,<sup>22,23</sup> metastatic breast cancers,<sup>24</sup> lung cancer,<sup>25</sup> and neuroblastoma<sup>26</sup> and this netrin-1 overexpression has been shown as a selective advantage for tumor progression.<sup>23-26</sup> The netrin-1/dependence receptors pair represents therefore a relevant mechanism to control tumor development and cellular escape. While several evidences show that, at genetic or epigenetic level, the downregulation of dependence receptor expression occurs through loss of heterozygosity or promoter methylation,<sup>17,20,27</sup> the mechanism allowing netrin-1 upregulation is not completely understood. Recently, we found that netrin-1 is a transcriptional target of NFκB,<sup>22</sup> a master transcription factor that is well known to link cell survival and tumor development. Here we describe the regulation of the netrin-1 gene expression by NFκB, and more specifically during inflammation-driven colorectal tumorigenesis.

## Intestinal Inflammation and Colorectal Cancer

Inflammation is a fundamental response to the loss of cellular and tissue homeostasis due to stress, injury and infection.<sup>28,29</sup> An inflammatory site is characterized by local expression of pro-inflammatory cytokines, chemokines and adhesion molecules, which regulate the coordinated recruitment of leukocytes to the site of infection or injury.<sup>30</sup> The first line of defense against pathogens and tissue damage is the innate immune system.<sup>31</sup> Initiation of the innate response for microbial infection is triggered by pathogen recognition receptors (PRRs), such as the membrane-bound Toll-like receptors (TLRs) and the cytoplasmic receptors that belong to the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family.<sup>32,33</sup> A normal inflammatory response has to be self-limited and involves the downregulation of pro-inflammatory gene expression and increased expression of anti-inflammatory genes. The molecular mechanisms that regulate the resolution of inflammation are not completely understood, but it has been shown that persistent inflammation increases the risk of cancer development.<sup>30</sup> During the 19<sup>th</sup> century, the German pathologist Rudolf Virchow first noted leukocytes in cancer tissue and first reported a possible link between inflammation and cancer.<sup>34</sup> This association has been confirmed by a substantial number of epidemiologic, gene association and molecular studies, leading to the elucidation of the precise role of inflammation in cancer. Indeed, it appears that chronic inflammation stimulates tumor development and plays a critical role in initiating, sustaining and promoting tumor growth.<sup>35,36</sup> Consistent with this hypothesis, many of the receptors and cytokines involved in innate immune responses, such as TNF $\alpha$ , IL-1, IL-6 and IL-8, have also been linked to tumor progression processes.

Among the different organ systems affected by chronic inflammation, the gastrointestinal tract represents an important entry site for pathogens, since it is the largest surface of contact between the body and the external environment.<sup>37,38</sup> Furthermore, the gastrointestinal lumen

contains a very large number of commensal bacteria. As the intestinal mucosa holds the highest number of immune cells in the body,<sup>39</sup> it is not surprising that the gut constitutes the largest lymphoid organ in the body.<sup>38</sup> Indeed, the intestinal immune system has to prevent infection while avoiding the development of destructive inflammatory responses to the normal microbiota. This requirement is possible as a result of the intestinal epithelium cellular composition, constituted primarily by intestinal epithelial cells (IECs), which form an impermeable barrier between the body and the luminal contents. Besides their role in importing luminal nutrients, IECs play an active role in immunity by producing antimicrobial peptides and pro-inflammatory cytokines following activation of PRRs by microbes.<sup>40</sup> The importance of the IEC dual function against pathogens (i.e., direct antimicrobial function and a sentinel function to alert the immune system) could be appreciated by several mouse models showing that disrupted signaling into IECs increases susceptibility to intestinal inflammation.<sup>40</sup> In addition to the immune function provided by IECs, populations of immune cells can be found within the intestinal tissue, such as dendritic cells (DCs) that sample the gut luminal contents,<sup>41</sup> and intraepithelial lymphocytes (IEL)<sup>38</sup> residing in the lamina propria, a layer of connective tissue supporting the epithelium. The interplay between IECs and inflammatory cells is likely to be a crucial process during inflammation-associated tumor development. This is particularly true for inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease, that are characterized by chronic intestinal inflammation induced by deregulated response to the intestinal flora.<sup>38</sup> IBD patients show a major risk of colorectal cancer and, even if the mechanisms that link these chronic inflammatory state and tumorigenesis are partially unknown, it has been suggested that pathogenic microbes initiate human IBD. Since IBD is associated with inappropriate inflammation in the presence of normal commensal bacteria, several groups have considered the possible involvement of TLRs and NODs, the two major forms of innate immune system sensors. The important

