

# Interfering with multimerization of netrin-1 receptors triggers tumor cell death

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Netrin-1 was recently proposed to control tumorigenesis by inhibiting apoptosis induced by the dependence receptors DCC (Deleted in colorectal cancer) and UNC5H. Although the loss of these dependence receptors' expression has been described as a selective advantage for tumor growth and progression in numerous cancers, recent observations have shown that some tumors may use an alternative strategy to block dependence receptor-induced programmed cell death: the autocrine expression of netrin-1. This alternative strategy has been observed in a large fraction of aggressive breast cancers, neuroblastoma, pancreatic adenocarcinoma, and lung cancer. This observation is of potential interest regarding future targeted therapy, as in such cases interfering with the ability of netrin-1 to inhibit DCC or UNC5H-induced cell death is associated with apoptosis of netrin-1-expressing tumor cells *in vitro*, and with inhibition of tumor growth or metastasis in different animal tumor models. The understanding of the mechanism by which netrin-1 inhibits cell death is therefore of interest. Here, we show that netrin-1 triggers the multimerization of both DCC and UNC5H2 receptors, and that multimerization of the intracellular domain of DCC and UNC5H2 is the critical step to inhibit the proapoptotic effects of both of these receptors. Taking advantage of this property, we utilized a recombinant specific domain of DCC that (i) interacts with netrin-1 and (ii) inhibits netrin-1-induced multimerization, to trigger apoptosis in netrin-dependent tumor cells.

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Dependence receptors form a group of receptors that share the ability to induce cell death when expressed in settings in which their trophic ligands are unavailable.<sup>1</sup> The survival of cells that express such receptors is hypothesized to be dependent on the suppression of the receptor-mediated cell death by the receptors' ligands. This functional receptor family includes the common neurotrophin receptor p75<sup>NTR</sup>,<sup>2</sup> the GDNF receptor RET,<sup>3</sup> the Sonic Hedgehog receptor Patched,<sup>4</sup> some integrins,<sup>5</sup> the RGM receptor neogenin,<sup>6</sup> ALK,<sup>7</sup> the NT-3 receptor TrkC,<sup>8</sup> the EphrinB3 receptor EphA4<sup>9</sup> and the netrin-1 receptors, DCC<sup>10</sup> and UNC5H.<sup>11</sup>

Netrin-1 was initially discovered as a laminin-related molecule, produced by the floor plate, that attracts commissural axons as they migrate from the dorsal to the ventral spinal cord during the development of the nervous system.<sup>12,13</sup> It was predicted from genetic screens that the *Caenorhabditis elegans* netrin-1—UNC6—interacted with UNC40 and with UNC5.<sup>14</sup> Four orthologues of UNC5 were identified in mammals: UNC5H1, H2, H3, and H4; and UNC40 was found to be the orthologue of the vertebrate DCC (Deleted in Colorectal Cancer).<sup>15</sup> The DCC gene was initially discovered by Vogelstein and colleagues as a gene located in the minimal region of chromosome 18q, which is deleted in most colorectal cancers.<sup>16,17</sup> The fact that DCC expression is lost in the majority of colorectal cancers, but also in many

other tumors, prompted it to be considered as a tumor suppressor gene.<sup>16</sup> However, although an initial series of reports supported the fact that DCC acted as a tumor suppressor (for a review, see Mehlen and Fearon<sup>17</sup>), doubts have arisen, mainly because of the rarity of point mutations in the DCC coding sequence and because of the lack of tumor predisposition in DCC hemizygous mice.<sup>18</sup> The observation that DCC is a dependence receptor and, as such, is able to trigger cell death in the absence of netrin-1 strengthened the initial hypothesis. Indeed, from this point of view, DCC would act as a tumor suppressor, limiting tumor development by inducing apoptosis of tumor cells that would otherwise proliferate in the environmental settings of netrin-1 absence.<sup>19</sup> Interestingly, the UNC5H netrin-1 receptors were also recently proposed to be tumor suppressors: UNC5H genes were shown to be downregulated in many cancers, especially in colorectal cancer,<sup>20</sup> and inactivation of UNC5H3 in mice is associated with intestinal tumor progression.<sup>21</sup> Moreover, ectopic expression of netrin-1 in the mouse intestine is associated with decreased apoptosis in the intestinal epithelium, as well as tumoral predisposition.<sup>22</sup> Thus, UNC5H (and probably DCC, as well) actually represent a novel class of tumor suppressors—conditional tumor suppressors—which suppress tumor formation in a milieu-dependent manner: in the absence of trophic support from netrin-1, they induce cell death and tumor

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**Abbreviations:** DCC, Deleted in colorectal cancer; CAM, chick chorioallantoic membrane; DCC-EC, complete extracellular domain of DCC

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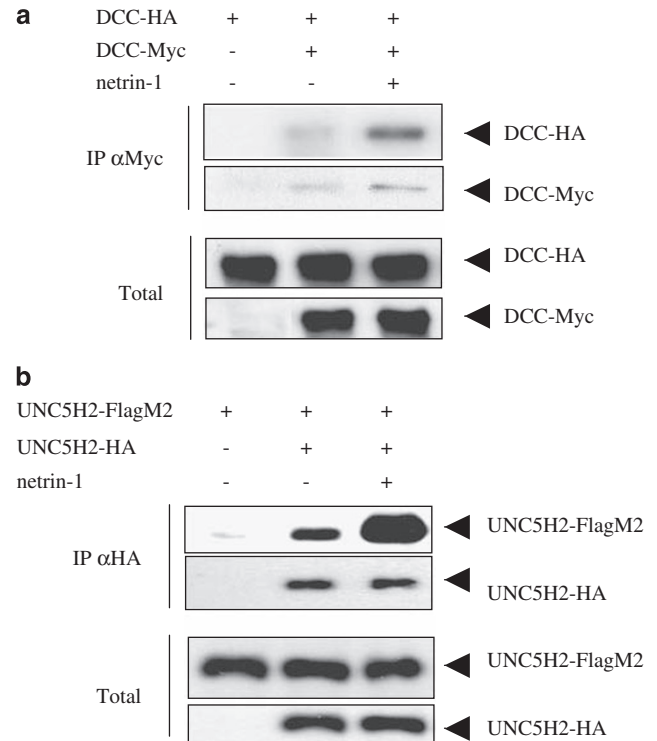
suppression; however, in the presence of high concentrations of netrin-1, they support tumor development.<sup>19,22</sup>

