Deleted in Colorectal Cancer (DCC) Pathfinding: Axon Guidance Gene Finally Turned Tumor Suppressor

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Abstract

Loss of heterozygosity (LOH) at human chromosome 18q, which includes the gene *Deleted in Colorectal Cancer (DCC)*, has been linked to colorectal and many other human cancers. *DCC* encodes the receptor for the axon guidance molecule Netrin (Net) and functions during neural development in a variety of organisms. However, since its discovery in the 1990s, the status of DCC as a tumor suppressor has been debated, primarily due to a lack of support for this hypothesis in animal models. A recent study from our laboratory capitalized on the genetic tractability of *Drosophila melanogaster* to demonstrate that this gene functions as an invasive tumor suppressor, thereby providing the first direct link between *DCC* loss and metastatic phenotypes in an animal model for cancer. Two subsequent studies from other laboratories have demonstrated that DCC suppresses tumor progression and metastasis in murine colorectal and mammary tumor models. Combined, these findings have prompted the rebirth of DCC as a tumor suppressor and highlighted the need for continued analysis of DCC function in animal models for human cancer.

Keywords

Apoptosis; axon guidance; cancer; DCC; *Drosophila melanogaster*; metastasis; netrin; tumor suppressor

LOH AT 18Q AND CANCER

LOH at chromosome 18q was identified in greater than 70% of human colorectal cancers [1, 2]. These findings triggered the search for a tumor suppressor gene in this chromosomal region, which led to the cloning of *DCC*. Expression of *DCC,* which encodes a cell surface receptor, was found to be altered in colorectal cancers, supporting the notion that *DCC* might be the 18q tumor suppressor [3]. Although it was initially hypothesized that DCC might function as a tumor suppressor gene, the tumor suppressor status of this gene has been debated since its discovery [4–7]. A chronological list of evidence both in support of and against this hypothesis is presented in (Table 1) and summarized below.

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DCC expression is reduced in a large percentage of colorectal tumors [3, 6]. However, point mutations in *DCC* are not often associated with tumors [5]. Furthermore, following cloning of *DCC*, *Smad4,* which is also located at 18q, was shown to function as a tumor suppressor gene, suggesting that this gene might in fact be the 18q locus of interest [8]. Moreover, the *DCC* knockout mouse did not demonstrate an increased incidence of tumor susceptibility [9]. This mouse did however exhibit axon guidance abnormalities [9], which have been associated with loss of *DCC* in a variety of other organisms, from *C. elegans* [10] to *D. melanogaster* [11], and more recently vector mosquitoes [12]. For these reasons, much of the focus of DCC research initially centered on its role as an axon guidance molecule [13].

DCC FUNCTIONS AS A RECEPTOR FOR NETRIN DURING AXON GUIDANCE

Similarities between the *DCC* and *Net* loss of function axon guidance phenotypes in *C. elegans* [10, 14], *D. melanogaster* [11, 15, 16], and mice [9, 17] suggested that DCC might function as a receptor for Net proteins, a notion that was confirmed by biochemical experiments [18]. Members of the Net family are laminin-related diffusible proteins that promote the outgrowth and guidance of commissural axons toward the midline in a variety of organisms [13, 19]. Upon their discovery in the chick [19], sequence comparisons indicated that chick *net-1* and *net-2* are related to *C. elegans unc-6*, which had previously been shown to direct the circumferential migration of axons to the midline in nematodes [10, 14]. *NetA* and *B* genes were thereafter isolated in *Drosophila,* where they regulate proper nerve cord formation by acting as short-range guidance cues that promote midline crossing of axons [15, 16, 20]. Likewise, inactivation of *Net-1* in mice results in pathfinding defects that prevent most commissural axons from reaching the floor plate [17].

Axon guidance functions of Net proteins are mediated by various Net receptors, including members of the DCC family [21]. *DCC* family genes, including the *C. elegans* homolog *unc-40* and the *Drosophila* homolog *frazzled (fra),* encode transmembrane proteins that have immunoglobulin domains and fibronectin type III repeats [7] (Fig. 1). DCC receptors are expressed on commissural axons and growth cones, where they function as cell-surface receptors for Net family proteins during axon guidance [22]. DCC has also been shown to regulate Net protein spatial distribution, and it has been suggested that this regulation of Net protein distribution guides neighboring axons through the creation of positional information for other Net receptors [23]. Mutation or inhibition of *DCC* receptors results in the failure of many commissural axons to reach the midline [9–11]. For example, the mouse *DCC* knockout exhibits axonal projection defects comparable to those observed in *Net-1* deficient mice [9]. Although characterization of the phenotype of mice lacking *DCC* supported a role for this gene in Net-1-mediated axon guidance, it raised questions concerning the status of DCC as a colorectal tumor suppressor. Loss of murine *DCC* does not affect mouse intestinal growth, differentiation, or morphogenesis, and it does not result in increased tumorigenesis [9]. For this reason, although the axon guidance roles of DCC were intensely studied during the late 1990's [13], its status as a tumor suppressor remained controversial at the turn of the century.

DCC: A DEPENDENCE RECEPTOR, BUT STILL NOT A TUMOR SUPPRESSOR?