role of TLR signaling in maintaining the intestinal homeostasis is one of the emerging themes in innate immunity in the gut.<sup>31</sup> In normal settings, TLR receptors protect the intestinal epithelial barrier and confer commensal tolerance. In abnormal settings, aberrant TLR signaling may stimulate different inflammatory responses leading to acute and chronic intestinal inflammation.<sup>42</sup> Along this line, although TLR receptors are weakly expressed and detected in primary IECs, thus minimizing recognition of luminal bacteria in the healthy intestine,<sup>43,44</sup> TLR4 is increased in primary IECs during active UC.<sup>45</sup> It has also been shown that changes in DC function may also contribute to the pathogenesis of IBD.<sup>46</sup> With respect to NODs, cytoplasmic proteins that were among the first NLRs reported, a strong association of Nod2 gene mutations and Crohn's disease was reported<sup>47,48</sup> even if the mechanisms by which Nod2 mutations confer an increased susceptibility to Crohn's disease are unknown. It has been shown that patients carrying Nod2 polymorphisms have increased levels of antibodies against luminal microbes,<sup>49,50</sup> suggesting that defective handling of the luminal flora plays a role in IBD.

### NF $\kappa$ B as a Link between Inflammation and Cancer

One of the key players in inflammatory process is nuclear factor  $\kappa$ B (NF $\kappa$ B) transcription factor family, composed by a number of closely related protein dimers that bind a common sequence motif. So far, five distinct NF $\kappa$ B subunits have been identified in mammalian cells: NF $\kappa$ B1 (p50/p105), NF $\kappa$ B2 (p52/p100), RelA (p65), RelB and c-Rel.<sup>51,52</sup> The different heterodimers bind to specific promoter and initiate transcription of a wide range of genes involved in inflammation, cell death, survival and tissue repair.<sup>53</sup> In resting cells, NF $\kappa$ B dimers are sequestered in the cytoplasm by members of the inhibitor of  $\kappa$ B (I $\kappa$ B) family, structurally related proteins, among which the isoform I $\kappa$ B $\alpha$  is the most abundant.<sup>54</sup> The classical and the alternative pathways represent the two distinct known NF $\kappa$ B activation pathways.<sup>55</sup> The former is triggered mainly by bacterial and viral infection, through

pro-inflammatory cytokines, all of which activate the I $\kappa$ B kinase (IKK) complex. This complex is composed by two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, IKK $\gamma$  (also known as NEMO).<sup>52,56</sup> Activated IKK complex phosphorylates NF $\kappa$ B-bound I $\kappa$ B proteins and targets them for polyubiquitination and degradation.<sup>57</sup> I $\kappa$ B-free-NF $\kappa$ B dimers can thus translocate to the nucleus and mediate transcription of several hundred target genes.<sup>58</sup> The activation of NF $\kappa$ B dimers is fast, an essential feature to allow host response to pathogens. However, the ability to switch-off NF $\kappa$ B signaling is equally important. The principal mechanism used to attenuate NF $\kappa$ B activation entails the NF $\kappa$ B-mediated upregulation of I $\kappa$ B proteins. Degraded I $\kappa$ B proteins are replaced by NF $\kappa$ B-dependent de novo synthesis of these inhibitors, generating a negative feedback loop for NF $\kappa$ B activity. The alternative NF $\kappa$ B activation pathway, triggered by certain members of the TNF family,<sup>55,58</sup> is independent of IKK $\beta$  and IKK $\gamma$  and involves the upstream kinase NF $\kappa$ B-inducing kinase (NIK), which activates IKK $\alpha$ . The alternative pathway results in specific activation of p52-RelB heterodimers, through specific IKK $\alpha$  phosphorylation of p52-p100 (NF $\kappa$ B2). This protein binds RelB through its I $\kappa$ B-like domain and retains itself and RelB into the cytoplasm.<sup>59</sup> Activation of IKK $\alpha$  results in the degradation of p100 and nuclear entry of p52-RelB dimers. The target genes of these two pathways are different and therefore mediate different functions.<sup>55</sup>

