SI Materials and Methods

Cell Line, Transfection procedure and Reagents. 4T1 and 67NR cells were a kind gift from F. Miller (Karmanos Cancer Institute, Wayne State University, Detroit, MI). Human breast cell lines listed were cultured using standard procedures and were obtained from D. Birnbaum (Centre de Recherche en Cancérologie, INSERM UMR 599, Marseille 13009, France). 67NR and 4T1 cells were stably transfected using the lipofectamine reagent (Invitrogen) and puromycine (Sigma) selection. 4T1-luc cells were obtained by stable transfection of a CMV-luciferase vector bearing hygromycine resistance. Clones were selected by luminescence intensity using the luminoskan Ascent Station (Labsystems). DCC-EC-Fc was obtained from R & D Systems and Netrin-1 and UNC5H2-EC-Fc from Apotech /Axxora.

Human Breast Tumor Samples. Fifty-one human breast cancer samples were provided by the tumor bank of the Centre Léon Bérard. Fresh tissue of the tumor was obtained during breast surgery prior any systemic therapy and snap-frozen in liquid nitrogen.

Plasmid Constructs, siRNA, and DCC-5Fbn Production. The dominant-negative mutant for UNC5H (pCR-UNC5H2-IC-D412N) has been described (1). PGNET-1, encoding chick netrin-1, has been described (2). Ps974-DCC-5Fbn allowing bacterial expression of the fifth fibronectin type III domain of DCC was obtained by inserting a

Pst1/BamH1 DNA fragment generated by PCR using pDCC-CMV-S as a template. DCC-5Fbn production was performed using a standard procedure. Briefly, BL21 cells were forced to express DCC-5Fbn in response to imidazole and the BL21 lysate was subjected to affinity chromatography using Flag-sepharose (Sigma). GST-DCC-5Fbn and GST-FaDD were from Apotech /Axxora. For cell culture use, scramble and netrin-1 siRNAs were designed by Santa Cruz Biotechnology as a pool of three target-specific 20-25nt siRNAs. For the *in vivo* approach two different single netrin-1, DCC, UNC5H1, or UNC5H2 siRNAs were produced (Sigma-Proligo).

DCC/Netrin-1 ELISA Assays. DCC-EC-Fc (1.25ng/ml) or UNC5H2-EC-Fc (0.5ng/ml) was adsorbed on 96-well maxisorp plate (Nunc) according to manufacturer instruction. Flag-tagged Netrin-1 (0.5ng/ml) was then added together with increased concentrations of DCC-EC-Fc. After a 1-hour incubation, plates were extensively washed, and bound netrin-1 was detected by immunolabelling using an anti-flagM2 antibody (Sigma) and a HRP-goat-anti-mouse (Jackson). Colorimetric measurement was performed on the multilabel Victor station (Wallac).

Immunoprecipitation. Coimmunoprecipitations were carried out on HEK293T cells transfected with various tagged constructs as described previously (3). Briefly, HEK293T cells were lysed in 50 mM HEPES pH 7.6, 125 mM NaCl, 5 mM EDTA and 0.1% NP-40 in the presence of protease inhibitor, and further incubated with anti-HA (Sigma), anti-c-myc antibody (Sigma), anti-FlagM2 (Sigma) and protein-A Sepharose (Sigma). Washes were done in 50 mM HEPES pH 7.6, 125 mM NaCl, 5 mM EDTA.

Cell Death Assays. 67NR, 4T1, T47D, SKBR7 or MDAMB231 cells were grown in serum-poor medium and were treated (or not) with DCC-EC-Fc or DCC-5Fbn or transfected with siRNA using Lipofectamine 2000 (Life Technologies). Cell death was analyzed using trypan blue staining procedures as described (2). The extent of cell death is presented as the percentage of trypan blue-positive cells in the different cell populations. Cell survival was also measured 48h post-transfection/treatment by MTT assay using Vybrant MTT assay kit (Molecular Probes) according to the manufacturer procedures. Relative Caspase activity was determined by flow cytometric analysis as follows: 2×10^5 treated cells were harvested, washed once in 1 ml of PBS, and resuspended in 200μl of staining solution containing FITC-VAD-fmk (CaspACE, Promega). After incubation for 60 min at 37°C, cells were washed in 1 ml PBS and resuspended in 300 μl PBS for flow cytometry analysis. Stained cells were counted using a FACS Calibur (Becton Dickinson) and CellQuest analysis software with excitation and emission settings of 488 nm and 525–550 nm (filter FL1), respectively. Caspase activity was also measured as described previously (2) using ApoAlert CPP32 kit from Clontech.

