Novel anti-netrin-1 antibody

The present invention relates to a netrin-1 binding polypeptide or antibody which is useful in inducing cell death or apoptosis of tumor cells bearing a netrin-1 receptor such as an UNC5 receptor in the presence of netrin-1, in particular the tumor express netrin-1, and its use for the treatment of cancer.

Netrin-1 is a member of the netrin family and is an axon navigation cue, both, in an attractive and repulsive context and plays a major role in the development of the nervous system. The main receptors for netrin-1 are DCC (Deleted in Colorectal Cancer) and UNC5 (UNC5A, UNC5B, UNC5C, UNC5D in human, UNC5H1, UNC5H2, UNC5H3, UNC5H4 in mice), which all belong to the dependence receptor family (Keino-Masu, 1996, Cell 87: 175-185 ; Ackermann, 1997, Nature 386: 838-842; Hong, 1999, Cell 97: 927-941; Mehlen, 1998, Nature 395: 801-804). Dependence receptors share the ability to induce apoptosis in the absence of their respective ligands, whereby this ability is blocked upon binding of the respective ligand (Mehlen, 2004, Cell Mol Life Sci 61: 1854-1866; Bredesen, 2005, Cell Death Differ 12: 1031-1043).

In various human cancers, reduction or loss of expression of DCC and, thus, reduction or loss of DCC-induced apoptosis has been observed (Kinzler, 1996, Proc Natl Acad Sci 100: 4173-4178). Furthermore, it has been observed that also UNC5 genes are downregulated in most colorectal tumors, indicating that the loss of dependence receptor UNC5 represents a selective advantage for tumor cells (Bernet, 2007, Gastroenterology 133: 180-1848; Shin, 2007, Gastroenterology 133: 1849-1857). However, not only down-regulation of the dependence receptor DCC and UNC5 enhances survival of various tumor cells, autocrine expression of their ligand netrin-1 has been observed. Particularly, it has been shown that the majority of breast tumors, i.e. metastatic breast cancers, exhibit increased expression of netrin-1 (Fitamant, 2008, Proc Natl Acad Sci 105: 4850-4855). Up to now, it has been demonstrated that the two Immunoglobulin (Ig) subdomains of the extracellular part of UNC5 are responsible for binding of netrin-1, but it is not clear whether both domains are necessary to full netrin-1 binding (Geisbrecht, 2003, J .Biol. Chem. 278 : 32561-32568 ; Kruger, 2004, J. Neur. 24 : 10826-10834).

As has been shown previously, neutralization of netrin-1 by the ectodomain of DCC or part of this ectodomain (acting as netrin-1 decoy protein) can induce apoptosis in tumor cells expressing dependence receptors DCC and/or UNC5 (EP-A1-1 989 546). This ectodomain or part of this ectodomain is capable to reduce metastasis of breast cancer cells into the lung (Fitamant et al. 2008). Furthermore, this ectodomain or part of this ectodomain has been also demonstrated to increase the cell death percentage of non-small cell lung cancer cells and neuroblastoma cells expressing high levels of netrin-1 (Delloye-Bourgeois, 2009, J Natl

Cancer Inst 101 : 237-247 ; Delloye-Bourgeois, 2009, JEM 206 : 833-847). WO2012025618 discloses DCC-fusion proteins having improved DCC decoy.

EP 1 989 546 (WO2007099133) discloses monoclonal or polyclonal antibodies directed specifically against netrin-1 or netrin-1 receptors, particularly directed to the extracellular domain of the netrin-1 receptors or to the netrin-1 fragment able to interact with the extracellular domain of said netrin-1 receptors, as a medicament.

Summary

In one aspect, the present invention refers to an isolated or purified polypeptide consisting of sequence SEQ ID NO: 3 comprising SEQ ID NO: 35, or encoded by the cDNA of sequence SEQ ID NO: 4 or SEQ ID NO: 36, or a variant by virtue of the degeneracy of the genetic code. In another aspect, the present invention refers to use of a polypeptide as disclosed herein for the preparation of a monoclonal antibody. In yet another aspect, the present invention refers to a netrin-1 binding antibody which comprises a CDR1-H of sequence SEQ ID NO: 5, a CDR2-H of sequence SEQ ID NO: 6, a CDR3-H of sequence SEQ ID NO: 7, and a CDR1-L of sequence SEQ ID NO: 8, a CDR2-L of sequence YAS and a CDR3-L of sequence SEQ ID NO: 9, wherein the antibody has the property of binding to netrin-1 and inducing cell death or apoptosis of a tumor cell via an UNC5 receptor or DCC receptor. In a further aspect, the present invention refers to a netrin-1 binding antibody which comprises a CDR1-H of sequence SEQ ID NO: 28, a CDR2-H of sequence SEQ ID NO: 29, a CDR3-H of sequence SEQ ID NO: 30, and a CDR1-L of sequence SEQ ID NO: 31, a CDR2-L of sequence SEQ ID NO: 32 and a CDR3-L of sequence SEQ ID NO: 9, wherein the antibody has the property of binding to netrin-1 and inducing cell death or apoptosis of a tumor cell via an UNC5 receptor or DCC receptor. In another aspect, the present invention refers to a pharmaceutical composition comprising an antibody as disclosed herein, and a pharmaceutically acceptable vehicle, carrier or diluent. In yet another aspect, the present invention refers to use of a pharmaceutical composition as disclosed herein. in the manufacture of a medicament for treating cancer, wherein the cancer has tumoral cells expressing a netrin-1 receptor, in the presence of netrin-1. In a further aspect, the present invention refers to use of a pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating cancer, wherein the cancer has tumoral cells expressing a netrin-1 receptor and netrin-1 or a tumoral cells expressing netrin-1 within stroma cells expressing netrin-1. In another aspect, the present invention refers to use of a pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating cancer. In a further aspect, the present invention refers to use of the netrin-1 binding antibody as disclosed herein in the manufacture of a medicament for treating cancer.

The Applicant has now determined a linear or substantially linear epitope on netrin-1 that likely corresponds to the specific binding region of netrin-1 to receptors, in particular of the UNC5 class, especially UNC5B and UNC5A, or alternatively corresponds to a region nearby the specific binding region of netrin-1 to receptor that when bound to an antibody prevents netrin-1/receptor interaction. This determination of a linear epitope allows the Applicant to produce antibodies binding to netrin-1 and interfering with netrin-1/receptors interaction, thereby inducing apoptosis or cell death of tumour cells expressing or overexpressing netrin-1 and at least one netrin-1 receptor, owing the fact that this interaction inhibits netrin-1 binding to a receptor and the multimerization of the receptor. The Applicant also produced a murine monoclonal antibody directed against this epitope and various humanized forms thereof.

The full-length amino acid sequence of netrin-1 is given as SEQ ID NO: 1 and a cDNA coding therefore is given at SEQ ID NO: 2. A linear epitope has been characterized in the second EGF-like domain of netrin-1 and is depicted on SEQ ID NO: 3 and more particularly on SEQ ID NO:35. A cDNA encoding this epitope is depicted on SEQ ID NO: 4, respectively 36.

A first object of the invention is thus a polypeptide representing a linear epitope of netrin-1 or a fragment or variant of said linear epitope. More specifically, the invention relates to:

- an isolated or purified polypeptide of sequence SEQ ID NO: 3,
- a variant polypeptide having at least 85, 90, 95, 96, 97, 98 or 99 % of identity to SEQ ID NO: 3,
- a variant polypeptide of at most 200, 150, 100, 90, 80, 70, 60, 50 amino acids and comprising SEQ ID NO: 3 or variant sequence as described above, such as a variant polypeptide consisting of the 22 consecutive amino acids of SEQ ID NO: 3 from position 9 (A) to position 30 (C), including these amino acids, which variant is presented as SEQ ID NO: 35,
- a variant polypeptide comprising at least 20, 25 or 30 consecutive amino acids of SEQ ID NO: 3 such as a variant polypeptide of sequence SEQ ID NO: 35, or variant sequence as described above,
- a polypeptide of sequence SEQ ID NO: 35, or variant sequence as described above,

- an isolated or purified polypeptide encoded by the cDNA of sequence SEQ ID NO: 4 or 36.

Another object of the invention is a cDNA coding for said polypeptide representing a linear epitope of netrin-1 or a fragment or variant of said linear epitope. More specifically, the invention relates to:

- a cDNA of sequence SEQ ID NO: 4,

- a variant cDNA encoding the polypeptide of sequence SEQ ID NO: 3 or a variant thereof, by virtue of the degeneracy of the genetic code,

- a variant cDNA encoding a variant polypeptide as defined earlier, especially a cDNA encoding the variant polypeptide SEQ ID NO: 35, such as cDNA of SEQ ID NO: 36,

- a variant cDNA having at least 85, 90, 95, 96, 97, 98 or 99 % of identity to SEQ ID NO: 4 or 36.