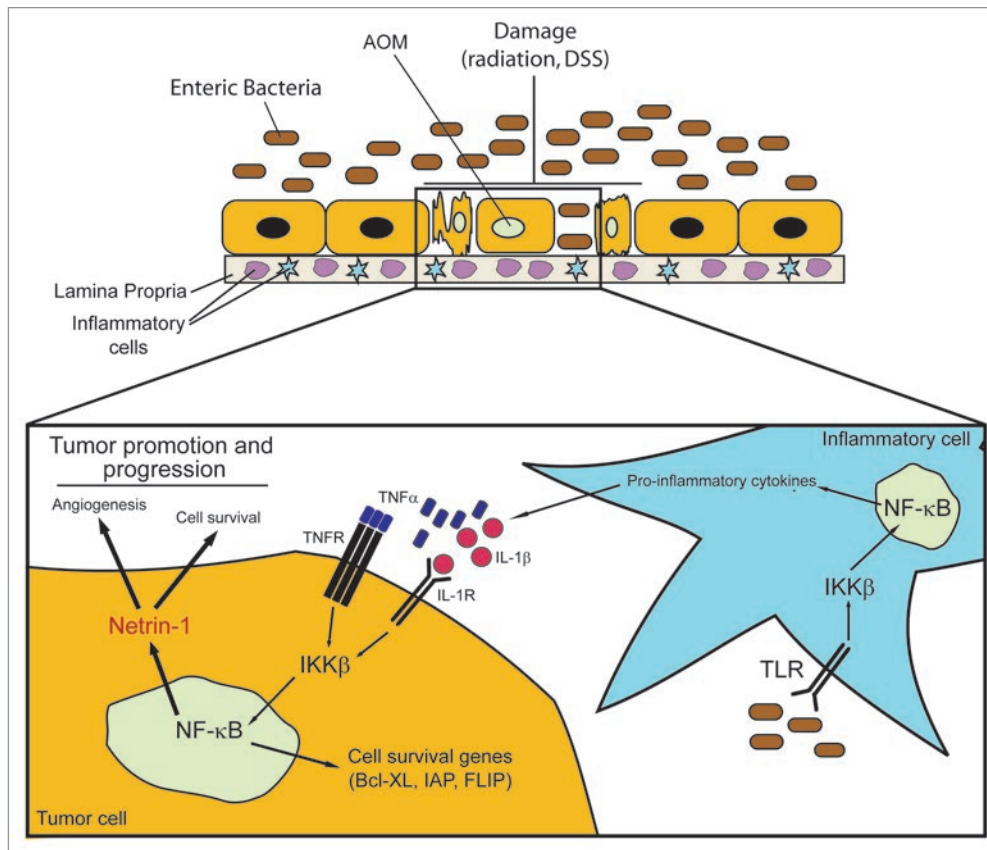
The implication of the classical IKK $\beta$ /NF $\kappa$ B activation pathway in acute inflammation and cell survival is well established.<sup>60,61</sup> NF $\kappa$ B activates the production of key pro-inflammatory cytokines and enzymes, including TNF $\alpha$ , IL-1 $\beta$ , IL-6 and COX-2,<sup>62</sup> as well as the expression of anti-apoptotic genes,<sup>63,64</sup> such as Bcl-X<sub>L</sub>,<sup>65</sup> FLICE-like inhibitory protein (FLIP)<sup>66,67</sup> and members of the inhibitor of apoptosis (IAP) family.<sup>68,69</sup> These findings support the view that NF $\kappa$ B has a dual function: it mediates cell survival and at the same time protects the local environment from infection and injury. As discussed above, to activate an appropriate immune response, the host must first recognize the presence

of pathogens, mainly through PRR receptors, such as TLRs. Ligand binding to these receptors induces the recruitment or the activation of adapter proteins through the Toll/IL-1 Receptor (TIR) domain, leading to the activation of IKK complex, degradation of I $\kappa$ B $\alpha$  and liberation of NF $\kappa$ B heterodimers.<sup>70</sup> Moreover, NF $\kappa$ B activation could also be achieved by cytoplasmic PRRs, such as NOD receptors, which are able to survey the cytosol for the presence of bacterial peptidoglycan and then detect intracellular pathogens.<sup>33</sup> Activation of NF $\kappa$ B triggers intestinal inflammation responses in either IECs or intestinal hematopoietic cells, such as macrophages or DCs,<sup>70,71</sup> and the consequent upregulation of adhesion molecules and chemokines leads to the recruitment and activation of effector cells—i.e., neutrophils and then later macrophages and other leukocytes. NF $\kappa$ B is also responsible for the production of antimicrobial effector molecules, such as  $\alpha$ -defensins, into the intestinal lumen,<sup>72</sup> and as such play an important role for the survival of recruited neutrophils in an inflammatory environment.<sup>73</sup> Finally, NF $\kappa$ B also regulates the expression of matrix metalloproteinases (MMPs), that are crucial mediators of local inflammation and leukocyte recruitment.<sup>74,75</sup>