The discovery that DCC functions as a dependence receptor (Fig. 2a, b) which induces cell survival in the presence of ligand prompted renewed interest in its putative functions in cancer [5]. Research demonstrated that in the absence of ligand, Caspase-9 is recruited into a complex with Caspase-3 and DCC. This in turn leads to caspase activation and cleavage of

the death domain of DCC at position 1290, which exposes the DCC proapoptotic addiction dependence domain (ADD) and promotes cell death (Fig. 2b). Thus, in the absence of ligand, DCC promotes apoptosis. Conversely, in the presence of ligand, DCC-induced apoptosis is suppressed, and cells expressing DCC become dependent upon the presence of Net for their survival [24, 25]. It was proposed that this dependence receptor system could serve as a mechanism by which DCC functions as a tumor suppressor, as pretumorous or metastatic cells growing in regions of ligand unavailability would be eliminated by DCC-mediated apoptosis [5, 26].

In mice, the ability of Net-1 to inhibit the proapoptotic function of DCC was shown to be important for tumor progression in the digestive tract [27]. During intestinal development in mice, Net-1 promotes maintenance, integrity, migration, and renewal of the intestinal epithelium by promoting cell survival in proliferating intestinal crypt progenitors that are exposed to high Net-1 levels. When mouse intestinal cells differentiate, they migrate to the surface of the intestinal luminal epithelium where lack of Net-1 results in DCC-induced apoptosis [5, 28]. Ectopic expression of Net-1 in the mouse gastrointestinal tract results in the spontaneous formation of hyperplastic and neoplastic lesions, and overexpression of Net-1 in an *APC* mutant background promotes intestinal tumor development. It was proposed that these tumors result from inhibition of DCC-induced apoptosis [27]. Likewise, elevated Net-1 levels have also been associated with metastatic breast cancer [26], aggressive neuroblastomas [29], and non-small cell lung cancer [30], suggesting that the abilities of Net-1 to prevent cell death and promote cell invasion may be critical in a number of different cancers. Additionally, Flannery *et al.* [31] demonstrated that Net-DCC signaling induces cellular growth and expression of key cellular growth regulators, including *myc, cdk4, PCNA,* and activated ERK. Thus, in addition to promoting cell survival and invasion, ectopic Net expression may also promote tumor cell growth (Fig. 2c).

Although a number of investigations suggested that DCC functions as a dependence receptor that promotes metastatic cancer when Net-1 ligand is present, these studies still failed to demonstrate that DCC functions as a classical tumor suppressor [5, 32]. While DCC had been shown to induce cell death when ligand is limiting (Fig. 2b), it was also found to promote tumor progression by mediating constitutive Net-1 signaling, which can confer a selective advantage on tumor cells (Fig. 2c). Furthermore, *DCC* deficiency had still not been shown to promote tumor progression in any animal model for cancer. For these reasons, many still deemed it to be a conditional tumor suppressor [5, 32].

ANALYSIS OF D. MELANOGASTER FRA/DCC PROVIDES THE FIRST DIRECT LINK BETWEEN LOSS OF DCC FUNCTION AND A METASTATIC PHENOTYPE IN AN ANIMAL MODEL FOR CANCER

D. melanogaster is an excellent system in which to study tumor suppressor gene function, as many of the hallmarks of human cancer, including self-sufficiency in growth and proliferative signals, insensitivity to anti-proliferative signals, evasion of apoptosis, and invasion/metastasis can be found in this animal model for cancer [13, 33, 34]. One advantage of this system is that it permits use of FLP/FRT recombination [35] to create clones of genetically distinct somatic cells that model human tumors. The genetic tractability of the *Drosophila* model also allows for multiple genetic manipulations to be performed simultaneously in such clones, a notable advantage given that multiple mutations typically cooperate to generate metastatic tumors in humans [13, 33].

In a recent investigation from our laboratory, Van-Zomeren-Dohm *et al.* [36] exploited the advantages of the *D. melanogaster* system to study the roles of Fra, the *Drosophila* homolog

of DCC. It was anticipated that the genetic tractability of this system might shed light on the controversial status of *DCC* as a tumor suppressor. In these studies, the authors investigated the function of Fra/DCC during development of the eye-antennal imaginal disc, a tissue in which the functions of many tumor suppressors have been analyzed [33]. Recent investigations have highlighted the importance of studying cancer genes in a clonal context [37, 38], and for this reason the authors chose to generate somatic loss of function *fra/DCC* mutant clones. They found that most *fra/DCC* loss of function clones generated during imaginal disc development are eliminated by the end of the third larval instar. Although most *fra/DCC* mutant clones do not persist, in ~1% of adult flies in which *fra/DCC* loss of function mutant clones were generated, mutant clone cells formed overgrowths. These exciting observations suggested that it would be useful to develop a strategy that would permit more efficient analysis of *fra/DCC* loss of function clones.

The authors chose to use the mosaic analysis with a repressible cell marker (MARCM) system [39, 40] to drive expression of the caspase inhibitor P35 in *fra/DCC* loss of function mutant clones generated in the developing eye. Although the majority of these flies did not survive to adulthood, this strategy (which is often employed in *Drosophila* for cases in which loss of a gene results in cell lethality) permitted analysis of clones in third instar larval eye-antennal discs and occasional analysis of adults that escaped death. A striking phenotype was observed in these adult flies: the detection of red-pigmented *fra/DCC* mutant eye cells throughout their bodies (Fig. 3). In the most dramatic case, *fra/DCC* mutant clone cells generated in the developing eye could be detected on the adult wing (Fig. 3a). These *fra/DCC* mutant eye cells appeared to differentiate, at least in part, as they expressed the neural differentiation marker Elav in third instar eye discs and displayed red pigmentation in adults (Fig. 3). These observations led to the hypothesis that Fra/DCC may have invasive tumor-suppressing functions in *Drosophila* (Fig. 2d).