The tumor suppressive effects of UNC5H and DCC, coupled with the observation that their expression is decreased or lost in numerous cancers, raise the possibility that these may be therapeutic targets. Theoretically, tumor development and metastasis can be enhanced either by the loss of the proapoptotic effect of these receptors or by the gain of the ligand's antiapoptotic effect, and both strategies have been observed: in some cancer types—for example, colorectal cancer—netrin-1 receptors expression is lost or markedly decreased; in contrast, in a large fraction of other tumor types, such as metastatic breast cancer,<sup>23</sup> lung cancer,<sup>24</sup> neuroblastoma,<sup>25</sup> and pancreatic adenocarcinoma,<sup>26</sup> we and others have observed that netrin-1 is upregulated. This upregulation of netrin-1 has been shown to confer a selective growth advantage similar to that conferred by the loss of netrin-1 receptors.<sup>23,24</sup> Furthermore, this autocrine production of netrin-1 is a mechanism that lends itself more readily to therapeutic attack than does the receptor loss: indeed, we have found that its inhibition, either by inhibiting netrin-1 production through RNA interference or by extracellular titration of netrin-1, is associated with cell death *in vitro* and tumor growth/metastasis inhibition *in vivo*.<sup>23,25</sup> The search for compounds that may interfere with netrin-1's ability to block DCC/UNC5H-induced cell death is consequently of great potential for the development of alternative anticancer strategies.

The finding that the cognate ligands for DCC, UNC5H, and other dependence receptors block their cell death induction raises the question of how this is achieved mechanistically. DCC has been shown to homomultimerize in the presence of netrin-1, an important feature for the positive signaling of the receptor during axon guidance.<sup>27</sup> Because classic death receptors, such as TNF $\alpha$  or Fas, promote apoptosis in the presence of ligand by homomultimerization, and because dependence receptors have been suggested to mirror death receptors functionally,<sup>28</sup> we analyzed whether netrin-1-induced DCC and/or UNC5H multimerization is a critical step for DCC and/or UNC5H-mediated proapoptotic activity. We show here that DCC and/or UNC5H multimerize in response to netrin-1, and that this event is sufficient to inhibit apoptosis. We also demonstrate that a domain of DCC called DCC-5Fbn that triggers cell death *in vitro* and tumor inhibition in animal models<sup>23,24</sup> does so by interfering with netrin-1-induced multimerization.

## Results and Discussion

To analyze whether DCC is primarily monomeric in the absence of netrin-1, we transiently co-expressed an HA-tagged full-length DCC together with Myc-tagged full-length DCC in HEK293T cells. Immunoprecipitation was then performed using an anti-Myc antibody, and as shown in Figure 1a, despite good expression of both HA and Myc-tagged DCC, DCC-HA was only modestly included in the DCC-Myc pull-down in the absence of ligand, suggesting that DCC was mainly present as a monomer when expressed in HEK293T in the absence of netrin-1. However, under the same experimental conditions, when netrin-1 was added to



**Figure 1** Netrin-1 mediates DCC and UNC5H2 multimerization. **(a)** DCC multimerization in the presence of netrin-1 in HEK293T cells. Lysates of HEK293T cells transiently transfected with DCC-HA and/or DCC-Myc expressing constructs treated or not with recombinant netrin-1 (300 ng/ml) were subjected to Myc pull-down (IP  $\alpha$ Myc). DCC-HA presence was revealed with an anti-HA antibody. Immunoprecipitated DCC-Myc is also shown. As a control, DCC surface expression in the presence or absence of netrin-1 was analyzed by FACs analysis and showed no significant change (not shown). **(b)** UNC5H2 dimerization in the presence of netrin-1 in HEK293T cells. Cell transfection and cell lysate preparation were done as in **(a)** but with UNC5H2-HA and/or UNC5H2-Flag-expressing constructs. Cell lysates were subjected to HA pull-down (IP  $\alpha$ HA). UNC5H2-Flag presence was revealed with an anti-Flag M2 antibody. Immunoprecipitated UNC5H2-HA is also shown. Total: western blot on lysate before pull-down

the culture medium (Figure 1a) or when a netrin-1 expression construct was co-expressed with the DCC-expressing constructs (not shown and Figure 3c), DCC-HA was clearly included in the DCC-Myc pull-down, demonstrating that netrin-1 triggers dimerization or multimerization of DCC. This result is in agreement with data from Tessier-Lavigne and colleagues,<sup>27</sup> who first reported netrin-1-induced multimerization, even though in our culture and immunoprecipitation conditions, DCC displayed a modest level of multimerization in the absence of netrin-1. This constitutive, low multimerization level could either be attributed to the low affinity of DCC receptors for themselves in the absence of ligand or to the system used, which is based on forced expression of high levels of transmembrane receptors.