Due to its central role in inflammation responses, it is not surprising if NF $\kappa$ B has been rapidly considered as a bridge between chronic inflammation and cancer.<sup>56,76</sup> Activated NF $\kappa$ B is indeed detected in colonic mucosa of patients suffering from IBD, as compared to healthy subjects.<sup>77-79</sup> Similarly, high levels of nuclear p65 and c-Rel accumulation has been observed in inflamed tissues of Crohn's disease patients, suggesting enhanced NF $\kappa$ B transcriptional activity in these patients.<sup>80</sup> Activation of NF $\kappa$ B in IBD could result of an aberrant PRR signaling, that seems to be occur in this disease, and this could contribute to tumor formation in patients affected by IBD. Indeed, NF $\kappa$ B has been linked with cellular transformation and it is constitutively active in most tumor cells. In tumor cells, it regulates the expression of a large fraction of anti-apoptotic genes, but also of genes involved in tumoral proliferation—such as c-myc and cyclin D1,—invasion,

angiogenesis and metastasis—such as MMPs, adhesion molecules and vascular endothelial growth factor (VEGF).<sup>81</sup> Therefore, it was reasonable hypothesis to predict that NF $\kappa$ B activation in chronic inflammation could also promote tumor development.<sup>82</sup> Cancer is the result of uncontrolled cellular growth that occurs together with the invasion of surrounding tissue and the spread of malignant cells.<sup>52</sup> Tumorigenesis can be divided into three phases: initiation, promotion and progression.<sup>83</sup> Initiation involves genomic alterations (by chemical or physical agents), leading to the activation of oncogenes and/or the inactivation of tumor-suppressor genes. Promotion is characterized by clonal expansion of transformed cells, due to increased proliferation and/or reduced cell death. Progression is characterized by an increase in size tumor, invasion and metastasis. It is now well accepted that inflammation and NF $\kappa$ B could affect each of these three stages, holding pro-tumorigenic effects. In chronic inflammatory diseases, such as IBD, it is thought that pro-inflammatory factors cause accumulating DNA damage in quiescent pre-malignant cells, e.g., IECs in the case of intestinal chronic inflammation, thereby pushing these cells over the threshold to become malignant. In the same time, NF $\kappa$ B activation that could be due to PRR engagement, carcinogens or follow oncogene activation, prevents apoptosis of pre-malignant cells, while in inflammatory cells, such as macrophages, DCs and neutrophils, recruited to the tumor microenvironment, triggers the production of cytokines and growth factors, as well as angiogenic factors. These proteins allow tumor cells to proliferate, invade and eventually metastasize. This suggests that NF $\kappa$ B-activated inflammatory cells are important mediators of tumor promotion and progression.<sup>34,84</sup>

A definitive evidence for the role of NF $\kappa$ B in bridging inflammation and cancer was provided by the seminal work by Karin and colleagues. They have shown, using a mice model of CAC (colitis-associated cancer), that the IKK $\beta$ -dependent NF $\kappa$ B activation pathway represents a critical molecular link between inflammation and cancer.<sup>85</sup> Mice were injected with the pro-carcinogen azoxymethane