To test this hypothesis, *fra/DCC* loss of function clones were studied in the developing eye-antennal disc, where clones were generated with an eye-specific driver. Although the *eyeless* promoter [41] used in the investigation was designed to be very tight, the investigators restricted all analyses to the eye-antennal disc in order to guard against any minor leakiness of the promoter and so that the point of clone origin (the eye) would be certain. Elav-positive *fra/DCC* mutant photoreceptor cells were detected outside of the developing eye field in the optic stalk and other regions of the eye-antennal disc that are not normally populated with photoreceptor cell bodies. In such cases, *fra/DCC* mutant Elav-positive foci were isolated and often located outside of mutant clone boundaries, suggesting that they had become invasive. Comparable results were obtained even in the absence of P35-rescue, and when loss of function clones were generated for two separate alleles of *fra/DCC* (fra^3 or fra^4, [11]). These findings indicated that *fra/DCC* mutant cells are invasive. However, one could alternatively argue that *fra/DCC* mutant cells located outside of the eye field had undergone a transformation toward an eye cell fate. The authors ruled out this possibility for a number of reasons (see [36] for details), mainly due to the fact that *fra/DCC* loss of function clones generated in the developing wing are not transformed into eye cells.

Immunohistochemical characterization of *fra/DCC* mutant cells in the third instar developing eye disc indicated that they express the tumor cell markers phospho-ERK and phospho-JNK. The clone cells were also found to exhibit changes in expression of E-cadherin, reorganization of the actin cytoskeleton, and loss of apical-basal polarity, characteristics typical of invasive tumor cells. Even though *fra/DCC* mutant cells retained expression of the neural differentiation marker Elav, a proportion of these cells were found to simultaneously express the mitotic marker phosphorylated histone H3. The authors also showed that loss of *fra/DCC* induces expression of Matrix metalloproteinase-1 (Mmp-1), basement membrane degradation, and invasion. Furthermore, by marking the *fra/DCC*

mutant cells with GFP expression (Fig. 3), the investigators demonstrated through live imaging experiments that *fra/DCC* mutant cells are invasive. Their movies captured projections extending and retracting from motile *fra/DCC* mutant cells as they exited the eye disc. Combined, these data indicated that Fra/DCC functions as an invasive tumor suppressor during *Drosophila* development, suggesting that loss of *DCC* can promote metastatic cancer in humans [36] (Fig. 2d).

VanZomeren-Dohm *et al.* [36] then attempted to identify suppressors of the loss of function *fra/DCC* metastatic phenotype. They hypothesized that JNK, a mediator of metastasis [42] that is upregulated in *fra/DCC* mutant cells, might drive the invasion of these cells. However, inhibition of JNK signaling did not suppress basement membrane degradation or invasion of *fra/DCC* mutant cells. In fact, inhibition of JNK signaling in *fra/DCC* mutant cells appeared to block JNK-mediated cell death, thereby promoting enhanced overgrowth and invasion of *fra/DCC* mutant cells (Fig. 2f). These results were initially somewhat surprising, as JNK-induced Mmp1-dependent degradation of the basement membrane was previously determined to be critical for invasion of cells in other *Drosophila* metastatic cancer models [42]. However, others have shown that JNK activity is context dependent and does not always function to promote cell invasion [43, 44]. Furthermore, JNK-driven apoptosis has been observed in the context of other *Drosophila* tumor suppressor mutations [44], and it is therefore perhaps not unexpected that it would function in the apoptotic removal of *fra/DCC* mutant cells.

The authors then examined if inhibition of Rho1, which has also been implicated in the invasive behavior of wing and eye imaginal disc cells [37, 45], could block *fra/DCC* mutant cell invasion. They found that inhibition of Rho1 signaling through expression of dominant negative-Rho1 in *fra/DCC* mutant cells represses basement membrane degradation and invasion. The repression of basement membrane degradation and invasion by dominant negative-Rho1 was substantial, albeit incomplete, suggesting that this process may also be mediated by additional unknown factors. Our lab has recently pursued global analysis of gene expression in *fra/DCC* mutant cells (Sarro and Duman-Scheel, unpublished), as well as a genetic interaction screen for enhancers of the *fra/DCC* loss of function phenotype (Sarro, Tessier, and Duman-Scheel, unpublished). These studies are elucidating additional molecules that may modulate and mediate *fra/DCC* mutant cell metastasis.

It should be noted that comparable eye cell invasive metastatic adult phenotypes are not typically reported in *Drosophila.* An exception is the Ferres-Marco *et al.* [46] *eyeful* study, in which the combined manipulation of *Delta, lola,* and *pipsqueak* generated a metastatic eye phenotype. It is interesting that both the *fra/DCC* and *eyeful* studies resulted in the generation of invasive cells which were at least partially differentiated yet still dividing. Zhai *et al.* [47] discuss the delicate balance between differentiation, proliferation, cell death, and invasion in their recent analysis of *Drosophila* Cut, a transcription factor with tumor suppressing capabilities. Loss of *Cut* enhances the *eyeful* phenotype and contributes to a high frequency of long range eye cell metastasis. Their data argue that Cut normally functions to remove cells that are not able to fully differentiate, thereby preventing them from developing into cancer cells. Cut accomplishes this through coordinate regulation of multiple cellular processes, including apoptosis, differentiation, cell adhesion, and proliferation, the combined regulation of which is required for proper cell fate specification and maintenance of a differentiated state. The results of this investigation suggest that Cut functions in an evolutionarily conserved cancer prevention regulatory network [47]. It is possible that Fra/DCC functions in a comparable cancer-preventing regulatory network.