Quantitative RT-PCR. To assay netrin-1, DCC, UNC5H receptors expression in human breast tumors, total RNA was extracted from biopsies of patients undergoing surgery for breast cancer using the Nucleospin RNAII kit (Macherey-Nagel) and 1 μg was reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). Real-time quantitative RT-

PCR was performed on a LightCycler 2.0 apparatus (Roche) using the Light Cycler FastStart DNA Master SYBERGreen I kit (Roche). Reaction conditions for all optimal amplification, as well as primer selection of netrin-1, were determined as described (4). The ubiquitously expressed human HMBS (PBGD), TBP and mouse RPLP0 genes showing the least variability in expression between normal and breast tumoral tissues (5, 6) were used as internal controls. The sequences of the primers are available upon request.

Immunohistology and Immunohistochemistry. Paraffin embedded tumors tissues fixed in formalin were used for analysis. The pathologist selected representative areas from breast carcinomas (N+M0, M+) or liver or uterus metastasis. The blocks were sectioned at a thickness of 4 μm. After deparaffinization and rehydratation, tissue sections were boiled in buffer Ph9 using a water bath at 97°C for 50 minutes. For blocking endogenous peroxidases, the slides were incubated in 5% hydrogen peroxide in sterile water. The slides were then incubated at room temperature for 1 hour with a polyclonal goat antibody recognizing the human netrin-1(R & D Systems). This antibody was diluted using an antibody diluent solution (ChemMate; Dako) at 1/800. After rinsing in phosphate buffer saline, the slides were incubated with a biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (Lsab+ Kit; Dako). Bound antibody was revealed by adding the substrate 3,3-diamino benzidine. Regarding netrin-1 immunostaining in 4T1 cells, 1 x 10^5 cells were cytospun on coverslips and fixed in 4% paraformaldehyde. The slides were then incubated at room temperature for 1 hour with a mouse netrin-1 antibody (R & D Systems). After rinsing in phosphate buffer

saline, the slides were incubated with an Alexa-Fluor 488 donkey anti-rat antibody (Molecular Probes). Nuclei were visualized with Hoescht staining.

Tumor Mouse Models. Female BALB/cByJ mice of 6-8 weeks of age from The Jackson Laboratory were used for surgery. For mammary gland injection of 67NR cells, mice were anesthetized with 2,2,2-tribromoethanol and 10^6cells in 50 μl of PBS were injected into the mammary gland and mice were killed when the tumor exceeded 1.5 cm and caused impediment to the movement of the animal. For intravenous injection, 10^5 (unless indicated in the figure legend) 4T1-luc cells in 150 μl of PBS were injected into a tail vein, and mice were either killed at day 13 or analyzed using luminescence recording. When animals were killed, lungs were removed, and metastatic nodules counted. Four-micron-thick lung or liver sections were also prepared and stained with hematoxylin-eosin-saffron. Histological classification and grading of neoplastic lesions was performed in a blinded fashion and according to standard procedures. For *in vivo* imaging of metastasis using 4T1-luc cells, the light resulting from the bioluminescent oxidation of the intraperitonaly injected endotoxin-free luciferin (Promega) (120 mg/kg of body weight) was detected and quantified (10 minutes after injection) with a NightOWL LB 981 NC 100 system from Berthold Technologies, using an anesthesia system with gaseous isoflurane from TEM SEGA. Daily i.p injections of scramble siRNA or netrin-1 siRNA (4μg per mouse) were performed according the procedure established by SeleXel Corp. DCC-5Fbn treatments were performed according the Fig. 3 legend.