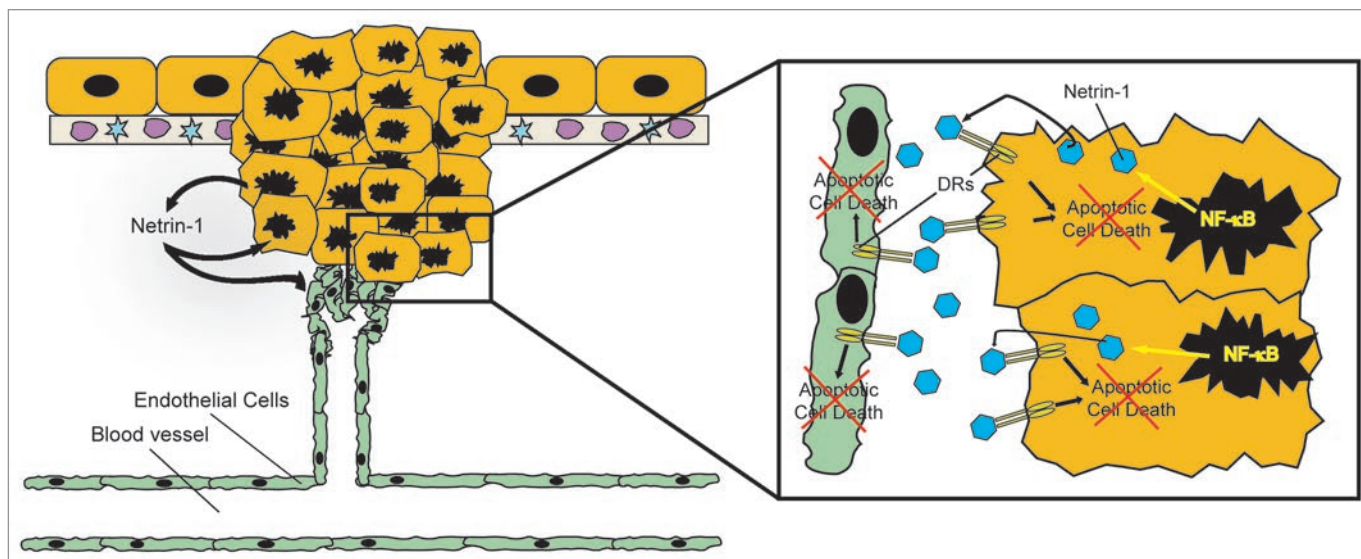


**Figure 2.** The central role of NF $\kappa$ B during inflammation-associated colorectal tumorigenesis. Intestinal exposition to AOM or other pro-carcinogenic initiates malignancy transformation in intestinal epithelial cells. DSS and other damaging agents affect the integrity of the mucosal barrier, causing the exposition of inflammatory cells, residing in the lamina propria, to the enteric bacteria. Following TLR receptors activation and nuclear translocation of NF $\kappa$ B through IKK $\beta$ , activated NF $\kappa$ B in inflammatory cells induces the expression of several secreted pro-inflammatory cytokines, such as TNF $\alpha$  and IL-1 $\beta$ , which enhance the growth and the survival of carcinoma cells. Activation of NF $\kappa$ B in the latter results in increased expression of survival genes. As we have shown, NF $\kappa$ B-dependent upregulation of netrin-1 in malignant cells is fundamental to tumor promotion and progression. AOM, azoxymethane; DSS, dextran-sulfate sodium salt; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; TNFR, TNF $\alpha$  receptor; IL1R, IL-1 $\beta$  receptor.

(AOM), which undergoes metabolic activation in IECs, leading to mutational activation of the  $\beta$ -catenin pathway. AOM causes cancer with low incidence, but this can be augmented by oral administration of dextran-sulfate sodium salt (DSS), which induces acute colitis characterized by bloody diarrhea, ulceration and infiltrations with granulocytes.<sup>86,87</sup> It is believed that DSS is directly toxic to gut epithelial cells of the basal crypts and therefore affects the integrity of the mucosal barrier.<sup>87</sup> This causes the exposition of lamina propria's macrophages to normal enteric bacteria and consequently the activation of NF $\kappa$ B in these cells through TLR signaling, leading to the production of pro-inflammatory cytokines that in turn activate NF $\kappa$ B in IECs (Fig. 2). The importance of NF $\kappa$ B signaling in this model has been

shown by enterocyte-specific ablation of IKK $\beta$ , leading to decreased NF $\kappa$ B activity in IECs. This selective inactivation resulted in a 80% decrease in CAC tumor incidence, without affecting the size and composition of tumors or the induction of oncogenic mutations, indicating that the IKK $\beta$ -dependent NF $\kappa$ B activation pathway in IECs operates during early tumor promotion.<sup>85</sup> Deletion of IKK $\beta$  in enterocytes does not affect DSS-induced inflammation, indicating that the pro-tumoral effect of NF $\kappa$ B in these cells is not due to the activation of pro-inflammatory cytokines, but rather to its ability to suppress apoptotic cell death in pre-malignant precursors.<sup>56,85</sup> However, another important factor in inflammation-promoted colon cancer is the production of pro-inflammatory mediators by myeloid cells, such

as mature macrophages, DCs and neutrophils. The role of NF $\kappa$ B in these cells has been decrypted by ablating IKK $\beta$  specifically in myeloid cells, which do not undergo malignant transformation. This deletion causes a decrease not only in the tumor number, but also in the tumor size probably as a consequence of decreased proliferation in transformed IECs which require growth factors produced by myeloid cells.<sup>85</sup> These findings allow to propose that IKK $\beta$ -dependent NF $\kappa$ B activation affects CAC development through two distinct cell type-specific mechanisms (Fig. 2): in enterocytes, NF $\kappa$ B activates anti-apoptotic genes, preventing the elimination of pre-neoplastic cells, while in myeloid cells it promotes the production of cytokines and growth factors for the pre-malignant enterocytes.<sup>52</sup>