In accordance with the invention, a variant polypeptide is able to generate an antibody which still keeps the ability to specifically bind to the linear epitope on netrin-1 and inhibit the interaction of netrin-1 to its receptor, in particular UNC5 or DCC, especially UNC5B and UNC5A, and induce apoptosis or cell death of the tumour cell expressing or overexpressing netrin-1 and a netrin-1 receptor. A variant cDNA codes for the polypeptide of SEQ ID NO: 3 or of SEQ ID NO: 35 or for a variant polypeptide.

Another object of the invention is the use of the polypeptide of SEQ ID NO: 3 or 35 or a variant thereof, to produce a monoclonal antibody, and the monoclonal antibodies so produced are also an object of the invention. The person skilled in the art is aware of the methods allowing producing monoclonal antibodies, using the plasmocyte and hybridoma technique. The invention encompasses the use of these methods to produce antibodies specifically directed against the polypeptide of SEQ ID NO: 3 or 35 or a variant thereof. It is also within the scope of the invention to produce polypeptides or monoclonal antibodies through genetic engineering based on nucleic acid sequences coding for the specific polypeptide or antibody, owing the determination and disclosure of the CDRs of the VH and VL. It is also within the scope of the invention to provide for antibody fragments and/or humanized antibodies or antibody fragments with a variable region specific to the linear epitope of the invention, a fragment or a variant thereof. The term “binding polypeptide” will be used herein to encompass antibodies and antibody variants, fragments and combination that keep the binding function of the antibody.

The present invention thus also relates to a netrin-1 binding polypeptide which specifically binds to a polypeptide having the amino acid sequence SEQ ID NO: 3 or 35 or a variant thereof. The binding polypeptide has the property of binding to netrin-1 and induce cell death or apoptosis of a tumor cell via an UNC5 or DCC receptor. The fact is that free or

active netrin-1 does no longer exist or at an insufficient level, so that the apoptosis signaling of the netrin-1 receptor is activated.

The present invention also relates to a netrin-1 binding polypeptide which comprises one or more complementarity-determining region(s) (CDR(s)) having an amino acid sequence SEQ ID NO: 7, SEQ ID NO: 30 or SEQ ID NO: 9, wherein the binding polypeptide has the property of binding to netrin-1 and inducing cell death or apoptosis of a tumor cell via an UNC5 or DCC receptor.

The polypeptide may be an antibody or an epitope-binding fragment thereof.

Using the IMGT definition, the present invention thus relates to a netrin-1 binding polypeptide which comprises one or more complementarity-determining region (CDR) having an amino acid sequence SEQ ID NO: 7 (CDR3-H), SEQ ID NO: 9 (CDR3-L), and preferably both.

More specifically, the polypeptide may be further defined by the additional presence of CDR1, CDR2 or the CDR1 and CDR2 of the VH and/or VL. Therefore, the polypeptide may comprise one or more CDR(s) having the amino acid sequences SEQ ID NO: 7 and SEQ ID NO: 5; SEQ ID NO: 7 and SEQ ID NO: 6; SEQ ID NO: 9 and the sequence YAS; and/or SEQ ID NO: 9 and SEQ ID NO: 8.

An object of the invention is a polypeptide comprising a CDR1-H of sequence SEQ ID NO: 5, a CDR2-H of sequence SEQ ID NO: 6, a CDR3-H of sequence SEQ ID NO: 7.

An object of the invention is a polypeptide comprising a CDR1-L of sequence SEQ ID NO: 8, a CDR2-L of sequence YAS and a CDR3-L of sequence SEQ ID NO: 9.

The polypeptide of the invention preferably comprises a CDR1-H of sequence SEQ ID NO: 5, a CDR2-H of sequence SEQ ID NO: 6, a CDR3-H of sequence SEQ ID NO: 7, a CDR1-L of sequence SEQ ID NO: 8, a CDR2-L of sequence YAS and a CDR3-L of sequence SEQ ID NO: 9.

Using the Kabat definition, the present invention thus relates to a netrin-1 binding polypeptide which comprises one or more complementarity-determining region (CDR) having an amino acid sequence SEQ ID NO: 30 (CDR3-H), SEQ ID NO: 9 (CDR3-L), and preferably both.

More specifically, the polypeptide may be further defined by the additional presence of CDR1, CDR2 or the CDR1 and CDR2 of the VH and/or VL. Therefore, the polypeptide may comprise one or more CDR(s) having the amino acid sequences SEQ ID NO: 30 and SEQ ID NO: 28; SEQ ID NO: 30 and SEQ ID NO: 29; SEQ ID NO: 9 and the SEQ ID NO: 32; and/or SEQ ID NO: 9 and SEQ ID NO: 31.

An object of the invention is a polypeptide comprising a CDR1-H of sequence SEQ ID NO: 28, a CDR2-H of sequence SEQ ID NO: 29, a CDR3-H of sequence SEQ ID NO: 30.

An object of the invention is a polypeptide comprising a CDR1-L of sequence SEQ ID NO: 31, a CDR2-L of sequence SEQ ID NO: 32 and a CDR3-L of sequence SEQ ID NO: 9.

The polypeptide of the invention preferably comprises a CDR1-H of sequence SEQ ID NO: 28, a CDR2-H of sequence SEQ ID NO: 29, a CDR3-H of sequence SEQ ID NO: 30, a CDR1-L of sequence SEQ ID NO: 31, a CDR2-L of sequence SEQ ID NO:32 and a CDR3-L of sequence SEQ ID NO: 9.

These polypeptides are netrin-1 binding polypeptides, wherein the binding polypeptide has the property of binding to netrin-1 and induce cell death or apoptosis of a tumor cell via an UNC5 or DCC receptor. These polypeptides are preferably antibodies, especially monoclonal antibodies. Various forms of binding polypeptides or antibodies (including fragments and combination thereof) will be described later herein.

In a first series of embodiments, the polypeptide or antibody of the invention comprises an amino acid sequence SEQ ID NO: 10, 11, 12 or 13. Typically, it comprises both sequences SEQ ID NO: 10 and 11, or SEQ ID NO: 12 and 13.

In a second series of embodiments, the polypeptide or antibody is humanized and comprises an amino acid sequence selected from the group of SEQ ID NO: 14 to 19 and/or from the group of SEQ ID NO: 20 to 27. Typically, the polypeptide or antibody is humanized and comprises an amino acid sequence selected from the group of SEQ ID NO: 14 to 19 and an amino acid sequence selected from the group of SEQ ID NO: 20 to 27.

Specific embodiments are the following humanized antibodies. The first listed in this table correspond to the grafting of the murine CDRs into a human IgG1. The others called HUM are monoclonal antibodies having variable human framework regions. The table gives also a reference for the CH and CL of a human IgG1. The other allotypes may be used as well.

	VH SEQ ID NO:	Constant heavy chain	VL SEQ ID NO:	Constant light chain
CDR graft (murine CDRs grafted into human IgG1)	19	Human IgG1 (GenBank: AEL33691.1 modified R97K)	27	Human IgG1 (GenBank: CAC20459.1)
HUM01	20	Human IgG1	14	Human IgG1
HUM02	21	Human IgG1	15	Human IgG1
HUM03	22	Human IgG1	16	Human IgG1
HUM04	23	Human IgG1	17	Human IgG1
HUM05	24	Human IgG1	17	Human IgG1
HUM06	25	Human IgG1	16	Human IgG1

HUM07	26	Human IgG1	17	Human IgG1
HUM08	22	Human IgG1	17	Human IgG1
HUM09	25	Human IgG1	18	Human IgG1
HUM10	21	Human IgG1	16	Human IgG1

Another object of the invention is a pharmaceutical composition comprising at least one netrin-1 binding polypeptide or antibody according to the invention and a pharmaceutically acceptable vehicle or excipient. In an embodiment, the polypeptide or antibody is humanized.

Still another object of the invention is a method of treatment of cancer wherein a subject in need thereof is administered with a therapeutically effective amount of a pharmaceutical composition comprising at least one netrin-1 binding polypeptide or antibody according to the invention and a pharmaceutically acceptable vehicle or excipient. In an embodiment, the polypeptide or antibody is humanized.

Thus the composition and method may comprise any one of the features or combination of features as disclosed with respect to the polypeptide or antibody as disclosed herein.

According to a feature, the cancer is one wherein tumoral cells express or over-express a netrin-1 receptor, in particular of the UNC5 class, especially UNC5B and/or UNC5A, and/or DCC. Typically the tumoral cells escape netrin-1 receptor related apoptosis owing binding of netrin-1 to said receptor, in particular of the UNC5 class, especially UNC5B and/or UNC5A, and/or DCC, in the presence of netrin-1. According to a feature, the cancer is one wherein tumoral cells or stromal cells express or over-express netrin-1.