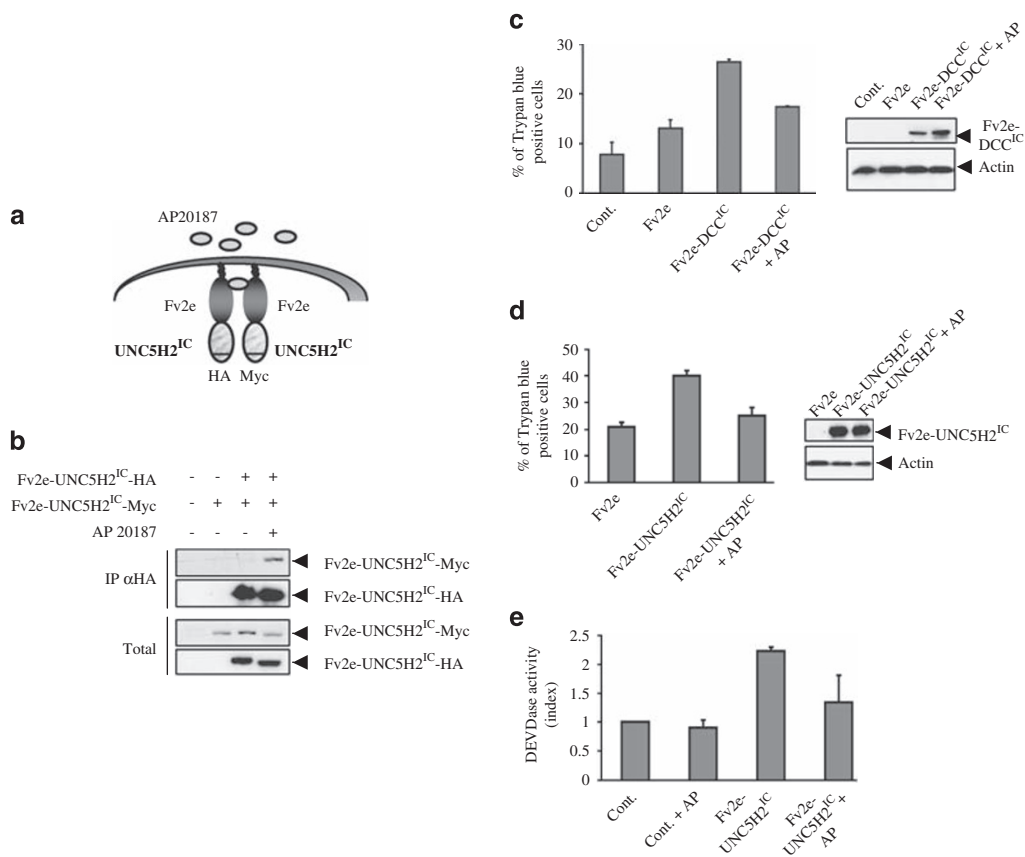
We then investigated whether the other netrin-1 receptors, UNC5H, share a similar behavior. HEK293T cells were transiently transfected with an HA-tagged full-length UNC5H2 together with Flag-tagged full-length UNC5H2 in the presence or absence of netrin-1. Immunoprecipitation was then performed using an anti-HA antibody. As shown in Figure 1b, the presence of netrin-1 triggers an efficient immunoprecipitation of UNC5H2-Flag with UNC5H2-HA.

Thus, although in the absence of netrin-1, DCC and UNC5H2 are mainly monomeric, both DCC and UNC5H2 show an increased propensity to multimerize in the presence of netrin-1.

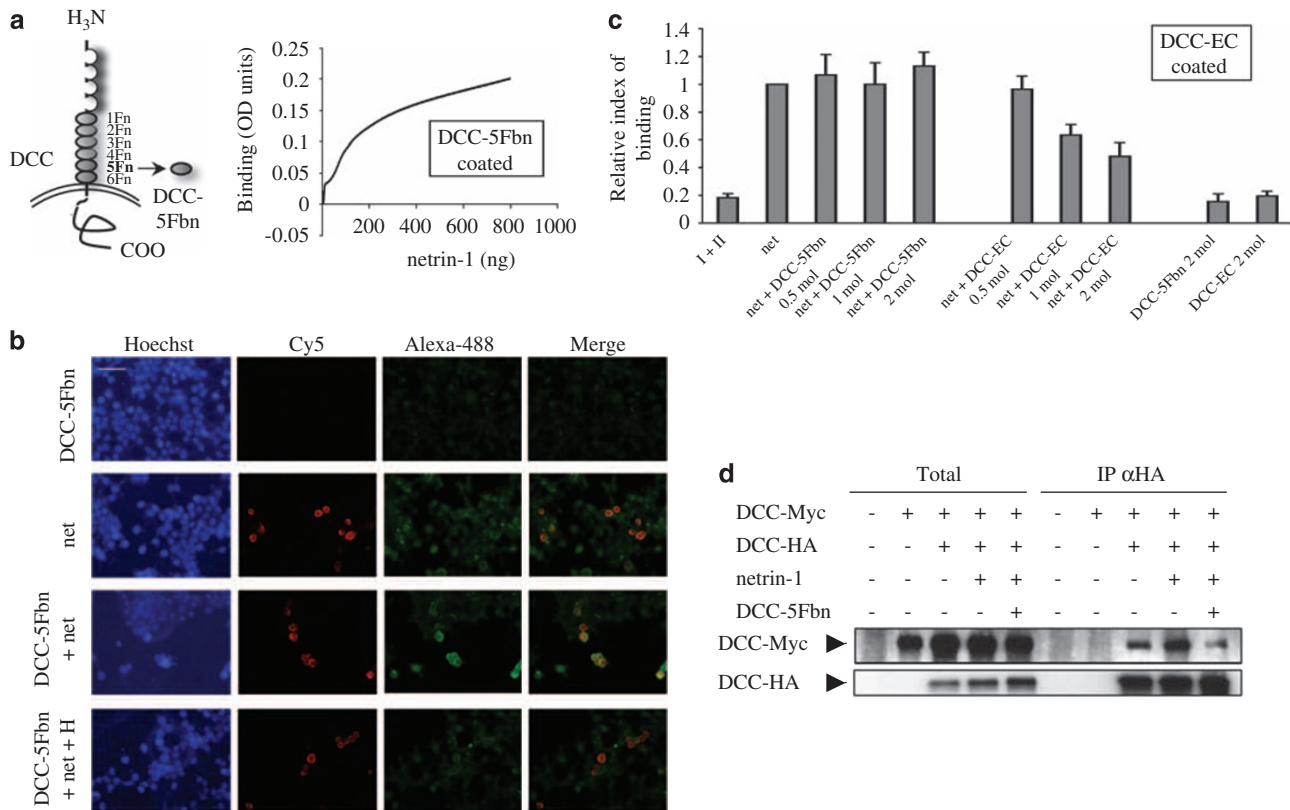
To determine whether netrin-1-induced multimerization is the crucial step for inhibiting DCC/UNC5H2 proapoptotic cell death, we developed a chimeric system in which protein dimerization can be induced by a chemical agent. This system was successfully used to show both the role of caspase-8 dimerization in caspase-8 activation<sup>29</sup> and the importance of p75<sup>ntr</sup>-multimerization in blocking p75<sup>ntr</sup> proapoptotic activity.<sup>28</sup> This system is derived from the ability of the Fk1012 compound to cross-dimerize the FkBP motif. DCC and UNC5H2 intracellular domains were fused in their N-termini to derived Fv2e FkBP motifs, and dimerization was induced using the AP20187 chemical compound (Figure 2a). We first analyzed whether the developed system recapitulates netrin-1-induced multimerization of the UNC5H2 intracellular domain. HEK293T cells were co-transfected with

an HA-tagged Fv2e-UNC5H2<sup>IC</sup> together with Myc-tagged Fv2e-UNC5H2<sup>IC</sup> and co-immunoprecipitations were performed using an anti-HA antibody. As shown in Figure 2b, without addition of AP20187, Fv2e-UNC5H2<sup>IC</sup>-Myc was barely detectable in the Fv2e-UNC5H2<sup>IC</sup>-HA pull-down, supporting the notion that Fv2e-UNC5H2<sup>IC</sup> is expressed in HEK293T cells mainly as a monomer. As expected, the addition of AP20187 led to the efficient pull-down of Fv2e-UNC5H2<sup>IC</sup>-Myc with Fv2e-UNC5H2<sup>IC</sup>-HA. Similar results were obtained with Fv2e-DCC<sup>IC</sup> (not shown). Thus, this dimerization system recapitulates the dimerization of the intracellular domain of the netrin-1 receptors DCC and UNC5H2.