**Figure 3.** Schematic representation of netrin-1 role in colorectal tumor promotion and progression. NFκB-dependent netrin-1 expression in colorectal cells is able to affect tumor promotion and progression by blocking apoptotic cell death induced by its receptors. Thus, netrin-1 expression and secretion renders cancer cells independent of the normal limiting netrin-1 settings, allowing cells to survive, abnormally proliferate and metastasize. Moreover, netrin-1 sustains tumor progression affecting angiogenesis. Paracrine netrin-1 expression by NFκB-activated tumor cells allows survival of endothelial cell forming the blood vessels, which in turn are attracted, through the release of angiogenic factors, by both inflammatory and tumor cells. DRs, dependence receptors.

### Netrin-1 in the Inflammation-Driven Colorectal Tumorigenesis

As discussed above, netrin-1 has been proposed to modulate tumorigenesis by acting as a survival factor.<sup>7</sup> Netrin-1 exerts these effects by blocking apoptosis pathways triggered by dependence receptors, notably DCC and UNC5H1-H4. The dependence effect upon netrin-1 has been suggested to act as a mechanism to eliminate tumor cells that would develop to an inadequate environment, in which ligand availability is the limiting factor.<sup>16,88,89</sup> Therefore, it has been proposed that a netrin-1 gain during tumorigenesis could represent a selective advantage for tumor cells (Fig. 3). These hypothesis have been confirmed by several studies that show an overexpression of netrin-1 in a wide range of human cancers.<sup>22,24-26</sup> In breast tumor, it has been shown that netrin-1 expression may be considered as a marker of a breast tumor's ability to disseminate, since most of the breast primary tumors with metastasis abilities showed an upregulation of netrin-1 expression.<sup>24</sup> This overexpression allows breast tumor cells to escape netrin-1-dependence receptor-induced apoptosis and, consequently, to survive independently

of netrin-1 availability. Along the same line, it has been demonstrated that a large fraction of primary human non-small cell lung cancer (NSCLC) tumors overexpress netrin-1 and that this expression confers a selective advantage for lung cancer epithelial cell survival.<sup>25</sup> Moreover, there is also strong evidence that an autocrine production of netrin-1 could allow apoptosis escape in a large fraction of aggressive neuroblastoma.<sup>26</sup>

Colorectal cancers have been important to the establishment of the dependence receptor concept. Indeed, the prototypic and main receptor to be recognized as a "dependence" receptor was DCC,<sup>8</sup> whose allele, located in chromosome 18q, has been shown to be deleted in 70% of human colorectal cancers.<sup>17,18</sup> Furthermore, UNC5H genes are downregulated in the vast majority of colorectal tumors, particularly UNC5H1 and UNC5H3, whose expression downregulation seems to be due to promoter methylation.<sup>20,21</sup> With respect to netrin-1, both mRNA and protein are detected in gastrointestinal tracts, mainly in crypts of the small intestine and at the bases of the colonic epithelial crypts.<sup>7</sup> This restricted expression of netrin-1 suggests the presence of a netrin-1 gradient in the colonic crypts, with higher concentration

at the proximal part of the intestinal villus, in which is present a site of intense cellular proliferation, than at the distal tip of the villus, a site of cell death and shedding.<sup>7</sup> Moreover, in mice forced overexpression of netrin-1 all along the intestinal epithelium inhibits cell death and is associated with tumor initiation and progression.<sup>7</sup> Despite the obvious importance of netrin-1/dependence receptor pair in the control of colorectal tumorigenesis, a clear correlation between netrin-1 overexpression and colorectal cancer development has not been found. On the opposite, in the human pathology netrin-1 is rarely overexpressed in colorectal cancer, a loss of receptors expression occurring in more than 90% of the tumor tested. We, however, recently have observed that a small fraction (10%) of colorectal tumors shows netrin-1 upregulation—rather than netrin-1 dependence receptors downregulation.<sup>22</sup> Interestingly, this high netrin-1 expression seems to correlate with elevated expression of COX-2 and IκBα genes, that are known molecular targets of NFκB,<sup>90</sup> whereas the vast majority of "sporadic" tumors shows low levels for these genes. This suggests that high netrin-1 levels are found in tumors showing high NFκB activity. Along this line we demonstrated that upon NFκB