Some embodiments of cancers include metastatic breast cancer, non-small cell lung cancer, aggressive neuroblastoma, pancreatic adenocarcinoma, primary melanoma, melanoma metastasis, ovarian cancers, glioblastoma, acute myeloid leukaemia, chronic lymphocytic leukaemia, aggressive B-cell lymphoma, sarcoma, renal adenocarcinoma, head and neck cancers, testicular cancers (e.g. embryonal carcinoma, teratoma, yolk sac tumors), kidney cancers, stomach cancers, uterus cancers

Methods of determining whether a given cell expresses netrin-1 dependence receptors DCC and/or UNC5 on the surface or shows significant up-regulation of netrin-1 gene expression are well known in the art and comprise, but are not limited to, IHC (Immunohistochemistry) of FACS (Fluorescence activated cell sorting), quantitative PCR (e.g. with hexamer primed cDNA) or alternatively Western Blot paired with chromogenic dye-based protein detection techniques (such as silver or coomassie blue staining) or fluorescence- and luminescence-based detection methods for proteins in solutions and on gels, blots and microarrays, such as immunostaining, as well as immunoprecipitation, ELISA, microarrays, and mass spectrometry. In the context of the present invention, examples for

cancers to be treated are listed herein including refractory versions of any of the mentioned cancers. Netrin-1 overexpression may thus be measured through RT-PCR using suitable primers as those disclosed and provided herein, with respect to normal tissue or to a similar cancer which does not overexpress netrin-1.

In an embodiment of the invention, the compositions and methods are for the treatment of cancers expressing or over-expressing netrin-1, wherein this expression or over-expression is linked to the cancer itself, or is induced by a chemotherapeutic drug treatment alone, or both.

An object of the invention is a method of combined anti-cancer treatment comprising the administration to a patient in need thereof of a chemotherapeutic drug and of a polypeptide or antibody as disclosed herein. The chemotherapeutic drug and the polypeptide or antibody is in effective amount.

Another object of the invention is a composition comprising a polypeptide or antibody as disclosed herein for use as an anti-cancer medicament to be used in combination with a chemotherapeutic drug in a patient. The invention also relates to a composition comprising a polypeptide or antibody as disclosed herein for use as an anti-cancer medicament in a patient who is treated with a chemotherapeutic drug.

Another object of the invention is a composition comprising a chemotherapeutic drug for use as an anti-cancer medicament to be used in a patient in combination with a polypeptide or antibody as disclosed herein. The invention also relates to a composition comprising a chemotherapeutic drug for use as an anti-cancer medicament in a patient who is treated with a polypeptide or antibody as disclosed herein.

Another object of the invention is a composition or kit of parts comprising a chemotherapeutic drug and a polypeptide or antibody as disclosed herein, for a simultaneous, separate or sequential administration to a patient.

Another object of the invention is a composition or kit of parts comprising a chemotherapeutic drug and a polypeptide or antibody as disclosed herein, for a simultaneous, separate or sequential administration to a patient, for use as an anti-cancer medicament or anti-cancer treatment.

Another object of the invention is a composition comprising a chemotherapeutic drug and a polypeptide or antibody as disclosed herein, in a pharmaceutically acceptable carrier or vehicle.

Another object of the invention is a composition comprising a chemotherapeutic drug and a polypeptide or antibody as disclosed herein, in a pharmaceutically acceptable carrier or vehicle, for use as an anti-cancer medicament.

Still another object is the use of a polypeptide or antibody as disclosed herein for the preparation of an anti-cancer medicament intended for a combined treatment of a patient

with a chemotherapeutic drug.

Still another object is the use of a chemotherapeutic drug for the preparation of an anti-cancer medicament intended for a combined treatment of a patient with a polypeptide or antibody as disclosed herein.

Still another object is the use of a polypeptide or antibody as disclosed herein and a chemotherapeutic drug for the preparation of a combined anti-cancer medicament.

Still another object is the use of a polypeptide or antibody as disclosed herein and a chemotherapeutic drug for the preparation of a combined anti-cancer medicament composition or kit of parts, for a simultaneous, separate or sequential administration to a patient.

In accordance with a feature of the invention and as further explained below, the chemotherapeutic drug is a drug which induces an over-expression of netrin-1 in cancer cells and the polypeptide or antibody as disclosed herein promotes netrin-1 receptors-induced apoptosis or cell death despite this overexpression.

The chemotherapeutic drug is in particular a drug which induces an over-expression of netrin-1 in cancer cells. The determination that a drug induces a netrin-1 over-expression may be easily performed on any cancerous cell, such as cell line or cells from a biopsy. In an embodiment, the assay is performed on cells from the cancer to be treated, for example from a biopsy. In another embodiment, the assay is performed on a cell, such as a cell line, which is representative for the cancer to be treated. In another embodiment, the assay is made on a A549 or H460 cell line. The assay may comprise comparing the netrin-1 gene expression between the cells treated with the chemotherapeutic drug and the cells not treated. The expression may be measured by PCR, especially quantitative RT-PCR, for example using the primers disclosed and provided herein (SEQ ID NO: 33 and 34) as described in PCT/EP2013/068937, the whole content of which is incorporated by reference or the skilled person may refer. The classification of a drug in the family of those inducing this over-expression may simply be performed in accordance with the method described in the following Material and Method on a A549 or H460 cell line, as described in PCT/EP2013/068937.

The chemotherapeutic drug is especially a cytotoxic drug. In some preferred embodiments, the drug is doxorubicin, 5-fluorouracil (5FU), paclitaxel (e.g. Taxol), or cisplatin.

In an embodiment, the drug is a cytotoxic antibiotic. The cytotoxic antibiotic may be actinomycin, an anthracycline, bleomycin, plicamycin or mitomycin. The anthracycline may be doxorubicin, daunorubicin, valrubicin, idarubicine or epirubicine.

In an embodiment, the drug is an alkylating agent. The alkylating agent may be a platinum derivative, such as cisplatin, carboplatin, oxaliplatine or other alkylating agents such

as cyclophosphamide, ifosfamide, melphalan, thiotepa. Other classes include-epipodophylotoxines, e.g. etoposide, topoisomerase inhibitors (camptotecines), e.g. irinotecan, topotecan , alkylating agents of the minor groove of DNA , e.g. Trabectedine (YONDELIS), methotrexate, pemetrexed, raltitrexed.

In an embodiment, the drug is a taxane or other tubulin targeting agents. The taxane may be paclitaxel or docetaxel, or eribuline (recently approved for breast cancer).

In an embodiment, the drug is an antineoplastic agent such as :

- breast hormonotherapy agents: e.g. tamoxifene, letrozole, anastrozole, exemestane, faslodex;
- prostate hormonotherapy agents:e.g. LHRH agonists, bicalutamide, abiraterone;
- monoclonal antibodies : e.g. cetuximab, panitumumab, bevacizumab;
- kinase inhibitors : e.g. imatinib, nilotinib, dasatinib, erlotinib, gefitinib, afatinib, sunitinib, sorafenib, pazopanib, crizotinib, axitinib.

Definitions and further embodiments, variants and alternatives of the invention:

As used herein, a sequence "at least 85% identical to a reference sequence" is a sequence having, on its entire length, 85%, or more, in particular 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or 100% sequence identity with the entire length of the reference sequence.

A percentage of "sequence identity" may be determined by comparing the two sequences, optimally aligned over a comparison window, wherein the portion of the polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.* gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison is conducted by global pairwise alignment, *e.g.* using the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443. The percentage of sequence identity can be readily determined for instance using the program Needle, with the BLOSUM62 matrix, and the following parameters gap-open=10, gap-extend=0.5.

In the context of the invention, a "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain group with similar chemical properties (*e.g.*, charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2)

aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfur-containing side chains: cysteine and methionine. Conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine-tryptophane, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

Throughout the instant application, the term "comprising" is to be interpreted as encompassing all specifically mentioned features as well optional, additional, unspecified ones. As used herein, the use of the term "comprising" also discloses the embodiment wherein no features other than the specifically mentioned features are present (*i.e.* "consisting of").

An "antibody" may be a natural or conventional antibody in which two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains. The light chain includes two domains or regions, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR). The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin and consists of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions (FR) influence the overall domain structure and hence the combining site.

"Complementarity Determining Regions" or "CDRs" refer to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. The light and heavy chains of an immunoglobulin each have three CDRs, designated CDR1-L, CDR2-L, CDR3-L and CDR1-H, CDR2-H, CDR3-H, respectively. A conventional antibody antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region.

"Framework Regions" (FRs) refer to amino acid sequences interposed between CDRs, *i.e.* to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved among different immunoglobulins in a single species. The light and heavy chains of an immunoglobulin each have four FRs, designated FR1-L, FR2-L, FR3-L, FR4-L, and FR1-H, FR2-H, FR3-H, FR4-H, respectively.

As used herein, a "human framework region" is a framework region that is substantially identical (about 85%, or more, in particular 90%, 95%, 97%, 99% or 100%) to the framework region of a naturally occurring human antibody.

In the context of the invention, CDR/FR definition in an immunoglobulin light or heavy chain is to be determined based on IMGT definition (Lefranc *et al.* (2003) *Dev Comp Immunol.* **27**(1):55-77; www.imgt.org).

As used herein, the term "antibody" denotes conventional antibodies and fragments thereof, as well as single domain antibodies and fragments thereof, in particular variable heavy chain of single domain antibodies, and chimeric, humanised, bispecific or multispecific antibodies.