Regarding xenograft of fresh human breast tumor in nude mice, 7- to 9-week old

females Hsd:Athymic Nude-Foxn1^{nu} mice obtained from Harlan were xenografted using human breast tumors according the protocol developed by Xentech. Netrin-1 and netrin-1 receptors expression was analyzed by QRT-PCR as in Fig.1*A* in a panel of 20 breast tumors, and the tumor BC174 was selected on the basis of high netrin-1 level and detected UNC5H receptors expression. Xenografted mice breeding was performed in the Centre d'Exploration et de Recherche Fonctionnelle Expérimentale (CERFE) (Evry, France) and in XenTech company (Pépinière Genopole Entreprises/CCIE, Evry, France). For each xenograft when tumors reached 62mm^3 PBS or 1.25 μg per g of bodyweight DCC-5Fbn were administered i.v every day. Animals were killed by cervical dislocation when the tumor reached a volume of 2,000 mm^3 or because of deteriorated health.

SiRNA Detection in Lung. The detection of netrin-1 siRNA in lung was performed by SeleXel (Paris, France) according the protocol described in ref.7.

Fig. 5. Netrin-1 and CXCR4 expression in human breast cancer. (*A*) Expression profile of *CXCR4* examined with QRT-PCR using *HMBS* gene expression as referenced in the human breast tumors described in Fig.1*A*. QRT-PCR was performed using total RNA extracted from 51 tumor biopsies. *CXCR4* expression is given as the ratio between *CXCR4* expression in each sample and the average of CXCR4 expression in the N0 samples as indicated by a horizontal bar. Nonparametric statistical significance tests (Mann-Whitney) were used, the *P* values are indicated. (*B*) The percentage of samples showing a netrin-1 expression higher than the average expression in N0 biopsies, 5-fold higher or 15-fold higher is indicated, as is the range of the overexpression. (*C*) Netrin-1 amplification bias using the oligodT method generally used in microarray platforms. RNA from two primary tumor samples (N0, M+) were extracted and cDNA was prepared using either hexamers or oligodT. Netrin-1 or CXCR4 levels were determined and a ratio of amplification between hexamers and oligodT is presented. Note that although no change appears for CXCR4, a 8- to 10-fold difference exists between netrin-1 cDNA amplification by both techniques. (*D*) Netrin-1 immunohistochemistry on sections of breast metastasis detected in the liver (*UPPER*) or in the uterus (*LOWER*). Enlargement is indicated.

Fig. 6. Netrin-1 is not sufficient to confer metastatic propensity to 67NR cells. (*A*) Q-RT PCR was performed using total RNA extracted from 67NR and 4T1 mouse cell lines

with specific mouse netrin-1 and netrin-1 receptors primers and the mouse gene RPLP0 as standard. Histogram showing the number of mRNA molecules for netrin-1 and netrin-1 receptors. An index is presented as the ratio between each sample to the number of netrin-1 mRNA molecules present in 4T1 cells. (*B* and *C*) Mock-transfected 67NR cells or 67NR cells stably transfected with netrin-1 (67NR-net1) were submitted to netrin-1 expression analysis. (*B*) RT-PCR using specific chicken netrin-1 primers was performed using total RNA extracted from 67NR-net1 and 67NR-mock cells. (*C*) Western blot using anti-c-myc or anti-chick netrin1 antibodies was performed. (*D*) Lung and liver metastasis of fat pad-injected mock 67NR cells versus 67NR netrin-1-expressing cells. One control cell clone bearing puromycine resistance (67NR-mock), one netrin-1-expressing cell clone (67NRnet1), and one polyclonal population of netrin-1 stably transfected 67NR (67NR-net1-polyclonal) were injected in fat pads of mice, and metastasis was analyzed in the lung or liver environment.