activation, netrin-1 gene is activated via direct binding of p65 to netrin-1 promoter.<sup>22</sup> Stimulation of several cancer cell lines, including colorectal cancer cell lines, and mouse colonic crypts with NFκB inducers triggers a direct netrin-1 mRNA upregulation. Moreover, we have shown that NFκB activation is associated with secretion of netrin-1, an event that inhibits the pro-apoptotic activity of netrin-1 receptor UNC5H2.<sup>22</sup> These findings suggest that netrin-1 could be upregulated during colorectal inflammation and possibly promote tumorigenesis. To more formally address the role of netrin-1 during inflammation-driven colorectal tumorigenesis, we used the AOM/DSS mouse model of chronic inflammation and colorectal cancer development. As compared to control mice, we observed in these mice clear signs of inflammation and increased expression of COX-2 and IκBα genes, as well as netrin-1 upregulation both at the RNA and protein levels.<sup>22</sup> In AOM/DSS-treated mice, netrin-1 is strongly expressed in IECs of inflamed mucosa, in which neoplastic lesions progressively developed, including adenomas and adenocarcinomas, showing a marked netrin-1 expression at immunohistochemical examination.<sup>23</sup> Furthermore, strong netrin-1 expression appears to be a characteristic feature of neoplastic lesions found in human IBD. A strong expression of netrin-1 is detected in epithelial cells of IBD patients, especially in areas of inflammation. Moreover, while in the sporadic cancer netrin-1 is rarely upregulated, netrin-1 seems to be highly expressed in 70% of tumors derived from IBD patients.<sup>23</sup> To determine whether netrin-1 upregulation in IBD patients and in the mouse model of CAC is causal to the cancer pathology, we have developed a netrin-1 interfering agent, the peptide DCC-4Fbn, corresponding to the 4<sup>th</sup> fibronectin domain of DCC ectodomain (see Fig. 1), which has been shown to interact with netrin-1.<sup>91</sup> This compound inhibits the ability of netrin-1 to block death signals triggered by its receptors.<sup>23</sup> Interestingly netrin-1 interference does not affect the inflammation induced by DSS, since the inflammatory aspect of the intestinal epithelium appears similar in control versus DCC-4Fbn-treated mice. In contrast, injection of the netrin-1 interfering

agent in AOM/DSS mice decreased the number of early neoplastic lesions (61 versus 95), even if this effect seems to be modest, indicating that netrin-1 inhibition could partially affect colorectal tumor promotion. Moreover, the biologic has a dramatic effect on tumor progression, as it triggers an increased frequency of low grade adenoma associated with a decreased incidence of high-grade adenoma and adenocarcinoma.<sup>23</sup> The effect of netrin-1 inhibition in inflammation-driven tumorigenesis is quite similar to the specific ablation of IKKβ in IECs, with some difference: the effect of the netrin-1 blocking agent in tumor promotion (i.e., number of tumors) is modest, as compared to enterocyte-IKKβ knockout mice, indicating a partial inhibition of pre-malignant cell apoptosis by netrin-1. The mild effect of netrin-1 inhibition in tumor promotion was quite expected, as netrin-1 is not the only survival factor dependent on NFκB activation. On the other hand, the most interesting difference between netrin-1 interference and enterocyte-specific deletion of IKKβ is tumor progression effect. Indeed, netrin-1 titration affects both tumor size and composition, while this effect is found only in the mouse model of myeloid cell-specific, and not enterocyte-specific, inactivation of NFκB function. Although netrin-1 interference effect on tumor progression could be explained as a consequence of inhibition of both anti-apoptotic and proliferative signals triggered by netrin-1 in myeloid cells, leading to a decreased production of pro-tumoral cytokines, this hypothesis is contradicted by the observation that inhibition of netrin-1 does not affect inflammation induced by DSS. An anti-inflammatory function of netrin-1, due to its effects on cell migration, has been observed,<sup>92-94</sup> nevertheless this feature seems not to be involved in the case of DSS-induced inflammation, limiting netrin-1 interference action to IECs. Another intriguing hypothesis, that could explain the effect of netrin-1 inhibition in tumor progression in AOM/DSS-treated mice, involves the controversial role of netrin-1 during angiogenesis. Indeed, netrin-1 has recently been showed to act as a pro-angiogenic factor,<sup>95,96</sup> probably through its ability to inhibit UNC5H2-

induced apoptosis in endothelial cells<sup>96</sup> (even though other published studies have reported netrin-1 as an anti-angiogenic factor<sup>97,98</sup>). Based on these observations, we could hypothesize that upregulation of netrin-1 through NFκB activation in enterocytes during chronic inflammation could not only protect pre-neoplastic cells from apoptosis induced by its own receptors, but also contribute to tumor sustenance, promoting angiogenesis (Fig. 3). Therefore, netrin-1 titration in AOM/DSS-treated mice could allow apoptotic cell death of transformed enterocytes (decreased number of neoplastic lesions and tumor delay) and inhibit blood vessel maintenance and/or development, affecting tumor progression. This hypothesis is supported by the observation that angiogenesis plays an important role in many chronic inflammation diseases, including IBD.<sup>99,100</sup> Along this line, this disease has been listed among the several conditions characterized by excessive angiogenesis.<sup>101</sup>