Because this chemically inducible DCC/UNC5H2 dimerization system appears to work adequately to mimic netrin-1-induced DCC/UNC5H2 multimerization, we then assessed whether the dimerization of DCC/UNC5H2 was sufficient to inhibit DCC/UNC5H2 proapoptotic activity. HEK293T cells



**Figure 2** Forced DCC (vs UNC5H2) dimerization blocks DCC (UNC5H2) proapoptotic activity. **(a)** Schematic representation of Fv2e-UNC5H2<sup>IC</sup> fusion constructions showing the two constructs (one tagged HA, the other one tagged Myc) used to validate the artificial dimerization system. **(b)** Lysates of HEK293T cells transiently transfected with Fv2e-UNC5H2<sup>IC</sup> tagged HA or Myc with or without the dimerization drug (AP20187, 10 nM) were subjected to HA pull-down (IP αHA). Total: Western blot on lysate before pull-down. Fv2e-UNC5H2<sup>IC</sup>-Myc presence was revealed with anti-Myc antibody. Immunoprecipitated Fv2e-UNC5H2<sup>IC</sup>-HA is also shown. **(c)** DCC-induced cell death is inhibited by dimerization induced by AP20187, as measured by Trypan blue exclusion. HEK293T cells were transfected with mock plasmid (Cont.), Fv2e, Fv2e-DCC<sup>IC</sup> with or without 10 nM AP20187 (AP). In all conditions, cells were also transfected with the surface marker pK<sup>K</sup>. Transfected cells expressing the marker were magnetically labeled with MACSelect Microbeads and separated using a MACS Separator and Separation Columns. Trypan blue exclusion was assayed on these purified cells. A western blot was performed using anti HA antibody. Actin loading control is also shown. **(d)** UNC5H2-induced cell death is inhibited by dimerization induced by AP20187, as measured by Trypan blue exclusion as in **(c)**. Cells were transfected with pMACSKk and Fv2e, Fv2e-UNC5H2<sup>IC</sup> with or without AP20187 (AP). A western blot was carried out using anti-HA antibody. Actin loading control is also shown. **(e)** UNC5H2-induced caspase activation is inhibited by dimerization induced by AP20187, as measured by relative DEVDase activity. HEK293T cells were transfected with mock vector pCMV (Cont.), Fv2e, Fv2e-UNC5H2<sup>IC</sup> with or without 10 nM AP20187 (AP). Index of relative caspase activity is presented as the ratio between the caspase activity of the sample and that measured in HEK293T cells transfected with pCMV. Standard deviations are indicated ( $n = 3$ )



**Figure 3** The recombinant soluble fifth fibronectin domain of DCC (DCC-5Fbn) inhibits netrin-1-induced DCC-multimerization. **(a)** Scheme showing DCC-5Fbn as one of the six fibronectin domain (Fn) of DCC. Affinity curve of netrin-1 on DCC-5Fbn measured by ELISA test shows that DCC-5Fbn is able to bind netrin-1. DCC-5Fbn (100 ng) or IL3-R (600 ng) was coated and increasing doses of netrin-1 were added (0–800 ng). The values obtained with IL3 were subtracted from the one obtained with DCC-5Fbn. The approximate Kd of DCC-5Fbn/netrin-1 was estimated at 5 nM. **(b)** DCC-5Fbn colocalizes with secreted netrin-1. HEK293T cells were transfected or not with Myc-tagged netrin-1 and treated or not with FlagM2-tagged DCC-5Fbn (DCC-5Fbn) with or without addition of Heparinase (H). Addition of Heparinase disrupted DCC-5Fbn colocalization with netrin-1. Representative fields of netrin-1 immunohistochemistry (Cy5) and DCC-5Fbn (Alexa-488) are presented. Superimposed photographs (Merge) are shown. Hoechst staining shows cell nuclei. Scale bar represents 100  $\mu$ M. **(c)** Competition assay. As in **(a)** but the complete extracellular domain of DCC (DCC-EC, 150 ng) was coated instead of DCC-5Fbn and netrin-1 (net) was added (50 ng) in the presence of increasing concentrations of either DCC-5Fbn or the complete DCC-EC. Note that DCC-5Fbn failed to compete with DCC/netrin-1 interaction. I + II (primary and secondary antibody), DCC-5Fbn 2 mol, DCC-EC 2 mol: controls performed in the absence of netrin-1. **(d)** Netrin-1-induced DCC multimerization is inhibited by DCC-5Fbn. Lysates of HEK293T cells transiently transfected with DCC-HA and/or DCC-Myc and/or netrin-1 (1/3 of total DNA) expressing constructs with or without DCC-5Fbn (900 ng/ml) were subjected to HA pull-down (IP  $\alpha$ HA). DCC-Myc presence was revealed with anti-Myc antibody. Anti-HA immunoblot was also performed to detect DCC-HA precipitate. Total: western blot on lysate before pull-down

were forced to express Fv2e-DCC<sup>IC</sup> in the presence or absence of AP20187, and cell death was assessed by Trypan blue staining, as previously described, to measure DCC-induced cell death.<sup>10,30</sup> As shown in Figure 2c, expression of Fv2e-DCC<sup>IC</sup> was associated with increased cell death compared with the expression of the Fv2e motives without the DCC-IC fusion. Interestingly, when AP20187 was added, cell death induced by Fv2e-DCC<sup>IC</sup> was reduced (Figure 2c). Similarly, although Fv2e-UNC5H2<sup>IC</sup> triggered cell death (Figure 2d) and caspase activation (Figure 2e) when expressed in HEK293T in the absence of AP20187, the addition of the dimerizing drug was sufficient to reduce significantly Fv2e-UNC5H2<sup>IC</sup>-induced cell death (Figure 2d) and caspase activation (Figure 2e). Thus, although monomeric DCC-IC and UNC5H2-IC were proapoptotic, the multimeric forms of DCC-IC or UNC5H2-IC did not display proapoptotic activity. Therefore, the ability of netrin-1 to inhibit DCC/UNC5H2 proapoptotic activity is intrinsically linked to the ability of netrin-1 to multimerize DCC or UNC5H2, as this multimerization process is sufficient to block DCC and UNC5H2 proapoptotic activity.