ASSESSING THE TUMOR-REPRESSING FUNCTIONS OF DCC IN MURINE CANCER MODELS

Following publication of the *Drosophila frazzled/DCC* study [36], two groups assessed the tumor-suppressor functions of DCC in mouse models for colorectal [48] and metastatic breast [49] cancers. These studies, which are reviewed below, also concluded that DCC functions as a tumor suppressor. To investigate the putative function of DCC as a colorectal cancer tumor suppressor, Castets *et al.* [48] constructed a mouse model in which aspartic acid residue 1290 of *DCC* was mutated [(DCC(D1290N)]. While mutation of this residue did not appear to impact Net-1 signaling through DCC, it did allow the authors to assess the importance of DCC pro-apoptotic activity, which was compromised by this mutation (Fig. 2e). In the mouse intestine, epithelial cell death is typically observed at the tips of the villi where Net-1 ligand is not abundant [5, 28]. In mice in which DCC proapoptotic activity was silenced, apoptosis in the intestinal epithelium was significantly decreased. This reduction in apoptosis was accompanied by limited spontaneous intestinal tumor formation, with nearly 15% of mutant mice (as compared to 0% of control animals) displaying neoplastic transformations, including adenomas and adenocarcinomas. Based on these results, the authors proposed that DCC-mediated cell death may normally function to limit the initiation of malignant transformations (Fig. 2e) [48].

Castets *et al.* [48] hypothesized that given the high incidence of *DCC* loss observed in late-stage tumors, DCC may predominantly function as a tumor suppressor in late stages of tumorigenesis. To test this, they examined the effect of the *DCC(D1290)* mutation in an *APC*$^{+/1638N}$ background. *APC*$^{+/1638N}$ mice are known to develop a moderate level of intestinal tumors [50], but in the *DCC(D1290)* homozygous background, the incidence and aggressiveness of the adenocarcinomas significantly increased (Fig. 2f). A reduced incidence of apoptosis was also observed in these tumors. Based on their observations, the authors argued that inhibition of DCC-induced apoptosis may disrupt the balance between proliferation and death in low grade tumors and stimulate tumor progression by increasing the likelihood for additional mutations to accumulate over time. The authors concluded that the results of their investigation demonstrate that DCC functions as a tumor suppressor in the intestinal tract by acting as a gatekeeper that limits tumor progression through its ability to promote apoptosis.

Krimpenfort *et al.* [49] examined the putative tumor-suppressing functions of DCC in a *p53* deficient mouse mammary tumor model. They introduced a *Cre/loxP* conditional *DCC* mutant lacking a transmembrane domain into a background in which the *cytokeratin 14* promoter was used to ablate both copies of *p53* in mammary epithelial tissue. They chose this well-established model because it yields well-encapsulated tumors that do not typically metastasize [51, 52]. Furthermore, as expression of Unc5h (which also functions as a Net-1 receptor) is known to be reduced in *p53* deficient conditions [53], the authors argued that use of this model would guard against the potential for Unc5h to mask the effects of *DCC* loss. This was an important concern given that multiple Net receptors are found in mice [6, 7]. Their results suggested that *DCC* loss does not increase the incidence of *p53* deficient primary tumors, but that the additional loss of *DCC* promotes metastasis of *p53* deficient mammary tumors (Fig. 2f). The authors speculated that this increase in metastatic tumors might relate to the ability of DCC to induce cell death when Net-1 ligand is limiting, for example in cases where cells have disseminated from the primary tumor site. Through cell culture experiments with *p53* deficient mouse mammary tumor cells, they next demonstrated that DCC promotes Net-1-dependent cell survival. Consistent with these *in vitro* data, the authors found that mice intravenously injected with *DCC* deficient *p53* deleted cells had significantly more tumor cell clusters in the lungs as compared to those injected with *DCC* proficient *p53* deleted cells. The authors concluded that DCC functions as a tumor

suppressor which induces the death of cells that disseminate from the primary tumor mass [49].

FROM FLIES TO MICE

Together, our initial study in the developing *Drosophila* eye [36] and two subsequent studies in mice [48, 49] have provided convincing new evidence that DCC functions as a tumor suppressor in three animal models for cancer (Fig. 2d–f). These critical findings have prompted the rebirth of DCC as a tumor suppressor. While the three studies have certainly quieted a 15-year old debate, they raise new questions about the functions of DCC in cancer.