As used herein, antibody or immunoglobulin also includes "single domain antibodies" which have been more recently described and which are antibodies whose complementary determining regions are part of a single domain polypeptide. Examples of single domain antibodies include heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional four-chain antibodies, engineered single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, goat, rabbit and bovine. Single domain antibodies may be naturally occurring single domain antibodies known as heavy chain antibody devoid of light chains. In particular, Camelidae species, for example camel, dromedary, llama, alpaca and guanaco, produce heavy chain antibodies naturally devoid of light chain. Camelid heavy chain antibodies also lack the CH1 domain.

The variable heavy chain of these single domain antibodies devoid of light chains are known in the art as "VHH" or "nanobody". Similar to conventional VH domains, VHHs contain four FRs and three CDRs. Nanobodies have advantages over conventional antibodies: they are about ten times smaller than IgG molecules, and as a consequence properly folded functional nanobodies can be produced by in vitro expression while achieving high yield. Furthermore, nanobodies are very stable, and resistant to the action of proteases. The properties and production of nanobodies have been reviewed by Harmsen and De Haard (Harmsen and De Haard (2007) Appl. Microbiol. Biotechnol. **77**:13-22).

The term "monoclonal antibody" or "mAb" as used herein refers to an antibody molecule of a single amino acid composition that is directed against a specific antigen, and is not to be construed as requiring production of the antibody by any particular method. A

monoclonal antibody may be produced by a single clone of B cells or hybridoma, but may also be recombinant, *i.e.* produced by protein engineering.

"Fragments" of (conventional) antibodies comprise a portion of an intact antibody, in particular the antigen binding region or variable region of the intact antibody. Examples of antibody fragments include Fv, Fab, $F(ab')_2$, Fab', dsFv, $(dsFv)_2$, scFv, $sc(Fv)_2$, diabodies, bispecific and multispecific antibodies formed from antibody fragments. A fragment of a conventional antibody may also be a single domain antibody, such as a heavy chain antibody or VHH.

The term "Fab" denotes an antibody fragment having a molecular weight of about 50,000 Da and antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain, among fragments obtained by treating IgG with a protease, papaine, are bound together through a disulfide bond.

The term "$F(ab')_2$" refers to an antibody fragment having a molecular weight of about 100,000 Da and antigen binding activity, which is slightly larger than the Fab bound via a disulfide bond of the hinge region, among fragments obtained by treating IgG with a protease, pepsin.

A single chain Fv ("scFv") polypeptide is a covalently linked VH::VL heterodimer which is usually expressed from a gene fusion including VH and VL encoding genes linked by a peptide-encoding linker. The human scFv fragment of the invention includes CDRs that are held in appropriate conformation, in particular by using gene recombination techniques. Divalent and multivalent antibody fragments can form either spontaneously by association of monovalent scFvs, or can be generated by coupling monovalent scFvs by a peptide linker, such as divalent $sc(Fv)_2$.

"dsFv" is a VH::VL heterodimer stabilised by a disulphide bond.

"$(dsFv)_2$" denotes two dsFv coupled by a peptide linker.

The term "bispecific antibody" or "BsAb" denotes an antibody which combines the antigen-binding sites of two antibodies within a single molecule. Thus, BsAbs are able to bind two different antigens simultaneously. Genetic engineering has been used with increasing frequency to design, modify, and produce antibodies or antibody derivatives with a desired set of binding properties and effector functions as described for instance in EP 2 050 764 A1.

The term "multispecific antibody" denotes an antibody which combines the antigen-binding sites of two or more antibodies within a single molecule.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are

forced to pair with the complementary domains of another chain and create two antigen-binding sites.

In a particular embodiment, the epitope-binding fragment is selected from the group consisting of Fv, Fab, $F(ab')_2$, Fab', dsFv, $(dsFv)_2$, scFv, $sc(Fv)_2$, diabodies and VHH.

A "chimeric antibody", as used herein, is an antibody in which the constant region, or a portion thereof, is altered, replaced, or exchanged, so that the variable region is linked to a constant region of a different species, or belonging to another antibody class or subclass. "Chimeric antibody" also refers to an antibody in which the variable region, or a portion thereof, is altered, replaced, or exchanged, so that the constant region is linked to a variable region of a different species, or belonging to another antibody class or subclass.

The term "humanised antibody" refers to an antibody which is initially wholly or partially of non-human origin and which has been modified to replace certain amino acids, in particular in the framework regions of the heavy and light chains, in order to avoid or minimize an immune response in humans. The constant domains of a humanized antibody are most of the time human CH and CL domains. In an embodiment, a humanized antibody has constant domains of human origin. As used herein, the term "humanized antibody" refers to a chimeric antibody which contain minimal sequence derived from non-human immunoglobulin, e.g. the CDRs.

The term “polypeptide” or “netrin-1 binding polypeptide” is used to encompass all these kinds of antibodies, fragments or combination thereof.

The goal of humanization is a reduction in the immunogenicity of a xenogenic antibody, such as a murine antibody, for introduction into a human, while maintaining the full antigen binding affinity and specificity of the antibody. Humanized antibodies, or antibodies adapted for non-rejection by other mammals, may be produced using several technologies such as resurfacing and CDR grafting. As used herein, the resurfacing technology uses a combination of molecular modeling, statistical analysis and mutagenesis to alter the non-CDR surfaces of antibody variable regions to resemble the surfaces of known antibodies of the target host.

Antibodies can be humanized using a variety of other techniques including CDR-grafting (EP0239400; WO91/09967; U.S. Patent Nos. 5,530,101 and 5,585,089), veneering or resurfacing (EP0592106; EP0519596; Padlan (1991) *Molecular Immunology* **28**(4/5):489-498; Studnicka *et al.* (1994) *Protein Engineering* **7**(6):805-814; Roguska *et al.* (1994) *Proc. Natl. Acad. Sci U.S.A.* **91**:969-973), and chain shuffling (U.S. Patent No. 5,565,332). Human antibodies can be made by a variety of methods known in the art including phage display methods. See also U.S. Patent Nos. 4,444,887, 4,716,111, 5,545,806, and 5,814,318; and International patent application WO98/46645, WO98/50433, WO98/24893, WO98/16654, WO96/34096, WO96/33735, and WO91/10741.

In the context of the invention, the term "treating" or "treatment", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition.

By the term "treating cancer" as used herein is meant the inhibition of the growth of malignant cells of a tumour and/or the progression of metastases from said tumor. Such treatment can also lead to the regression of tumor growth, *i.e.*, the decrease in size of a measurable tumor. In a particular embodiment, such treatment leads to a partial regression of the tumor or metastasis. In another particular embodiment, such treatment leads to the complete regression of the tumor or metastasis.

According to the invention, the term "patient" or "patient in need thereof" is intended for a human or non-human mammal affected or likely to be affected with a malignant tumor.

In a particular embodiment, the patient to be treated may have been previously treated with other anti-cancer treatments. In particular, the patient to be treated may have been previously treated with an oxaliplatin-, cisplatin-, a carboplatin-, and/or a paclitaxel-docetaxel-based regimen.

By a "therapeutically effective amount" of the polypeptide or antibody of the invention is meant a sufficient amount thereof to treat said cancer disease, at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the polypeptide or antibody of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific polypeptide or antibody employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific polypeptide or antibody employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide or antibody employed; and like factors well known in the medical arts. In a particular embodiment, said therapeutically effective amount of the polypeptide or antibody administered to the patient is a dose ranging from 5 mg/m^2 to 500 mg/m^2, more particularly ranging from 150 mg/m^2 to 450 mg/m^2 of body surface area.

In a further embodiment, the polypeptide or antibody of the invention is administered repeatedly according to a protocol that depends on the patient to be treated (age, weight, treatment history, etc.), which can be determined by a skilled physician.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically

acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions including the polypeptide or antibody of the invention and the route of administration naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and gender of the patient, etc.