Although the mechanisms underlying netrin-1-induced receptor multimerization are yet to be described, the observation that netrin-1-induced DCC/UNC5H2 multimerization is sufficient to inhibit DCC/UNC5H2-induced cell death may represent an interesting tool to turn on DCC or UNC5H proapoptotic activity *in vivo*, in tumors expressing netrin-1 in an autocrine manner. Of relevance to this goal, we have demonstrated that netrin-1 overexpression in mouse gastrointestinal tract is associated with intestinal tumor development because of apoptosis inhibition,<sup>22</sup> and we and others have recently observed that netrin-1 is overexpressed in a large fraction of metastatic breast cancers,<sup>23</sup> lung cancer,<sup>24</sup> aggressive neuroblastoma,<sup>25</sup> and pancreatic cancers.<sup>26</sup> Therefore, the inhibition of DCC/UNC5H-induced dimerization might potentially represent a method to trigger tumor cell apoptosis.

To evaluate this possibility, we used the fifth fibronectin domain of DCC, which has been shown to be a domain of interaction with netrin-1 (Figure 3a and Geisbrecht *et al.*<sup>31</sup>), even though conflicting data have also been reported.<sup>32</sup> We

thus first assessed whether a recombinant soluble fifth fibronectin domain of DCC (DCC-5Fbn) could bind to recombinant netrin-1. ELISA assay demonstrated that DCC-5Fbn specifically binds to netrin-1, as opposed to the extracellular domain of an unrelated receptor, IL3-R (Figure 3a). The approximate K<sub>d</sub> for DCC-5Fbn/netrin-1 was estimated to be approximately 5 nM, in keeping with the order of magnitude of the described DCC/netrin-1 K<sub>d</sub>. To further analyze the specificity of the DCC-5Fbn interaction with netrin-1, we next performed a colocalization study of netrin-1 with DCC-5Fbn in netrin-1 transfected HEK293T cells. Although netrin-1 is known to be secreted, a large fraction is bound to the plasma membrane probably because of its binding to heparin.<sup>12</sup> Of interest, netrin-1 and DCC-5Fbn nicely colocalize at the plasma membrane but both netrin-1 and DCC-5Fbn immunostaining disappear upon heparinase treatment (Figure 3b). Thus, DCC-5Fbn specifically interacts with secreted netrin-1. We next investigated whether this domain was sufficient to displace DCC/netrin-1 interaction. As shown in Figure 3c, using an ELISA assay in which the extracellular domain of DCC was coated and netrin-1/DCC interaction was detected by netrin-1 immunoreactivity, we observed that, while as a positive control, the complete extracellular domain of DCC (DCC-EC) was sufficient to displace DCC/netrin-1 interaction, DCC-5Fbn failed to interfere. Thus, DCC-5Fbn interacts with netrin-1 but is not sufficient to inhibit DCC/netrin-1 interaction. We next investigated whether DCC-5Fbn could influence DCC multimerization. We performed co-immunoprecipitation in HEK293T transiently transfected with HA-tagged full-length DCC together with Myc-tagged full-length DCC in the presence or absence of netrin-1. As shown in Figure 3d (also in Figure 1a), the presence of netrin-1 triggered the immunoprecipitation of DCC-Myc with DCC-HA, demonstrating netrin-1-induced DCC multimerization. However, when the cells incubated with netrin-1 were also simultaneously treated with DCC-5Fbn, the DCC-HA/DCC-Myc interaction returned to netrin-1 untreated levels (Figure 3d). Thus, DCC-5Fbn interacts with netrin-1 and inhibits netrin-1-induced DCC-multimerization.

We therefore tested whether the ability of DCC-5Fbn to inhibit netrin-1-induced DCC-multimerization could trigger apoptosis in cells expressing netrin-1 and its receptors. To this end, HEK293T cells were transfected to express DCC in the presence or absence of netrin-1, with or without DCC-5Fbn, and cell death was determined by Trypan blue exclusion assay (Figure 4a) or caspase assay (not shown). As shown in Figure 4a, although DCC failed to trigger cell death in the presence of netrin-1, the addition of DCC-5Fbn was sufficient to block the inhibitory activity of netrin-1, thus leading to DCC-induced cell death. A similar effect was observed with UNC5H2 when cell death was measured by TUNEL staining (Figure 4b). Thus, DCC-5Fbn interfered with netrin-1-mediated receptor multimerization, and triggered cell death.

DCC-5Fbn not only triggered apoptosis in HEK293T cells ectopically expressing DCC/UNC5H2, but also was active in a more pathophysiologically relevant setting: previously we reported that DCC-5Fbn not only induces apoptosis of endogenously netrin-1 expressing metastatic breast cancer lines and non-small-cell lung cancer cell lines or neuroblastoma cell lines *in vitro*, but also inhibits tumor growth

and metastasis in different animal models.<sup>23–25</sup> Similarly, the colorectal cancer cell line, HCT116, expresses netrin-1 receptors and a high level of netrin-1 at the RNA level (Figure 5a), which is associated with the secretion of netrin-1 protein, as shown by netrin-1 immunohistochemistry performed on non-permeabilized cells (Figure 5b). When these HCT116 cells were treated with DCC-5Fbn, apoptotic cell death was induced, as measured by Trypan blue exclusion and caspase-3 activity assays (Figure 5c); furthermore, this death effect was specifically related to an inhibition of netrin-1 activity, as the addition of recombinant netrin-1 in excess blocked DCC-5Fbn-mediated killing. To provide further support for the *in vivo* efficacy of DCC-5Fbn to limit tumor progression,<sup>23–25</sup> HCT116 cells were grafted onto the chorioallantoic membrane (CAM) of 10-day-old chick embryos, a model that has been shown to recapitulate both tumor growth at a primary site—that is, within the CAM—as well as tumor invasion and dissemination at a secondary site—metastasis to the lung.<sup>25</sup> HCT116 cells grafted-embryos were treated on day 10 and day 13 with PBS or DCC-5Fbn, and 17-day-old chicks were analyzed for primary tumor size and metastases in the lung. Interestingly, although DCC-5Fbn failed to have any significant effect on primary tumor size (not shown), DCC-5Fbn dramatically reduced lung metastasis formation (Figure 5d).