The *Drosophila* study [36] provided evidence that loss of function mutations in *DCC/fra* alone could result in tumor-like outgrowths, albeit at a very low frequency (~1% of the animals in which mutant clones had been induced). This result differs from the initial *DCC* mouse knockout study [9], as well as the Krimpenfort *et al.* investigation [49] in which no increased incidence of primary tumors was found to be associated with loss of *DCC*. In addition to DCC, several other proteins, including Unc-5 and Neogenin, function as Net-1 receptors in mice [32]. Such redundancy, which is perhaps not an issue in the *Drosophila* eye given the metastatic phenotypes observed (Fig. 3), could explain the lack of cancer phenotypes in the *DCC* knockout mouse [9]. The Krimpenfort *et al.* [49] mammary tumor study partially addresses the receptor redundancy issue through choice of a model in which Unc-5 should not be a confounding factor, but it was not addressed in the Castets *et al.* [48] investigation. Furthermore, redundancy with additional Net-1 receptors [i.e. Down's syndrome cell adhesion molecule (DSCAM), Neogenin, A2b] could still be an issue in these and other mouse *DCC* deficient tumor studies.

The *Drosophila* study also differs from the Castets *et al.* [48] investigation where the authors used a mutant in which the proapoptotic functions of DCC were specifically disrupted. As discussed above, the authors found that mice homozygous for this mutation had a moderately increased incidence of spontaneous neoplastic transformations in the intestinal tract. However, they argued that the *DCC(D1290N)* mutation impacts only the proapoptotic functions of DCC while permitting Net-1-DCC signaling (Fig. 2e), as indicated by the viability of these animals and their lack of obvious brain defects. If Net-1-DCC signaling is in tact in these animals, then it is difficult to directly compare these experiments to the *Drosophila* study [36] in which loss of function *fra/DCC* mutations were utilized. A comparable analysis of the proapoptotic functions of Fra/DCC have not yet been performed in *Drosophila*, but pursuing this line of experiments might prove useful. An interesting observation in the VanZomeren-Dohm [36] investigation is that most *fra/DCC* mutant clones generated in the *Drosophila* eye-antennal disc do not persist beyond the third larval instar (Fig. 2d). Based on the dependence receptor model (Fig. 2a, b), and in light of the two murine studies [48, 49], one might have expected that loss of function mutations in *fra/DCC* would result in increased cell viability. One possible interpretation of the *Drosophila* result is that Net ligand, which is expressed in the eye-antennal disc, provides a survival cue for developing cells in this tissue. *fra/DCC* mutant cells lacking the ability to receive this survival cue could ultimately die, presumably through activation of JNK signaling [36].

Another critical difference between the three recent *fra/DCC* investigations discussed herein is that only the *Drosophila* study involved production of somatic clones of mutant cells (Figs. 2d, 3). Recent studies have highlighted the importance of studying cancer genes in a clonal context [37, 38]. In support of this notion, expression of *fra-RNAi* throughout the developing *Drosophila* eye does not generate metastatic eye phenotypes (although *fra-RNAi* clones, like *fra* loss of function clones, do not typically persist during development; C. Tessier and M. Duman-Scheel, unpublished observation). It is possible that using an

experimental design more comparable to the one employed by VanZomeren-Dohm *et al.* [36], one in which clones of *DCC* loss of function cells are generated (and perhaps also rescued from death), could generate primary tumor phenotypes in mice. However, if a comparable somatic clonal study were to be performed in mice, it may be very difficult to score enough adult animals to observe this effect. Even in the fly model, rescue of *DCC* mutant clones with P35 expression was employed to facilitate completion of the investigation. This has consequently raised the question of whether expression of P35 influenced the results. However, VanZomeren-Dohm *et al.* [36] controlled for use of P35. Moreover, they demonstrated that use of P35 did not appear to be a confounding factor in the investigation, as basement membrane degradation, invasion, and tumor-like overgrowths could all be observed in the absence of P35 rescue, albeit at a low frequency (Fig. 2d).

Since publication of the VanZomeren-Dohm *et al.* [36] manuscript, we have initiated a genetic interaction screen in the *Drosophila* eye that examines whether lesions in other signaling pathways, in the absence of P35 rescue, will enhance the *fra/DCC* loss of function phenotype. We have found that expression of *fra-RNAi* in combination with other genetic lesions results in a variety of eye abnormalities, ranging from rough eyes to dramatic metastatic adult eye phenotypes (J. Sarro, C. Tessier, M. Duman-Scheel, unpublished). The results of this screen, as well as the published observation that inhibition of JNK signaling enhances the *fra/DCC* loss of function phenotype [36], are in agreement with the recent murine studies, both of which illustrated that loss of *DCC* in conjunction with other mutations *(APC, p53),* can result in the formation of highly invasive carcinomas (Fig. 2f). Interestingly, interactions between *fra* and *APC* were uncovered in the *Drosophila* screen. Furthermore, recent microarray experiments in which changes in gene expression in response to gain and loss of Net-Fra/DCC signaling were assessed have uncovered multiple hits in the Wnt pathway, including *APC* (J. Sarro and M. Duman-Scheel, unpublished). These results are interesting given the Castets *et al.* [48] finding that silencing DCC-induced apoptosis is associated with increases in the number and aggressiveness of intestinal tumors in an *APC* mutant context [48].