The polypeptide or antibody of the invention can be formulated for a topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular administration and the like. In a particular embodiment, the polypeptide or antibody of the invention is administered intravenously

In particular, the pharmaceutical compositions including the polypeptide or antibody of the invention may contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

To prepare pharmaceutical compositions, an effective amount of the polypeptide or antibody of the invention may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like) and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants, stabilizing agents, cryoprotectants or antioxidants. The prevention of the action of microorganisms can be brought about by antibacterial and antifungal agents. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the

basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

Table 1: Description of the sequences:

SEQ ID NO:	Description	Sequence
1	Netrin-1 amino acid (aa) sequence (seq.) with signal peptide in bold and linear epitope mapping in bold and underlined	***MMRAVWEALAALAAVACLVGAVRG***GPGLSMFAGQAAQ PDPCSDENGHPRRCIPDFVNAAFGKDVRVSSTCGRPPA RYCVVSERGEERLRSCHLCNASDPKKAHPPAFLTDLNNP HNLTCWQSENYLQFPHNVTLTLSLGKKFEVTYVSLQFCS PRPESMAIYKSMDYGRTWVPFQFYSTQCRKMYNRPHRA PITKQNEQEAVCTDSHTDMRPLSGGLIAFSTLDGRPSAHD FDNSPVLQDWVTATDIRVAFSRLHTFGDENEDDSELARD SYFYAVSDLQVGGRCKCNGHAARCVRDRDDSLVCDCRH NTAGPECDRCKPFHYDRPWQRATAREANEC**<u>VACNCNL HARRCRFNMELYKLSGRKSGGVCLNCRHNTAGRHCH</u>**Y CKEGYYRDMGKPITHRKACKACDCHPVGAAGKTCNQTT GQCPCKDGVTGITCNRCAKGYQQSRSPIAPCIKIPVAPPT

		TAASSVEEPEDCDSYCKASKGKLKINMKKYCKKDYAVQIH ILKADKAGDWWKFTVNIISVYKQGTSRIRRGDQSLWIRSR DIACKCPKIKPLKKYLLLGNAEDSPDQSGIVADKSSLVIQW RDTWARRLRKFQQREKKGKCKKA
2	Netrin-1 nucleic acid seq.	ATGATGCGCGCAGTGTGGGAGGCGCTGGCGGCGCTG GCGGCGGTGGCGTGCCTGGTGGGCGCGGTGCGCGG CGGGCCCGGGCTCAGCATGTTCGCGGGCCAGGCGGC GCAGCCCGATCCCTGCTCGGACGAGAACGGCCACCC GCGCCGCTGCATCCCGGACTTTGTCAATGCGGCCTTC GGCAAGGACGTGCGCGTGTCCAGCACCTGCGGCCGG CCCCCGGCGCGCTACTGCGTGGTGAGCGAGCGCGGC GAGGAGCGGCTGCGCTCGTGCCACCTCTGCAACGCGT CCGACCCCAAGAAGGCGCACCCGCCCGCCTTCCTCAC CGACCTCAACAACCCGCACAACCTGACGTGCTGGCAG TCCGAGAACTACCTGCAGTTCCCGCACAACGTCACGCT CACACTGTCCCTCGGCAAGAAGTTCGAAGTGACCTAC GTGAGCCTGCAGTTCTGCTCGCCGCGGCCCGAGTCCA TGGCCATCTACAAGTCCATGGACTACGGGCGCACGTG GGTGCCCTTCCAGTTCTACTCCACGCAGTGCCGCAAG ATGTACAACCGGCCGCACCGCGCGCCCATCACCAAGC AGAACGAGCAGGAGGCCGTGTGCACCGACTCGCACAC CGACATGCGCCCGCTCTCGGGCGGCCTCATCGCCTTC AGCACGCTGGACGGGCGGCCCTCGGCGCACGACTTC GACAACTCGCCCGTGCTGCAGGACTGGGTCACGGCCA CAGACATCCGCGTGGCCTTCAGCCGCCTGCACACGTT CGGCGACGAGAACGAGGACGACTCGGAGCTGGCGCG CGACTCGTACTTCTACGCGGTGTCCGACCTGCAGGTG GGCGGCCGGTGCAAGTGCAACGGCCACGCGGCCCGC TGCGTGCGCGACCGCGACGACAGCCTGGTGTGCGACT GCAGGCACAACACGGCCGGCCCGGAGTGCGACCGCT GCAAGCCCTTCCACTACGACCGGCCCTGGCAGCGCGC CACAGCCCGCGAAGCCAACGAGTGCGTGGCCTGTAAC TGCAACCTGCATGCCCGGCGCTGCCGCTTCAACATGG AGCTCTACAAGCTTTCGGGGCGCAAGAGCGGAGGTGT CTGCCTCAACTGTCGCCACAACACCGCCGGCCGCCAC TGCCATTACTGCAAGGAGGGCTACTACCGCGACATGG

		GCAAGCCCATCACCCACCGGAAGGCCTGCAAAGCCTG TGATTGCCACCCTGTGGGTGCTGCTGGCAAAACCTGC AACCAAACCACCGGCCAGTGTCCCTGCAAGGACGGCG TGACGGGTATCACCTGCAACCGCTGCGCCAAAGGCTA CCAGCAGAGCCGCTCTCCCATCGCCCCCTGCATAAAG ATCCCTGTAGCGCCGCCGACGACTGCAGCCAGCAGCG TGGAGGAGCCTGAAGACTGCGATTCCTACTGCAAGGC CTCCAAGGGGAAGCTGAAGATTAACATGAAAAAGTACT GCAAGAAGGACTATGCCGTCCAGATCCACATCCTGAA GGCGGACAAGGCGGGGGACTGGTGGAAGTTCACGGT GAACATCATCTCCGTGTATAAGCAGGGCACGAGCCGC ATCCGCCGCGGTGACCAGAGCCTGTGGATCCGCTCGC GGGACATCGCCTGCAAGTGTCCCAAAATCAAGCCCCT CAAGAAGTACCTGCTGCTGGGCAACGCGGAGGACTCT CCGGACCAGAGCGGCATCGTGGCCGATAAAAGCAGCC TGGTGATCCAGTGGCGGGACACGTGGGCGCGGCGGC TGCGCAAGTTCCAGCAGCGTGAGAAGAAGGGCAAGTG CAAGAAGGCCTAGCG
3	Netrin-1 aa epitopic seq.	VACNCNLHARRCRFNMELYKLSGRKSGGVCLNCRHNTA GRHCH
4	Netrin-1 epitopic cDNA seq.	GTGGCCTGTAACTGCAACCTGCATGCCCGGCGCTGCC GCTTCAACATGGAGCTCTACAAGCTTTCGGGGCGCAA GAGCGGAGGTGTCTGCCTCAACTGTCGCCACAACACC GCCGGCCGCCACTGCCAT
5	aa seq. of CDR1-H (IMGT)	GYTFTSYN
6	aa seq. of CDR2-H (IMGT)	IYPGNGDT
7	aa seq. of CDR3-H (IMGT)	ARGGTGFAY
8	aa seq. of CDR1-L (IMGT)	QSVSND
-	aa seq. of CDR2-L (IMGT)	YAS
9	aa seq. of CDR3-L	QQDYSSPWT

	(IMGT et Kabat)	
10	aa sequence of mouse 4C11 VH	QAYLQQSGAELVRPGASVKMSCKAS**GYTFTSYN**MHWVK QTPRQGLEWIGA**IYPGNGDT**SYNQKFKGKATLTVDKSSS TAYMQLSSLTSEDSAVYFC**ARGGTGFAY**WGQGTLVTVS A
11	aa sequence of mouse 4C11 VL	SIVMTQTPKFLLVSAGDRVTITCKAS**QSVSND**VAWYQQK PGQSPKLLIY**YAS**NRYTGVPDRFTGSGYGTDFTFTISTVQ AEDLAVYFC**QQDYSSPWT**FGGGTKLEIK
12	Full aa sequence of 4C11 (VH + mouse IgG1 CH)	QAYLQQSGAELVRPGASVKMSCKAS**GYTFTSYN**MHWVK QTPRQGLEWIGA**IYPGNGDT**SYNQKFKGKATLTVDKSSS TAYMQLSSLTSEDSAVYFC**ARGGTGFAY**WGQGTLVTVS AAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVT VTWNSGSLSSGVHTFPAVLESDLYTLSSSVTVPSSPRPS ETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSV FIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDD VEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFK CRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAK DKVSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMN TNGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHNHHT EKSLSHSPGK
13	Full aa sequence of 4C11 (VL + mouse Kappa CL)	SIVMTQTPKFLLVSAGDRVTITCKAS**QSVSND**VAWYQQK PGQSPKLLIY**YAS**NRYTGVPDRFTGSGYGTDFTFTISTVQ AEDLAVYFC**QQDYSSPWT**FGGGTKLEIKRADAAPTVSIFP PSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNG VLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEAT HKTSTSPIVKSFNRNEC
14	VL aa sequence of humanized variant of 4C11	EIVMTQSPATLSVSPGERATLSCKAS**QSVSND**VAWYQQK PGKAPKLLIY**YAS**NRYTGIPPRFSGSGYGTDFTLTINNIES EDAAYYFC**QQDYSSPWT**FGQG
15	VL aa sequence of humanized variant of 4C11	DIQMTQSPSSLSASVGDRVTITCKAS**QSVSND**VAWFQQR PGQSPRRLIY**YAS**NRYTGVPSRFSGSGSGTDFTFTISSLE AEDAATYYC**QQDYSSPWT**FGQG
16	VL aa sequence of humanized variant of 4C11	DIQMTQSPSSLSASVGDRVTITCKAS**QSVSND**VAWYQQK PGQAPRLLIY**YAS**NRYTGIPPRFSGSGYGTDFTLTINNIES EDAAYYFC**QQDYSSPWT**FGQG