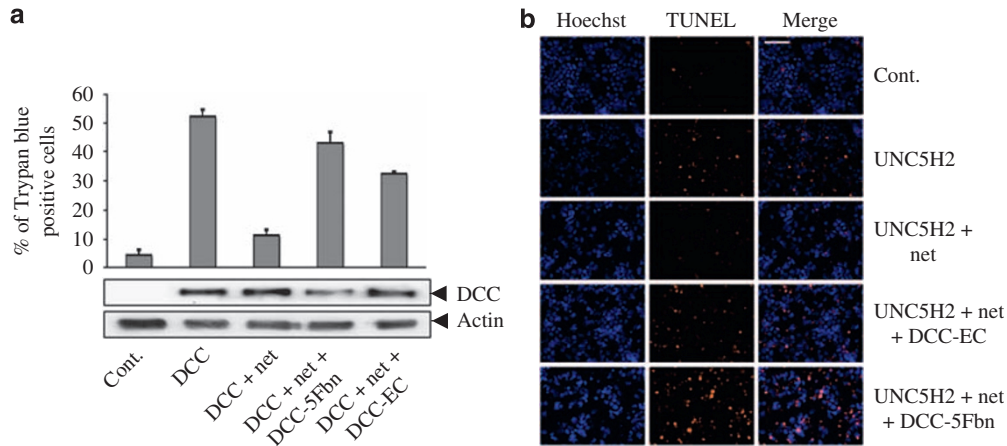
Taken together, these results demonstrate that the multimerization of the dependence receptors DCC and UNC5H is a sufficient mechanism to block their proapoptotic activity. Interestingly, this inhibitory mechanism appears to mirror what is observed with death receptors. Indeed, it is known that TNF $\alpha$  or Fas requires trimerization to induce apoptosis.<sup>33</sup> This intrinsic difference may therefore suggest a therapeutic strategy that uses dependence receptors. Indeed, the search for therapeutic molecules in the past has mainly led to hits that act on the inhibition of cellular processes – for example, kinase inhibitors, IAP inhibitors – rather than activators. As a consequence, inhibition of netrin-1 receptors' multimerization by the use of recombinant DCC-5Fbn or by any compound screened to interfere with receptor multimerization appears to be a tempting strategy for the treatment of cancers, in which netrin-1 autocrine expression has been acquired.

## Materials and Methods

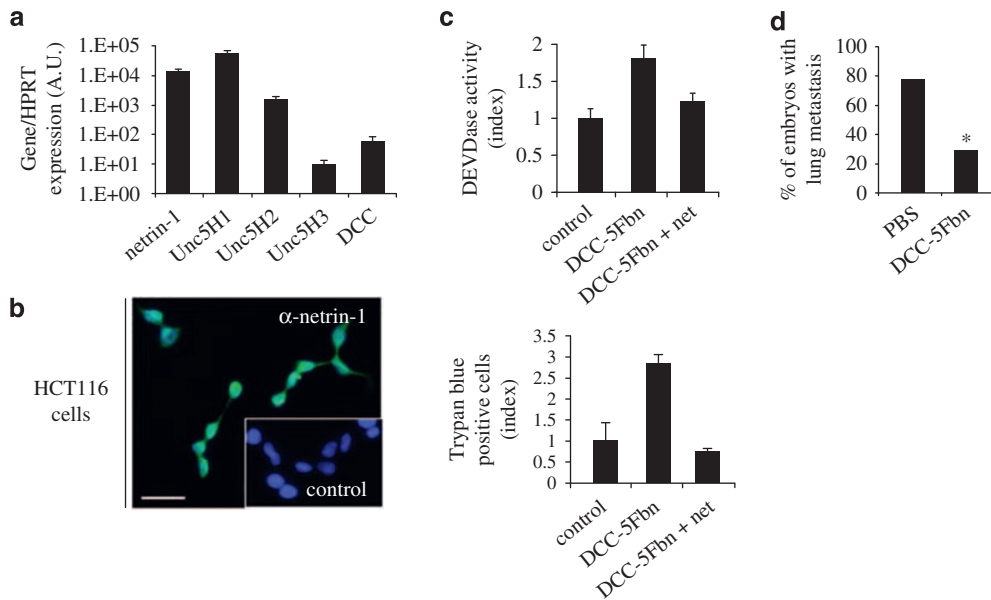
**Cell cultures, transfection procedures, reagents, and immunoblots.** Transient transfections of human embryonic kidney 293T cells (HEK293T) were performed as previously described,<sup>4</sup> according to a modified calcium phosphate procedure or using Lipofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Immunoblots were performed as described previously<sup>10</sup> using anti-Myc (Sigma, St Louis, MO, USA, 1/1000), anti-FlagM2 (Sigma, Santa Clara, CA, USA, 1/10000), anti-HA (Sigma, 1/5000), anti-DCC Ab20 (Tebu, Santa Cruz, CA, USA; 1/1000). The artificial dimerizing agent AP20187 was from Ariad Pharmaceuticals, Cambridge, MA, USA. The DCC-EC was purchased from R&D system, Minneapolis, MN, USA. Netrin-1 (with a FlagM2 tag) was from Apotech, Epalinges Switzerland. For cell death analysis, caspase activity measurement and immunoprecipitation, AP20187 was used at a final concentration of 10 nM, netrin-1 was used at a final concentration of 300 ng/ml.