Finally, it is interesting to consider the results of the Krimpenfort *et al.* [49] mammary tumor study in the context of the *Drosophila* [36] investigation. Based on their analysis of *p53* deficient mammary tumors, Krimpenfort *et al.* [49] suggest that DCC limits survival of disseminated tumor cells, as supported by both their cell culture and intravenous injection experiments. However, in light of the *fra/DCC* investigation [36], it is also seems likely that loss of *DCC* in *p53* deficient mammary tumor cells more directly contributes to the invasive properties of these cells (Fig. 2f). For example, given that inhibition of Rho signaling suppresses invasion of *fra/DCC* mutant cells [36], it is plausible that altered Rho signaling, which is known to correlate with breast tumor progression, metastasis, and poor prognosis [54], could contribute to the enhanced metastatic capacity of p53-deficient mammary tumors lacking *DCC.*

In addition to Rho signaling, microarray experiments recently performed in our laboratory suggest that components of a number of other cell signaling pathways that regulate development and cancer are differentially expressed in response to *fra/DCC* deletion (J. Sarro and M. Duman-Scheel, unpublished). We are in the process of determining if these signaling pathways function in invasive growth of *DCC* mutant cells. In light of the Krimpenfort *et al.* [49] study, it is anticipated that this work will promote a better understanding of metastatic breast cancers in which *DCC* is deficient. In support of this notion, bioinformatic analysis of the *fra/DCC* loss of function microarray hits uncovered significant breast neoplasm human disease gene networks. Colorectal neoplasm disease networks were also identified, further supporting the use of *Drosophila* to model the roles of Fra/DCC in colorectal cancer. Furthermore, these studies uncovered significant prostatic and

lung cancer disease networks (J. Sarro and M. Duman-Scheel, unpublished), suggesting that DCC may also function as a tumor suppressor in these tissues.

IMPLICATIONS FOR THERAPIES

It is well established for a number of cancers that gain of autocrine Net-1 signaling (Fig. 2c) confers a selective advantage on tumor cells and promotes metastatic cancers [32]. For this reason, therapy development to date has primarily focused on disrupting the interaction of Net-1 with its receptors. Studies in metastatic breast cancer cells [26], many of which are known to have elevated Net-1 levels, suggest that targeting Net-1 signaling has good therapeutic potential. Experimental reduction of Net-1 levels through small interfering siRNA (siRNA) has been shown to promote cell death in metastatic breast [26] and lung [30] cancers. siRNA silencing of Netrin-1 (Ntn1) was also found to inhibit tumor cell invasion of pancreatic ductal adenocarcinoma cells in a chick model [55]. Comparable strategies could be employed to combat the myriad of other human cancers that have been linked to activated Net-1 signaling [32].

Drug development has concentrated on biological agents that mimic the interaction of DCC with Net-1 [32]. For example, the fifth fibronectin domain of DCC, DCC-5fbn, is known to interact with Net-1. Although DCC-5fbn does not appear to block Net-1/receptor interactions, it prevents receptor multimerization which is required for Net-1 inhibition of apoptosis [56] and possibly for induction of other Net-1-DCC signaling responses [32]. While DCC-5fbn shows promise for treating both metastatic breast [26] and lung [30] cancers, little is known about the toxicity of this agent [32], a concern that must be addressed in future studies. Although autocrine Net-1 signaling has been linked to cancer, Net and DCC have many normal biological functions [13]. For this reason, if therapies targeting Net-1 or DCC are to be utilized, it may be critical to employ delivery strategies, such as nanoparticles, that limit delivery of the therapeutics to the tumor site.

The three recent investigations of the tumor suppressing functions of DCC highlighted in this review make it clear that loss of *DCC* can promote tumor progression (Fig. 2d–f). These studies indicate that targeting DCC, rather than the ability of Net-1 to interact with DCC, may in fact advance tumorigenesis in some contexts. In such cases, it may instead be more appropriate to activate the proapoptotic functions of DCC. In support of this, expression of DCC has been found to suppress tumorigenic growth of cells in culture and following transplantation of such cells in nude mice [57, 58]. Studying how signaling is altered in response to cellular loss of *DCC* may also reveal novel therapeutic approaches. In this regard, given that the genetic tractability of the *Drosophila* system was vital in providing the first direct link between *fra/DCC* deficiency and a metastatic phenotype in an animal model for cancer, it is likely that the genetic interaction screen and microarray approaches we are employing to study *fra/DCC* in the fly will be fruitful.