17	VL aa sequence of humanized variant of 4C11	DIQMTQSPSSLSASVGDRVTITCKAS**QSVSND**VAWYLQK PGQSPQLLIY**YAS**NRYTGVPSRFSGSGSGTDFTFTISSLE AEDAATYYC**QQDYSSPWT**FGQG
18	VL aa sequence of humanized variant of 4C11	DIVMTQTPLSLPVTPGEPASISCKAS**QSVSND**VAWYQQK PGQAPRLLIY**YAS**NRYTGIPPRFSGSGYGTDFTLTINNIES EDAAYYFC**QQDYSSPWT**FGQG
19	VL aa sequence of humanized variant of 4C11	EIVMTQSPATLSVSPGERATLSCRAS**QSVSND**VAWYQQK PGQAPRLLIY**YAS**NRYTGIPARFSGSGSGTEFTLTISSLQS EDFAVYYC**QQDYSSPWT**FGQG
20	VH aa sequence of humanized variant of 4C11	QVQLVQSGAEVKKPGASVKVSCKAS**GYTFTSYN**MHWVR QATGQGLEWMGA**IYPGNGDT**SYNQKFKGRVTITADKSTS TAYMELSSLRSEDTAVYYC**ARGGTGFAY**WGQG
21	VH aa sequence of humanized variant of 4C11	QVQLQQSGPGLVKPSQTLSLTCAIS**GYTFTSYN**MHWIRQ PPGKGLEWIGA**IYPGNGDT**SYNQKFKGRVTITADKSTSTA YMELSSLRSEDTAVYYC**ARGGTGFAY**WGQG
22	VH aa sequence of humanized variant of 4C11	QVQLQQSGPGLVKPSQTLSLTCAIS**GYTFTSYN**MHWVRQ ATGQGLEWMGA**IYPGNGDT**SYNQKFKGRLTISKDTSKNQ VVLTMTNMDPVDTATYYC**ARGGTGFAY**WGQG
23	VH aa sequence of humanized variant of 4C11	EVQLVQSGAEVKKPGESLRISCKGS**GYTFTSYN**MHWVR QATGQGLEWMGA**IYPGNGDT**SYNQKFKGRFTISRDDSK NTAYLQMNSLKTEDTAVYYC**ARGGTGFAY**WGQG
24	VH aa sequence of humanized variant of 4C11	QVQLQESGPGLVKPSQTLSLTCTVS**GYTFTSYN**MHWVR QAPGQGLEWMGA**IYPGNGDT**SYNQKFKGRVTISVDTSK NQFSLKLSSVTAADTAVYYC**ARGGTGFAY**WGQG
25	VH aa sequence of humanized variant of 4C11	QVQLVQSGAEVKKPGASVKVSCKAS**GYTFTSYN**MHWVR QATGQGLEWMGA**IYPGNGDT**SYNQKFKGRVTITADKSTS TAYMELSSLRSEDTAVYYC**ARGGTGFAY**WGQG
26	VH aa sequence of humanized variant of 4C11	QVQLQQSGPGLVKPSQTLSLTCAIS**GYTFTSYN**MHWVRQ ATGQGLEWMGA**IYPGNGDT**SYNQKFKGRVTITADKSTST AYMELSSLRSEDTAVYYC**ARGGTGFAY**WGQG
27	VH aa sequence of humanized variant of 4C11	QVQLVQSGAEVKKPGASVKVSCKAS**GYTFTSYN**MHWVR QAPGQGLEWMGA**IYPGNGDT**SYAQKFKGRVTMTRDTST STVYMELSSLRSEDTAVYYC**ARGGTGFAY**WGQ
28	aa seq. of CDR1-H (Kabat)	SYNMH
29	aa seq. of CDR2-H	AIYPGNGDTSYNQKFKG

	(Kabat)	
30	aa seq. of CDR3-H (Kabat)	GGTGFAY
31	aa seq. of CDR1-L (Kabat)	KASQSVSNDVA
32	aa seq. of CDR2-L (Kabat)	YASNRYT
33	Forward primer	aaaagtactgcaagaaggactatgc
34	Reverse primer	ccctgcttatacacggagatg
35	Netrin-1 aa epitopic seq.	ARRCRFNMELYKLSGRKSGGVC
36	Netrin-1 epitopic cDNA seq.	GCCCGGCGCTGCCGCTTCAACATGGAGCTCTACAAGC TTTCGGGGCGCAAGAGCGGAGGTGTCTGC

CDRs under IMGT are highlighted in bold in Table 1 where appropriate.

The present invention will now be described in further detail using examples that are to be considered as non-limiting embodiments.

Brief description of the Figures:

Figure 1 : ELISA binding assay of murine 4C11 to human Netrin-1. Various concentrations of 4C11 were incubated onto 96-well microtiter plate coated with FLAG-tagged Netrin-1 (APOTECH). Bound 4C11 was detected using a goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson Immunoresearch) and a chemiluminescent substrate (PIERCE ECL western blotting substrate). The luminescent was read on a Tecan Infinite F-500 luminometer.

Figure 2 : Inhibition of Netrin-1 binding onto UNC5B-Fc by 4C11. FLAG-tagged Netrin-1 (APOTECH) in presence of various concentrations of 4C11 were incubated onto 96-well microtiter plate coated with UNC5B-Fc (Netris). Bound Netrin-1 was detected using an anti-FLAG antibody conjugated with horseradish peroxidase (Sigma) and a chemiluminescent substrate (PIERCE ECL western blotting substrate). The luminescence was read on a Tecan Infinite F-500 luminometer.

Figure 3 : Linear epitope mapping of 4C11 was performed using an array of 590 15-amino acid linear peptides covering the whole sequence of human Netrin-1 with a peptide-peptide overlap of 14 amino-acids. Binding of 4C11 on the peptide was revealed using a peroxidase (POD)-labeled anti-mouse IgG and a chemoluminescent substrate.

Figure 4 : Scheme showing the amino acid sequence of the Netrin-1 epitope (located in the second EGF-like laminin domain) recognized by the murine 4C11 antibody.

Figure 5 : Induction of caspase 3 in A549 adenocarcinomic human alveolar basal epithelial cells in presence of the murine 4C11 antibody.

Figure 6 : Growth inhibition of human xenografts in nude mice treated with the murine 4C11 antibody. A549 human lung adenocarcinoma epithelial cells were injected subcutaneously to athymic (i.e. immunodeficient) nude mice. Once the tumor had reached approximately 100 mm^3, mice (n=10 per group) were treated intraperitoneally with 5mg/kg once a week with 4C11 or an isotypic control (MOPC21),.

Figure 7 : Growth inhibition of human xenografts in nude mice treated with the murine 4C11 antibody. GRANTA-519 human mantle cell lymphoma cells were injected subcutaneously to athymic (i.e. immunodeficient) nude mice. Once the tumor had reached approximately 100 mm^3, mice (n=10 per group) were treated intraperitoneally with 2mg/kg once a week or twice a week with 4C11 or with the vehicle (PBS).

Figure 8 : In vivo effect of humanized 4C11 antibody (hum03) in the growth of rat transplantable osteosarcoma. Rat osteosarcoma tumors were transplanted in paratibial position after denudation of the periosteum (n=7). The rats were treated twice a week by intra-peritoneal injection of 4.4mg/kg of humanized 4C11 hum03 or an isotypic control with or without doxorubicin (2mg/kg) or with PBS. The figure displays the tumor fold increase at day 17.

Figure 9: Pepscan analysis of the NET1 binding domain of the anti-netrine 1 humanized 4C11 monoclonal antibody (NET1-H-mAb) HUM03. Raw Pepscan data obtained by screening humanized 4C11 mAb against the over-lapping peptide library of 590 linear peptides (x-axis). The signal strength of the interaction is visualized on the y-axis. The peak corresponds to the following amino-acid sequence: ARRCRFNMELYKLSGRKSGGVC (SEQ ID NO: 35).

Example 1: Antibody generation, screen and humanization

HTP™ mice received 8 injections (everyday two days; first injection in presence of complete Freund adjuvant, the others with incomplete adjuvant) of 100µg Netrin 1-Fc (Adipogen). Hybridoma fusion was performed 2 weeks after the first immunization (Abpro, Lexington, MA). Hybridoma supernatants were screened for specific monoclonal anti-Netrin 1 antibodies using a dual-antigen ELISA assay (Netrin 1-Fc and irrelevant Fc chimeric protein). A secondary ELISA-type assay was used to select monoclonal antibodies able to block the interaction of Netrin 1 with DCC or UNC5h2. A murine monoclonal antibody (murine 4C11 or NET1-M-mAb was then selected. This antibody is made of sequences SEQ ID NO: 12 and 13.

Humanization was performed as follows: double stranded DNA fragments coding for the light chain and heavy chain CDR sequences of murine 4C11 were combined with pools

of human frameworks. Full length variable domains were then cloned into mammalian expression vectors. Light chain variable domains were cloned in frame with a secretion signal and a human kappa constant domain. Heavy chain variable domains were cloned in frame with a leader sequence and a human IgG1 constant domain. Diversity of the library and integrity of the LC and HC reading frames was checked by sequencing. Single clones were arrayed in 96 well format and plasmid DNA was prepped for transfection into CHO cells. The humanized library was transfected into CHO cells in 96 well format. Supernatants from transfected CHO cells were then collected at 48 hours post transfection and screened by Netrin-1 binding and competition ELISA assays. The light chain and heavy chain variable domains of the top 10 hits were sequenced, aligned, and analyzed. Humanized antibodies HUM01-10 as described above were generated.