Fig. 7. Disruption of the netrin-1 autocrine loop triggers breast cancer cell death via UNC5H receptors. (*A*) DCC-EC-Fc displaces DCC/netrin-1 and UNC5H2/netrin-1 interaction. ELISA assay with DCC-EC-Fc (*LEFT*) or UNC5H2-EC-Fc (*RIGHT*) coated, and quantification of bound netrin-1 using anti-Flag-netrin-1 antibody in the presence of increasing competitor (DCC-EC-Fc) concentration or of an unrelated soluble protein (IL3R). (*B*) HEK293T cells were transfected with DCC expressing vector and incubated for 15 minutes with 300 ng/ml netrin-1 in the presence of not of DCC-EC-Fc (2 μg/ml, 3 μg/ml). PhosphoErk and total Erk western blots were performed as described in ref. 8 and are shown. (*C*) T47D cells were treated with scramble siRNA (control) or netrin-1

siRNA and cell death was determined the day after by caspase activity assay. An index is presented as the ratio of fluorescence between netrin-1 siRNA-transfected T47D cells and scramble siRNA-transfected cells. Standard deviations are indicated (n = 3). (*D*) HEK293T cells were transfected with a mock or UNC5H2-expressing vector together or not with UNC5H2-IC-D412N encoding the intracellular portion of UNC5H2 mutated in its caspase site. Forty-eight hours after transfection, cell death was measured as described (1, 9) using TUNEL staining. Note that UNC5H2-IC-D412N fully inhibits cell death induced by full-length UNC5H2. Standard deviations are indicated (n = 3). Mann-Whitney tests were performed, and a *P* value is indicated. Similar results were obtained if UNC5H1 or UNC5H3 were expressed instead of UNC5H2, supporting the fact that UNC5H2-IC-D412N acts as a dominant-negative mutant for UNC5H proapoptotic activity (data not shown).

Fig. 8. The antimetastatic effect of netrin-1 siRNA depends on UNC5H1, and UNC5H2 is detected in lung after i.p. injection. (*A*) Netrin-1 siRNA is detected in the lung of netrin-1 siRNA i.p injected mice. Lungs were collected 30 minutes after i.p injection with netrin-1 siRNA or scramble siRNA and netrin-1 siRNA was analyzed according to the ref. 7 protocol. (*B*) 4T1 i.v. injected mice were treated daily with siRNA combination as indicated and as in Fig.3 *A*-*C*, and the number of metastasis was quantitated. In *A* and *B*, standard deviations are indicated (n = 3). Mann-Whitney tests were performed, and a *P* value is indicated.

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2. Mehlen P, *et al.* (1998) The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. *Nature* 395: 801-804.
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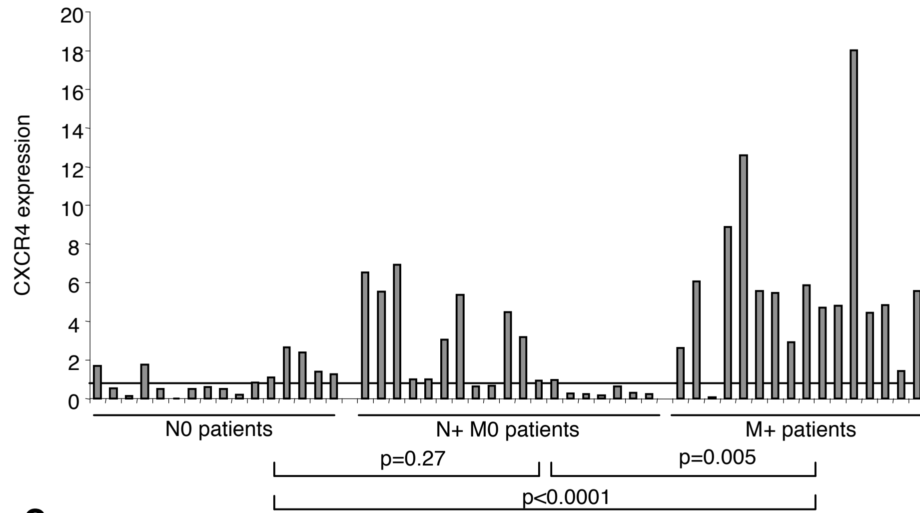
Fig. 5