Acknowledgments

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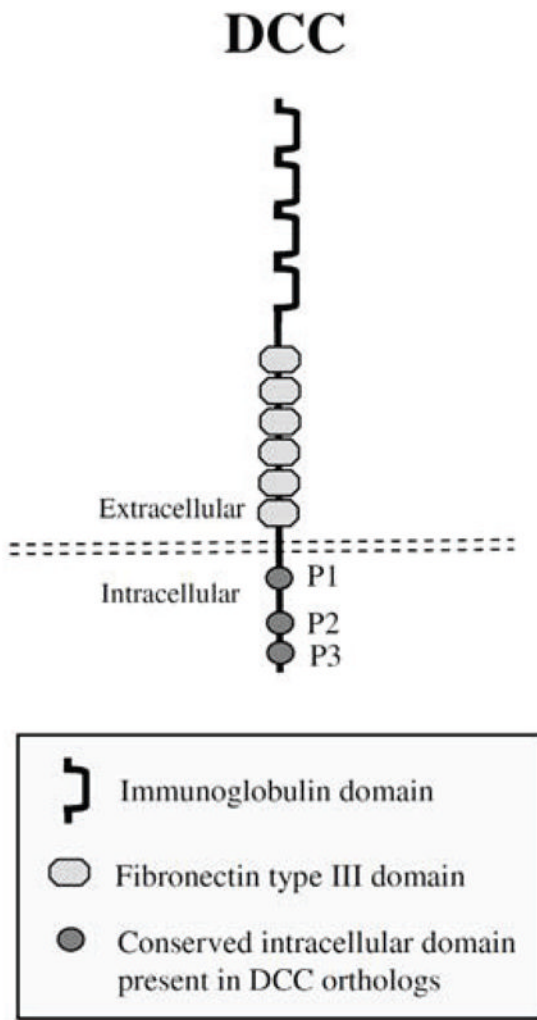
Fig. 1.
DCC protein structure. A schematic representation of DCC with the location of the immunoglobulin, fibronectin type III, transmembrane, as well as the conserved intracellular P1, P2, and P3 domains noted. The structure depicted is adapted from reference [7].
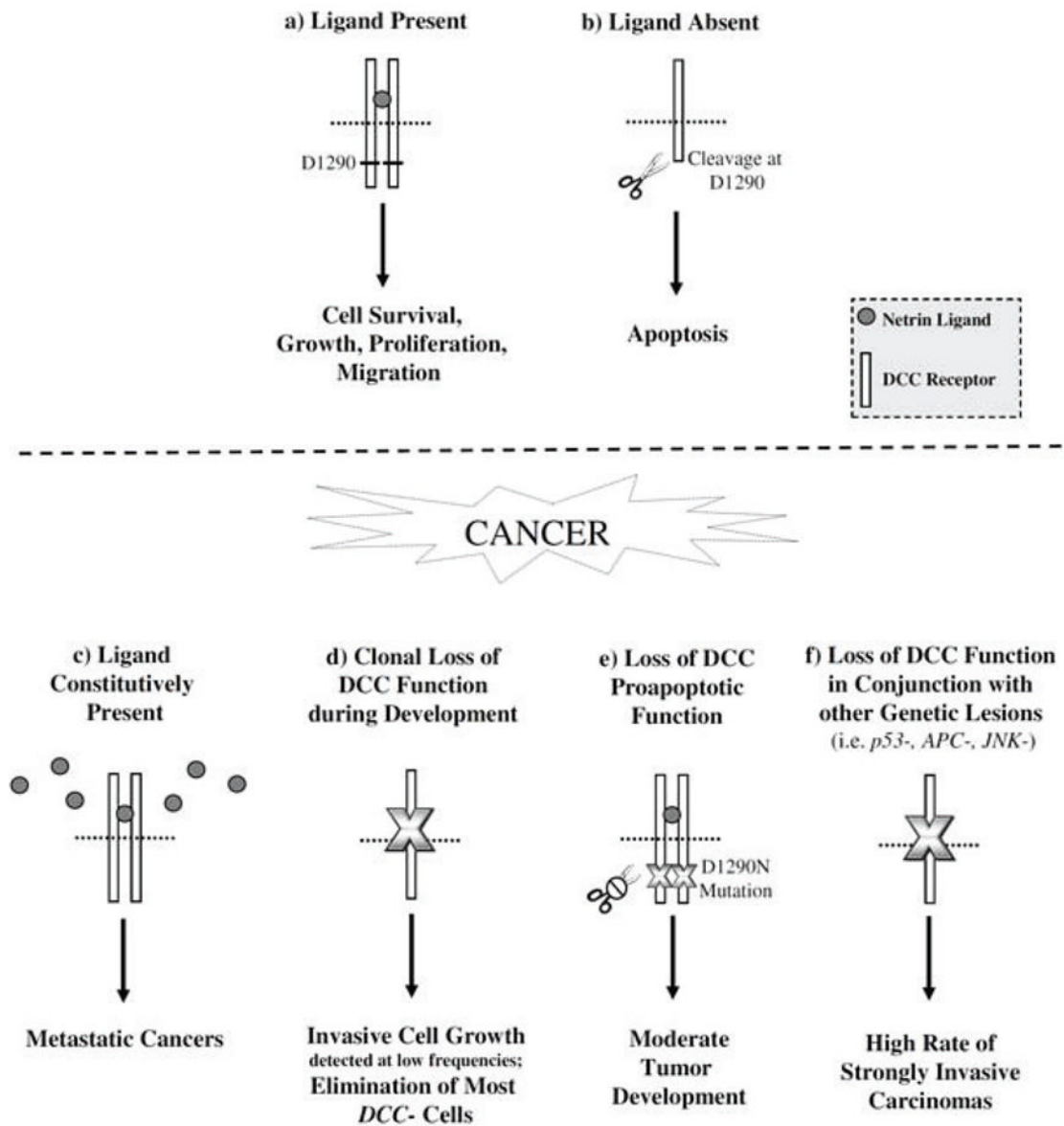
NORMAL DEVELOPMENT / HOMEOSTASIS