## Conclusions

From our work showing upregulation of netrin-1 in colorectal cancer from patients with IBD and NFκB-dependent netrin-1 expression together with the fact that NFκB activation contributes to IBD-associated colorectal cancer formation by providing anti-apoptotic signals to IECs, we propose the following sequence for colorectal tumor progression in IBD patients: in response to chronic inflammation, NFκB activation triggers netrin-1 upregulation in the normal and altered epithelium, providing, together with other tumor initiation mechanisms, the sufficient survival signal to epithelial cells for tumor progression (Figs. 2 and 3). Therefore, we propose that netrin-1 could be an important effector of the molecular pathways linking chronic inflammation, cancer development and NFκB activation. Consequently, netrin-1 inhibition could be an attractive target for therapeutic intervention. Indeed, drug compounds targeting netrin-1 are currently under development. Intense interest is prompt in the development of NFκB inhibitors for the treatment of inflammatory diseases, since this factor and the signaling pathways involved in its activation are

attractive target for cancer prevention and therapy. However, NFκB in inflammatory cells serves an important immune function, and its absence can result in several immunodeficiencies. In addition, although a number of studies confirmed that NFκB inhibition has anti-inflammatory effects *in vivo*,<sup>85,102-105</sup> recent studies indicate that inactivation of NFκB in certain tissues might have pro-inflammatory effects by disrupting physiological immune homeostasis. As an example, NF-κB inhibition in epidermal keratinocytes spontaneously triggers a strong inflammatory response in the skin and the development of squamous cell carcinoma.<sup>106-109</sup> Along the same line, in hepatocytes, NFκB provides survival signals that protect cells from toxic compounds, and its inhibition triggers liver inflammation and compensatory hepatocyte proliferation, resulting in the development of hepatocellular carcinoma.<sup>39,110</sup> For this reason, although strategies targeting NFκB have great potential for the treatment of inflammatory diseases, the potential side effects of NFκB blockade seem to be a major problem in bringing NFκB inhibitors to the clinic. Another possibility to treat chronic inflammation is to interfere with the initial causes of NFκB activation, through the use of anti-inflammatory drugs. Several clinical studies have shown that anti-cytokine therapy is beneficial in treatment of chronic inflammation diseases. However, immune-suppressing therapies might cause the development of malignancy: the immune system is classically believed to display a preeminent protective role and multiple conventional therapies have aimed at stimulating the pro-inflammatory functions of the immune system's patient that fail to recognize cancer cells as being detrimental.<sup>111</sup> Therefore, TNFα blocking agents have been applied for treatment of several chronic inflammation, such as Crohn's disease.<sup>112</sup> However, several severe side effects have been associated with these therapeutic agents, including infections, lymphoma and autoimmune diseases.<sup>113</sup> In the case of inflammation-associated colon cancers, the scenario is also complicated by the existing relationship between colon and commensal bacteria. TLRs have been described to have a protective role against epithelial tissue damage caused by

commensal bacteria,<sup>114</sup> mainly by NFκB activation, and therefore inhibition of NFκB in this tissue could potentially contribute to an increase in epithelial damage by commensal bacteria.<sup>60,114</sup> The challenge for future drug discovery and therapy development for chronic inflammation-associated cancers is therefore to develop drugs that prevent and/or inhibit tumor growth, while attempting to prevent the inhibition of tumor-specific immune response.

In light of these considerations, netrin-1 could be a suitable target for drug therapy in inflammation-driven cancers, particularly in colorectal cancers, as its inhibition, using titrating biologics, prevents tumor growth and does not affect inflammation, at least in the mouse models tested so far. Furthermore, we have shown that disruption of netrin-1 autocrine survival loop, using such biologics, is effective to decrease tumor growth and metastatic dissemination in different avian and mouse models.<sup>23-26</sup> Anti-netrin-1 drugs, such as small interfering peptides or neutralizing antibodies, could therefore complement the actual treatments of IBD, in order to inhibit progression of early neoplastic lesions. Moreover, these drugs could act also as a preventive treatment for IBD patients, which could be less invasive than colectomy, applied as preventive treatment in ulcerative colitis.

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