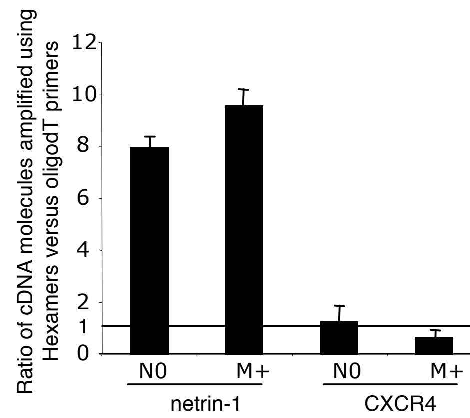
A



B

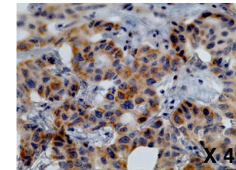
n=51		N0 Patients (n=16)	N+ M0 Patients (n=19)	M+ Patients (n=16)
% of breast tumors that over-express netrin-1		31	73.7	93.7
	more than 5 fold	0	31.5	62.5
	more than 15 fold	0	0	37.5
Range of netrin-1 over-expression		0.02-4.6	0.03-12.8	0.6-111.7

C



D

Liver metastasis



Uterus metastasis

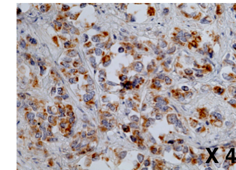
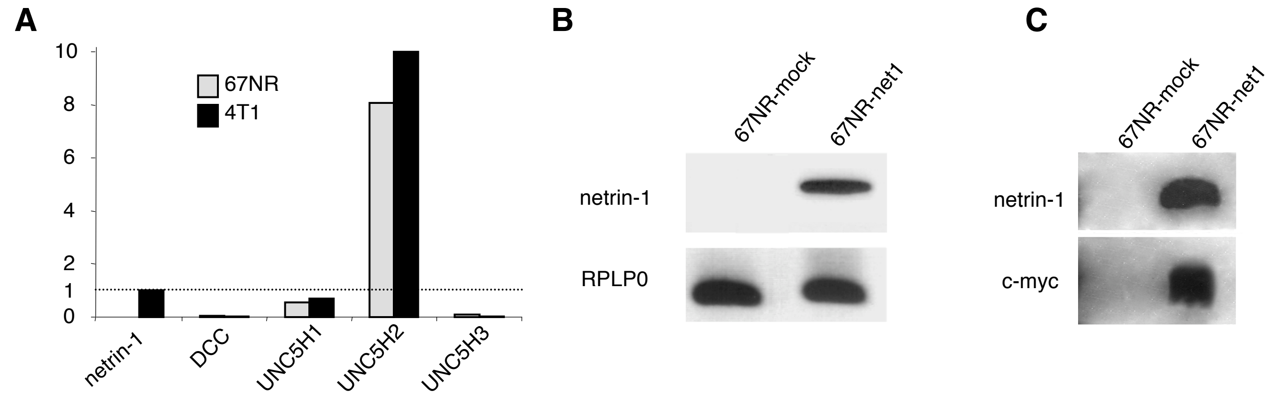


Fig. 6



D

Injected cells	Mice (n)	Primary tumors	Metastasis	Comment
67NR-mock	9	9	0	
67NR-net1	7	7	0	Suspicion of 1 micrometastase in liver
67NR-net1 polyclonal	12	12	0	Suspicion of 1 micrometastase in lung