Fig. 2.
DCC tumor suppressing functions. Top: DCC functions as a dependence receptor during normal development and homeostasis. **a**) In the presence of Net ligand, DCC induces a variety of cellular responses including survival, growth, proliferation, and migration. **b**) However, in the absence of ligand, DCC is cleaved at D1290 and promotes cell death. **c**) In human cancers (bottom), the constitutive presence of Net ligand promotes metastatic cancer. (**d**–**f**) Recent studies in *D. melanogaster* and murine cancer models suggest that loss of DCC function also promotes cancer. **d**) During *D. melanogaster* development, although most somatic *fra/DCC* loss of function clones are eliminated, *DCC* deficiency can result in excess cell growth and invasion. **e**) The D1290N mutation results in loss of the proapoptotic

function of DCC and moderate tumor formation in mice. **f**) In conjunction with other genetic lesions (i.e. mutation of *P53, APC,* or *JNK)*, loss of DCC function results in highly invasive carcinomas. Such metastatic cancers may result from loss of the proapoptotic functions of DCC and direct stimulation of growth, proliferation, and invasion in response to changes in cell signaling (i.e. activated Rho signaling) that occur when DCC function is compromised. See text for details and references to studies supporting this model.
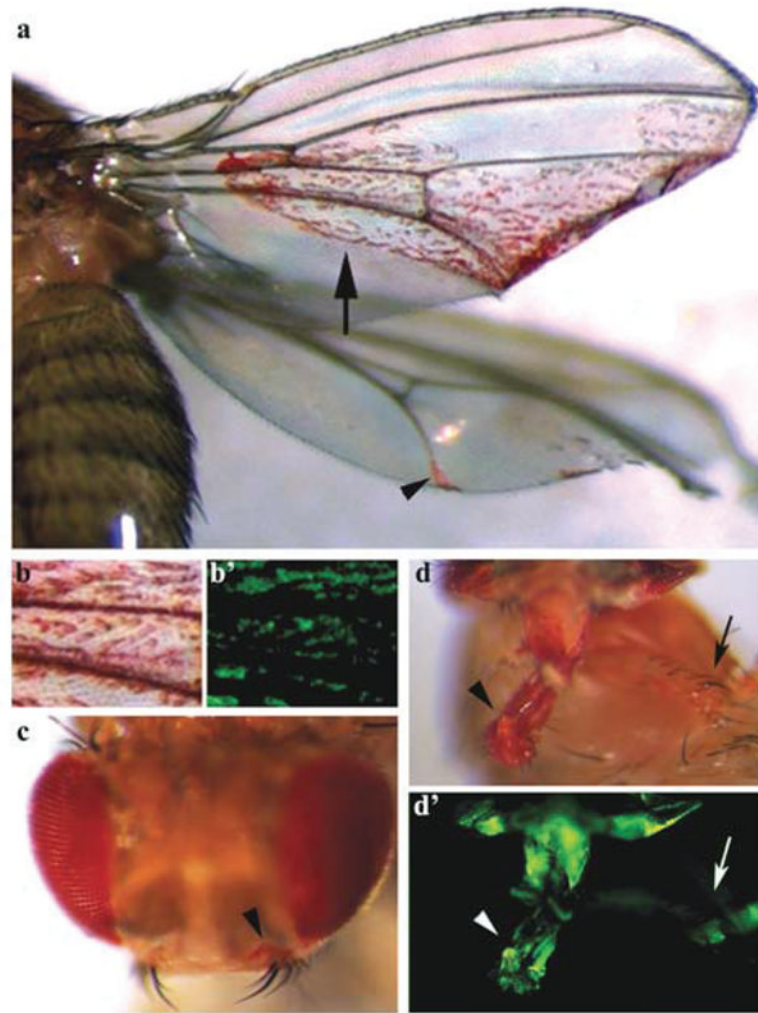
Fig. 3.
Loss of *fra/DCC* function during development of *D. melanogaster* results in metastatic phenotypes. Metastatic phenotypes resulting from somatic loss of function *fra/DCC* clones generated in the developing eye are shown. P35-rescued *fra/DCC* mutant red-pigmented eye cells generated in the developing eye have invaded the wing (arrow and arrowhead in **a**), head (arrowhead in **c**), proboscis (arrowhead in **d**) and leg (arrow in **d**). A high magnification view of the region marked by the arrow in **a** is shown in **b**. GFP expression (**b**′ and **d**′) used to positively mark mutant cells permitted live imaging of these invasive cells during development [36]. Panels **a**, **b**, and **b**′ were originally published in reference [36].

Table 1
DCC: Road to a Tumor Suppressor

A chronological list of key findings that either supported or refuted the DCC tumor suppressor hypothesis is provided.

Year	Finding	References[a]
1988	LOH at 18q is implicated in colorectal cancer.	[1, 2]
1990	Cloning of *DCC*, found to be altered in colorectal cancers	[3]
1996	DCC functions as a Netrin receptor.	[18]
1997	Mouse *DCC* knockout lacks a colorectal cancer phenotype.	[9]
1998	*Smad4* (which maps to 18q) functions as a tumor suppressor.	[8]
1998	DCC induces apoptosis.	[24]
1999–2000	Expression of DCC suppresses tumorigenic growth of cells in culture and following transplantation in nude mice.	[57, 58]
2001	Net-1 signaling through DCC promotes cell survival.	[25]
2004	Upregulated Net-1 induces tumor progression in the mouse intestine.	[27]
2008–2009	Upregulation of Net-1 in metastatic breast, non-small-cell lung, neuroblastoma, pancreatic adenocarcinoma, and IBD-associated cancers	[26, 30, 29], [55, 59]
2011	Fra/DCC functions as an invasive tumor suppressor in *D. melanogaster*.	[36]
2012	Mutation of *DCC* advances tumor progression in murine *APC* deficient colorectal and *p53* deficient mammary tumor models.	[48, 49]

[a]Refer to the numbered references cited in the text.