**Site-directed mutagenesis and plasmid construction.** pCMV and pGNET1 were described previously.<sup>10</sup> pK<sup>K</sup> was described by Llambi *et al.*<sup>34</sup> DCC-HA was obtained by introducing an HA tag in the template pCMV-DCC (described by Mehlen *et al.*<sup>10</sup>) by QuikChange site-directed mutagenesis system (Stratagene, Santa Clara, CA, USA) using the following primers: DCC-HA F:





**Figure 4** DCC-5Fbn triggers cell death by disruption of netrin-1-mediated inhibition of DCC/UNC5H2 proapoptotic activity. **(a)** HEK293T cells were transiently transfected with a mock (Cont.) or a full-length DCC construct (DCC) and incubated with or without netrin-1 (net) (150 ng/ml) and/or DCC-5Fbn (800 ng/ml) or the complete extracellular domain of DCC (DCC-EC) (200 ng/ml). Cell death was assessed by Trypan blue staining. Inset: DCC immunoblot showing DCC expression level in the different tested conditions. Actin loading control is also shown. **(b)** HEK293T cells were transiently transfected with a mock (Cont.) or a full-length UNC5H2 construct (UNC5H2) and incubated with or without netrin-1 (net) (500 ng/ml) and/or DCC-5Fbn (1000 ng/ml) or the complete extracellular domain of DCC (DCC-EC) (200 ng/ml). Cell death was measured by fluorescent TUNEL staining. Hoechst staining shows cell nuclei. For each condition, at least five randomly chosen fields were analyzed and photographed under epifluorescence microscopy. A representative field is presented from three independent experiments. Superimposed photographs (Merge) are shown. Scale bar represents 100  $\mu$ M



**Figure 5** DCC-5Fbn triggers HCT116 cell death *in vitro* and inhibits HCT116 cell metastasis in a chicken model. **(a)** Quantification of netrin-1 and netrin-1 receptors. Ratio of netrin-1 and netrin-1 receptors expression to HPRT housekeeping gene is presented. **(b)** Representative netrin-1 immunohistochemistry on HCT116 tumor cell line. Inset: Control without primary antibody is presented. Scale bar represents 50  $\mu$ M. **(c)** Quantitative analysis of cell death in HCT116 cell line treated with DCC-5Fbn, with or without addition of netrin-1 in excess to reverse the effect of DCC-5Fbn. Cell death was quantified by Trypan blue exclusion assay (below panel), while apoptosis was monitored by measuring relative DEVdase activity (upper panel). **(d)** HCT116 cells were grafted in chick chorioallantoic membrane at day 10 and DCC-5Fbn or PBS was injected on days 10 and 13. Tumors and lungs were harvested on day 17. Percentage of embryos with lungs invaded by human HCT116 cells after two injections (days 10 and 13) of either DCC-5Fbn or PBS is shown. \*Indicates a  $P < 0.05$  calculated using a  $\chi^2$ -test

5'-CACAGGCTCAGCCTTTTATCCATATGATGTACCGGATTATGCATAACATGTA TTTCTGAATG-3', DCC-HA R: 5'-CATTTCAGAAATACATGTTATGCATAATCCG GTACATCATATGGATAAAAGGCTGAGCCTGTG-3'. DCC-Myc was also obtained by introducing a Myc tag in the template pCMV-DCC by QuikChange using the following primers: DCC-Myc F: 5'-CACAGGCTCAGCCTTTGAGCAGAAGT TGATAAGTGAGGAAGATCTGTAACATGATTTCTGAATG-3'. DCC-Myc R: 5'-C ATTCAGAAATACATGTTACAGATCTTCTCACTTCTCAACTTCTGCTCAAAGGC TGAGCCTGTG-3'. Fv2e-HA encoding expression vector (in pC4M) from the Argent Regulated Homodimerization kit is from Ariad Pharmaceuticals. From this plasmid,

the Fv2e-DCC<sup>IC</sup>-HA plasmid was constructed. A PCR fragment of the intracellular domain of DCC (1122–1447) was obtained with the primers: F: 5'-TAT GTCGACCGACGCTCTTCAGCCAGCAGAGA-3' and R: 5'-TATGAATTCCTAG TCGAGTGCCTAGTCTGGTACGTCGTACGGATAAAAGGCTGAGCCTGTGATG GCATTAAG-3'. The reverse primer fused the HA tag to C-terminal end of DCC. The PCR fragment was subcloned in HA-Fv2e by *Sa*I and *Eco*RI restriction digestion. The Fv2e-DCC<sup>IC</sup>-Myc was obtained using the QuikChange site-directed mutagenesis system (Stratagen) with pC4M-Fv2e-DCC<sup>IC</sup>-HA as template and the following primers: primer F: 5'-CTTAATGCCATCACAGGCTCAGCCTTGA

ACAGAACTCATCTCTGAAGAGGATCTGAAGAATTCATAAAGGGCAAT-3' and primer R: 5'-ATTGCCCTTTATGAATTCCTACAGATCCTCTTCAGAGATGAGTTTC TGTTCAAAGGCTGAGCCTGTGATGGCATTAAAG-3'. UNC5H2-HA (in pcDNA3.1) has already been described;<sup>11</sup> the constructs encoding UNC5H2-FlagM2 was generated by cloning in p3xFlag-CMV-7.1 (Sigma) the *NotI*-*EcoRI* PCR fragment derived from UNC5H2-HA as template and the following primers: primer F: 5'-GCGCGCCGAGGGCCGAGCGGG-3' and primer R: 5'-CGGAA TTCTCAGCAATCGCCATCAGTGGTC-3'. Fv2e-UNC5H2<sup>IC</sup>-HA and Fv2Ee-UNC5H2<sup>IC</sup>-Myc in pC4M were generated by PCR amplification of the UNC5H2 intracellular domain using the following primers: UNC5H2-HA F: 5'-CGGTC GACGTGTACCGGAGAACTGC-3' and UNC5H2-HA R: 5'-GCGAATT CTCATGCATAATCCGGACATCATACGGATAGCAATCGCCATCAGTGGTC-3', and UNC5H2-Myc F: 5'-CGGTCGACGTGTACCGGAGAACTGC-3' and UNC5H2-Myc R: 5'-GCGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTG TTCGAATCGCCATCAGTGGTC-3', respectively. The PCR fragments were cloned in Fv2e-HA by *SalI* and *EcoRI* restriction digestion. The cDNA encoding the Fv2e-UNC5H2<sup>IC</sup>-HA and Fv2e-UNC5H2<sup>IC</sup>-Myc fusion proteins were then subcloned in pcDNA3.1-TOPO by PCR using the following primers: Fv2e F: 5'-CCACCATGGGGAGTAGCA-3' and UNC5H2-HA R: 5'-TCATGCAT AATCCGGACATCATACGGATAGCAATCGCCATCAGTGGTC-3', and Fv2e F: 5'-CCACCATGGGGAGTAGCA-3' and UNC5H2-Myc R: 5'-TCACAG ATCCTCTTCTGAGATGAGTTTTGTCGAATCGCCATCAGTGGTC-3', respectively and Fv2e-UNC5H2<sup>IC</sup>-HA and Fv2e-UNC5H2<sup>IC</sup>-Myc in pC4M as respective templates. pLIM01-DCC-5Fbn-His allowing bacterial expression of the fifth fibronectin type III domain of DCC was carried out by M Noirclerc-Savoie and B Gallet from the laboratory RoBioMol at the Institut de Biologie Structurale, Grenoble. This plasmid was created by using the Ligation Independent Cloning strategy (Novagen, San Diego, CA, USA). A PCR fragment of DCC-5Fbn was produced using plasmid pQE-Flag-DCC-5Fbn as template. The PCR product was inserted into pLIM01 by spontaneous hybridization. The pQE-Flag-DCC-5Fbn construct was carried out by Apotech by inserting human DCC-5Fbn fragment in *PstI* and *BamHI* sites in plasmid pQE-Flag.