EXAMPLE 2: MAb production and protein A purification

Methods for producing monoclonal antibodies based on the nucleic acid sequences coding for the heavy and light chains are known from the person skilled in the art. Based on the sequences disclosed herein, the person skilled in the art may produce various murine, humanized and fully humanized antibodies directed against the linear epitope of SEQ ID NO: 3 or 35 or any variant thereof, such as the murine 4C11, and the humanized HUM1-10 and HUM1'-10' antibodies.

Mammalian cells are the preferred as hosts for production of therapeutic glycoproteins, due to their capability to glycosylate proteins in the most compatible form for human applications *(Jenkins et al., Nat Biotech. 1996; 14:975-81).* Mammalian host cells that could be used include, human Hela, 283, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1 African green monkey cells, quail QC1-3 cells, mouse L cells and Chinese hamster ovary cells. Bacteria very rarely glycosylates proteins, and like other type of common hosts, such as yeasts, filamentous fungi, insect and plant cells yield glycosylation patterns associated with rapid clearance from the blood stream.

The Chinese hamster ovary (CHO) cells allow consistent generation of genetically stable, highly productive clonal cell lines. They can be cultured to high densities in simple bioreactors using serum-free media, and permit the development of safe and reproducible bioprocesses. Other commonly used animal cells include baby hamster kidney (BHK) cells, NSO- and SP2/0-mouse myeloma cells. Production from transgenic animals has also been tested *(Jenkins et al., Nat Biotech. 1996; 14:975-81).*

A typical mammalian expression vector contains the promoter element (early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses e.g. RSV, HTLV1, HIV1 and the early promoter of the cytomegalovirus (mCMV, hCMV), which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript (BGH polyA, Herpes

thimidine kinase gene of Herpes simplex virus polyA (TKpa), Late SV40 polyA and 3' UTR_Beta_Globin_polyA). Additional elements include enhancers (Eμ, hIE1), Kozak sequences, signal peptide and intervening sequences flanked by donor and acceptor sites for RNA splicing. Suitable expression vectors for use in practise in practising the present invention include, for examples, vectors such as pcDNA3.1, pcDNA3.3, pOptiVEC, pRSV, pEμMCMV, pMCMVHE-UTR-BG, pHCMVHE-UTR-BG, pMCMV-UTR-BG, pHCMV-UTR-BG, pMCMVHE-SV40, pHCMVHE-SV40, pMCMV-SV40, pHCMV-SV40, pMCMVHE-TK, pHCMVHE-TK, pMCMV-TK, pHCMV-TK, pMCMVHE-BGH, pHCMVHE-BGH, pMCMV-BGH, pHCMV-UTR-BGH).

The empty CHO Easy C cells are co-transfected with MAb expression vector for light and heavy chains following usual transient or stable transfection procedure. Secretion of H and L chains are enabled by the respective human IgH leader sequence. The coding regions for light and heavy chains are introduced into the MAb expression vector in the multiple cloning site. The transformants are analyzed for correct orientation and reading frame, the expression vector may be transfected into CHO cell line.

The harvested cell culture fluid produced from CHO cells is loaded onto the Hi Trap rProtein A column (GE Healthcare, Saint Cyr au Mont d'Or, France) that is equilibrated with Phosphate buffered saline, pH 7.2. The non-binding proteins are flowed through and removed by several washings with PBS buffer followed. The MAb is eluted off the Protein A column using a step of elution of 0.1 M citric acid at pH 3.0. Column eluent is monitored by A280. The MAb peak is pooled.

Example 3 : ELISA-type binding assay of 4C11 antibodies to Netrin-1 (Figure 1)

White 96-well microtiter plate (Costar 3912 Corning) was incubated overnight at 4°C with 100ng of His-tagged Netrin-1 (R&D 6419-N1) in 100μL of Phosphate buffer saline (PBS). After three washings with 300μL of PBS-0.05% Tween-20 (PBS-T), the plate was blocked by addition of 100μL of PBS-3% BSA and incubated 1 hour at room temperature. After three washings with 300μL of PBS-T, the plate was incubated with various quantities (10 ng to 1200ng) of the anti-netrin-1 antibody. After three washings with 300μL of PBS-T, 100μL of a relevant secondary antibody conjugated to horse radish peroxidase (e.g. goat anti-human IgG (Fc) Sigma A0170 or goat anti-mouse IgG light chain specific (kappa) Jackson Immunoresearch 115-035-174) diluted 1/10,000 in PBST-3% BSA was added and the plate was incubated 1 hour at room temperature. After three washings with 300μL of PBS-T, 100μL of a luminescent substrate of HRP (ECL western blotting substrate, PIERCE) is added. After 5 to 10 minutes, the luminescence was read on a Tecan Infinite F-500 luminometer.

Figure 1 shows the typical dose-dependent interaction of the 4C11 murine antibody to adsorbed netrin-1 in the ELISA-type assay.

The concentration of the antibodies allowing 50% binding (EC_{50}) are calculated from the sigmoidal binding curves as displayed in figure 1. Table 1 displays the EC_{50} values (in µg/mL and in nM) of the different murine (4C11 whole antibody as well as Fab and Fab'2 fragments) and humanized 4C11 antibodies.

Table 1: Potency of the various 4C11 variants to bind to netrin-1 (Example 3) or to impair the netrin-1 binding onto UNC5B (Example 5):

	Potency Data			
	Binding (EC50)		**Ligand binding inhibition (IC50)**	
	ng/mL	**pM**	**ng/mL**	**pM**
4C11 murine IgG1	57	380	75	502
4C11 murine IgG2a	63	420	85	567
4C11 Fab	650	13000	844	16880
4C11 Fab'2	35	350	89	890
HUM01	134	894	54	360
HUM03	166	1107	65	434
HUM09	153	1021	71	474
HUM08	134	894	77	514
HUM06	139	927	82	547
HUM05	151	1007	85	567
HUM07	125	834	89	594
HUM10	211	1407	110	734
HUM04	198	1321	199	1327
HUM02	260	1734	215	1434

HUM03 was selected for further experiments. HUM03 may sometimes be called Humanized 4C11 hereafter.

Example 4 : Antibody binding assay by surface plasmon resonance

The binding properties of the antibodies were analyzed using Biacore T I 00 (GE Healthcare) with the associated Software Biacore T100 Control Biacore TI 00 Evaluation, and the Chip:CM5-Chip as Assay format.

Murine 4C11 (IgG1) antibody (SEQ ID NO: 12 and 13) was captured via amine coupled capture molecules. A series with increasing concentrations of netrin-1 was injected. Chip surface with amine coupled capture molecule alone was used as reference control surface for correction of possible buffer-effects or non-specific binding of netrin-l.

Capture molecules: Anti-mouse IgG antibodies (from goat, Jackson Immuno Research).

Amine coupling of capture molecules. Standard amine coupling according to the manufacturer's instructions: running buffer: HBS- N buffer, activation by mixture of EDC/NHS, aim for ligand density of 10000 RU; the capture-antibodies were diluted in coupling buffer 10mM NaAc, pH 4.5, c = 30 µg/mL; finally remaining activated carboxyl groups were blocked by injection of 1 M ethanolamine.

4C11 antibody capture : Capturing of 4C11 antibody on flow cells 2 to 4: Flow 5 µL/min, contact time 72 seconds, c(Anti-mouse IgG antibodies) = 5nM. Capture buffer : PBS (pH7.4), 0.005% Tween 20

Analyte sample: Classical concentration series were measured at a flow rate of 50 µL/min by consecutive injection of the analyte in 5 or 6 increasing concentrations (c = 2-164 nM). Running buffer : 20mM Hepes pH7.4, 600mM NaCl, 0.005% Tween 20. The analyte was injected for 3 minutes followed by a dissociation phase of 90s.

Semi-quantitative surface plasmon resonance (SPR) analysis of Netrin-1 binding kinetics to captured 4C11 was performed using BIAcore analysis of binding of 4C11 murine antibody as provided and described in the present invention to human netrin-1. 4C11 antibody was captured on the chip surface via amine coupled anti-human IgG(Fc) molecules. A series with increasing concentrations of human netrin-1 was injected and the kinetic binding behaviour was monitored by SPR changes. Changes as relative units (RU) versus a control chip were recorded on the y-axis over time (x-axis). Representative association and dissociation curve of the captured analyte 4C11 at different concentrations of injected human netrin-1 was observed.

Kinetic parameters were then calculated by using the usual double referencing (control reference: binding of analyte to capture molecule; Flow Cell: netrin-1 concentration "0" as Blank) and calculation with model ' titration kinetics 1: 1 binding.