Fig. 7

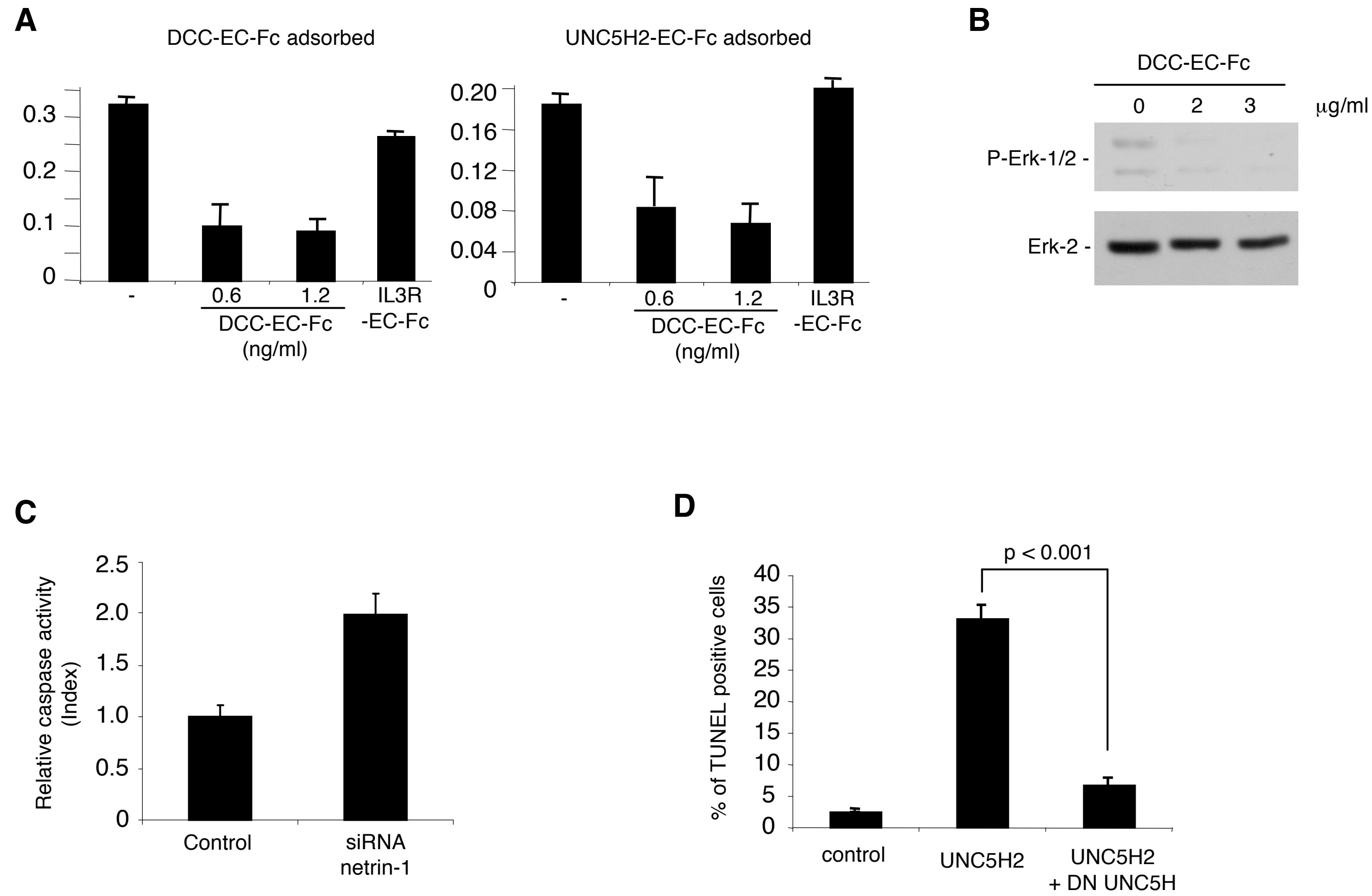
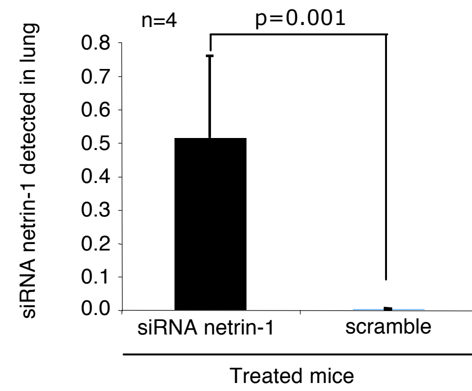


Fig. 8

A



B