**DCC-5Fbn production.** DCC-5Fbn production was performed using a standard procedure. Briefly, BL21 cells were forced to express DCC-5Fbn in response to IPTG and the BL21 lysate was subjected to affinity chromatography using His purification on Ni-NTA columns (Qiagen, Courtaboeuf, France) or Flag-agarose (Sigma) to respectively obtain His-tagged DCC-5Fbn or Flag-tagged DCC-5Fbn.

**Immunoprecipitation.** Co-immunoprecipitations were carried out on HEK293T cells transfected with various tagged constructs as described previously.<sup>30</sup> Briefly, HEK293T cells were lysed in 50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM EDTA, and 0.1% NP-40 in the presence of protease inhibitor and sonicated 2 pulses of 5 s, and further incubated with anti-HA (Sigma), anti-Myc antibody (Sigma), anti-FlagM2 (Sigma) and protein-A Sepharose (Sigma). Washes were performed in 50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM EDTA.

**Binding assay and ELISA competition assay.** DCC-5Fbn (100 ng) or IL3-R (R&D systems, 600 ng) was coated on maxisorp plate (Nunc, Rochester, NY, USA) and increasing doses of netrin-1 (Apotech) were added (0–800 ng) for binding assay. DCC-EC (R&D systems, 150 ng) was coated on maxisorp plate for ELISA competition assay. Netrin-1 (Apotech, 50 ng) and competitor DCC-EC or DCC-5Fbn were then added simultaneously in increasing concentration. After washes, for both binding assay or ELISA competition assay, residual netrin-1 still fixed was revealed with an anti-netrin-1 antibody (R&D systems) and OPD revelation (Sigma).

**Cell death analysis and caspase activity measurement.** Cell death was analyzed 48 h after transfection using Trypan blue staining procedures as described previously.<sup>10</sup> To select transfected cells, cells were co-transfected with the surface marker pK<sup>k</sup> and the plasmid encoding genes of interest. Transfected cells expressing the marker were magnetically labeled with MACSelect Microbeads and separated using a MACS Separator and Separation Columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Trypan blue exclusion was assayed on these purified cells. Caspase-3 activity was measured by using the Caspase-3 assay from BioVision, Mountain View, CA, USA. Caspase activity is presented as the ratio between the caspase activity of the sample and that measured in HEK293T cells transfected with pCMV. For cell death analysis and caspase activity measurement, AP20187 or/and netrin-1 or/and DCC-5Fbn were added in cell culture medium 20

and 1 h before collecting cells. TUNEL staining was carried out as previously described<sup>10,30</sup> but with fluorescent staining using streptavidine-conjugated Cy3 antibody (Jackson ImmunoResearch, Suffolk, UK; 1/1000) and Hoechst (Sigma, 1/1000).

**Immunohistochemistry.** HEK293T were transfected with Myc-tagged netrin-1 for 48 h. Cells were treated 24 h before harvesting by DCC-5Fbn (1  $\mu$ g/ml) and 1 h before with or without Heparinase III (Sigma, 2  $\mu$ g/ml). Cells were fixed in PFA 4% (30 min), permeabilized by PBS Triton 0.2% (20 min) and incubated for saturation in PBS-BSA 2%-Normal donkey serum 2% (2 h). Primary antibodies were added overnight (Myc-rabbit Sigma, 1/400; FlagM2-mouse Sigma, 1/400) and secondary antibodies were added for 1 h (Cy5-Donkey anti-rabbit, Jackson ImmunoResearch, 1/450; Alexa 488-Donkey anti mouse, Jackson ImmunoResearch, 1/450, respectively). Immunohistochemistry on HCT116 cells was performed as for HEK293T cells with netrin-1 primary antibody (R&D System, 1/150) for 2 h and Alexa 488-Donkey anti-rat secondary antibody (Jackson ImmunoResearch, 1/450) for 1 h.

**Metastasis quantification in chicken model.** HCT116 colorectal tumoral cells ( $2 \times 10^7$ ) were seeded on 10-day-old (day 10) chick CAM. HCT116 cells were suspended in 100  $\mu$ l of either DCC-5Fbn solution (0.05  $\mu$ g/ $\mu$ l) or PBS. A second injection of DCC-5Fbn or PBS was performed on the tumor at day 13 (100  $\mu$ l of DCC-5Fbn 0.05  $\mu$ g/ $\mu$ l or 100  $\mu$ l of PBS). To assess metastasis, the lungs were harvested from the tumour-bearing embryos and genomic DNA was extracted with the NucleoSpin Tissue kit (Macherey Nagel, Duren, Germany). Metastasis was quantified by PCR-based detection of the human Alu sequences using the primers 5'-ACG CCTGTAATCCCAGCACTT-3' (sense) and 5'-TCGCCAGGCTGGAGTGCA-3' (antisense) and the chick glyceraldehyde-3-phosphate dehydrogenase-specific primers (sense, 5'-GAGGAAAGGTTCGCCTGGTGGATCG-3'; antisense, 5'-GGTG AGGACAAGCAGTGAGGAACG-3') as controls. For both couples of primers, metastasis was assessed by polymerase activation at 95 °C for 2 min followed by 30 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. Genomic DNA extracted from the lungs of healthy chick embryos were used to determine the threshold between HCT116 cells invaded and non-invaded lungs.

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