Table 2 gives the affinity data measured by SPR (BIACORE® T100) at 25 °C in PBS:

	Ka (M-1s-1)	**Kd (s-1)**	**KD (nM)**
Exp 1	1,4 x 105	1,8 x 105	13,3
Exp 2	1,4 x 105	1,8 x 105	12,7

Example 5: 4C11 inhibits the binding of Netrin-1 to UNC5B (Figure 2)

White 96-well microtiter plate (Costar 3912 Corning) was incubated overnight at 4°C with either 100ng UNC5B-Fc (R&D 1006-UN-050) or DCC-Fc (R&D 844-DC-050) in 100µL of Phosphate buffer saline (PBS). After three washings with 300µL of PBS-0.05% Tween-20 (PBS-T), the plate was blocked by addition of 100µL of PBS-2% BSA and incubated 1 hour

at room temperature. After three washings with 300µL of PBS-T, the plate was incubated for 1 hour at room temperature, with 100µL of PBS-1% BSA containing 50ng/mL FLAG-tagged netrin-1 (Adipogen) and various quantities (0,2 ng to 3000ng) of the 4C11 antibody. After three washings with 300µL of PBS-T, 100µL of an anti-FLAG M2 monoclonal antibody conjugated to horse radish peroxidase (HRP) (Sigma A8592) diluted 1/5,000 in PBST-1% BSA was added and the plate was incubated 1 hour at room temperature. After three washings with 300µL of PBS-T, 100µL of a luminescent substrate of HRP (ECL western blotting substrate, PIERCE) is added. After 5 to 10 minutes, the luminescence was read on a Tecan Infinite F-500 luminometer.

Figure 2 shows the typical dose-dependent inhibition of netrin-1 binding to UNC5B by increasing amounts of the murine 4C11 murine antibody in an ELISA-type assay.

The concentration of the antibodies allowing 50% inhibition (IC_{50}) are calculated from the sigmoidal binding curves as displayed in figure 5. Table 1 gives the IC_{50} values (in µg/mL and in nM) of the different murine (4C11 whole antibody as well as Fab and Fab'2 fragments) and humanized 4C11 antibodies.

Example 6 : Netrin-1 epitope mapping of murine 4C11 (Figures 3 and 4)

Epitope mapping was performed using an array of 590 15-amino acid linear peptides covering the whole sequence of human Netrin-1 (without the signal peptide) with a peptide-peptide overlap of 14 amino acids. Linear peptides were synthesized using standard Fmoc-chemistry and deprotected using trifluoric acid. The 455-well credit-card format polypropylene cards containing the covalently linked peptides were incubated 30min at 25°C in PBST (PBS-1% Tween 80) containing 5% SQ (SQ, Super-Q, 4% horse serum (v/v), 5% ovalbumin (w/v) in PBST. After washing, the peptides were incubated with the 4C11 (1 µg/mL) PBST-0.1% SQ. After washing, the peptides were incubated with a 1/1000 dilution of anti-mouse antibody peroxidase conjugate (SouthernBiotech) for one hour at 25° C. After washing, the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 microlitres of 3 percent H2O2 are added. After one hour, the color development are quantified with a charge coupled device (CCD) – camera and an image processing system.

Figure 3 shows that the murine 4C11 interacts specifically with peptides included in the SEQ ID NO: 3: VACNCNLHARRCRFNMELYKLSGRKSGGVCLNCRHNTAGRHCH.

Figure 4 is a cartoon displaying the location of the epitope recognized by the murine 4C11 antibody. This epitope is carried by the second EGF-like domain of Netrin-1.

Pepscan epitope mapping using the spot array of 590 15-amino acid linear peptides covering the whole sequence of human NET1. As shown in Fig. 9, the antibody HUM03 binds 8 overlapping peptides corresponding to the amino acid sequence "ARRCRFNMELYKLSGRKSGGVC" (SEQ ID NO: 35) which is present within the V-2 domain of NET1. The binding epitope of the antibody thus overlaps with the NET1 domain involved in

the interaction with UNC5B. To confirm that the V-2 domain of NET1 is indeed responsible for the interaction with HUM03, we generated a collection of mutants and performed in vitro binding assays of these mutants with the antibody as well as with UNC5B. The point mutation K358L was sufficient to decrease interaction with the HUM03. The triple mutation R348A-R349A-R351A reduced the interaction.

Example 7 : 4C11-induced caspase-3 in human A549 lung adenocarcinoma epithelial cells (Figure 5)

On day 1, cells were plated in serum free medium (1.8 x 10^5 cells per well in six-well plates with 1mL per well). On day 2, the medium was replaced with 1mL fresh serum-free medium containing either vehicle (Ctrl), the mouse 4C11 antibody, or an murine IgG1,k irrelevant antibody (Ab) (10µg/mL). Treatments were performed in duplicate. On day 3, cells from the 2 identically treated wells were harvested and combined as one pool. After centrifugation, cell pellets were resuspended in 55µL of lysis buffer provided in the Caspase 3/CPP32 Fluorimetric Assay Kit (Gentaur Biovision, Brussels, Belgium). Apoptosis was then monitored by measuring caspase-3 activity using the above-mentioned kit. All values were normalized to the control.

Figure 5 shows that the murine 4C11 antibody induces caspase 3 activity in human A549 lung adenocarcinoma epithelial cells.

Example 8 : in vivo 4C11-induced tumor growth inhibition of A549 (Human lung adenocarcinoma epithelial cells) (Figure 6) and GRANTA (human mantle cell lymphoma cells) cells xenografts (Figure 7).

Seven-week-old (20-22 g body weight) female athymic nu/nu mice were obtained from Charles River animal facility. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. All tumors were implanted by s.c. injections of tumor cells (10^7 A549 cells or 10^6 GRANTA cells) in 200µL PBS into the right flank of the mice. Treatment with the 4C11 antibody started when tumors were established (V≈100mm^3, approximately 15-20 days post-injection). Mice received intraperitoneal injection of the 4C11 antibody (various doses and schedules), the vehicle (PBS), or an isotypic control (MOPC21) (n=10 mice). Tumor sizes were measured with a caliper. Tumor volumes were calculated with the formula $v = 0.5*(length*width^2)$.

Mice with A549 xenografts were treated with 5mg/kg of 4C11 once a week, while mice with GRANTA xenografts received lower dose of 4C11 (2mg/kg) once or twice a week.

Figures 6 and 7 demonstrated significant suppression of tumor growth of human A549 lung adenocarcinoma epithelial cells (A) and human GRANTA human mantle cell lymphoma (B) *xenografted in immunodeficient mice*.

The 4C11 antibody shows an inhibition of A549 and GRANTA tumor growth.

Example 9 : Synergy between humanized 4C11 (hum03) and doxorubicine in rat osteosarcoma (Figure 8).

A radiation-induced rat osteosarcoma has been transformed into a transplantable model gratfed in paratibial position after denudation of the periosteum (ref. Allouche M.et al. , 1980, *Int. J. Cancer 26, 777-782).* As no netrin-1 was expressed in this tumor, we stimulated netrin-1 and its receptors expression using a chemotherapy agent (Dox, Dorubicin) as recently published (*cf Paradisi et al.2013 EMBO Mol Med (2013) 5, 1821–1834*). Rats grafted with the osteosarcoma received twice a week, intra-peritoneal injection of humanized 4C11 hum03 (4.4mg/kg) or the vehicle (ctr). Some animals received in addition intra-peritoneal injection of doxorubicin (2mg/kg). Tumor sizes were measured with a caliper. Tumor volumes were calculated with the formula $v = 0.5*(length*width^2)$. In parallel, MRI is used to follow the growth pattern.

Figure 8 shows that an inhibition of osteosarcoma growth is achieved in animals treated simultaneously with 4C11 and doxorubicin.

Example 10 : Effet of murine 4C11 in Inflammatory model of spontaneous colon cancer generated by inflammation. (mouse model of IBD (Inflammatory Bowel Disease)-associated colorectal cancer)

(*cf Ref Proc Natl Acad Sci U S A. 2009 Oct 6;106(40):17146-51*).

Mice were treated with AOM + DSS and treated with PBS or 4C11 intraperitoneally 2mg/kg twice a week (n=7) as described previously (Ref : Neufert C et al (2007) Nat Protoc 2: 1998-2004). Briefly, pathogen-free 8-week old female Wild- Type Balb/C mice were injected intraperitoneally with 10 mg/kg body weight of AOM dissolved in PBS. The day after, 2.5% DSS was given in the drinking water over one week, followed by 2 weeks of regular water. Mice were treated with DSS for 1 week every 2 weeks until the tenth week of the experiment and were injected three times per week with 4C11 or with PBS. The animals were sacrificed at the beginning of the tenth week and the colon was removed for histological analysis. Table 3 clearly shows that the treatment of the mice with the 4C11 antibody prevents or slow-down the development of inflammatory-driven colon adenocarcinomas.

Table 3 : Effect of the anti-Netrin mAb 4C11 on inflammatory-driven colon tumors in vivo. Mouse model of IBD (Inflammatory Bowel Disease)-associated colorectal cancer was generated as already described (*Proc Natl Acad Sci U S A. 2009 Oct 6;106(40):17146-51*). Mice were first treated with azoxymethane (AOM) and Dextran sulfate sodium (DSS) to induce colorectal cancer. Mice were treated with PBS or 4C11 intraperitoneally 2mg/kg twice a week (n=7). Mice were sacrificed. The removed colon was fixed in formaldehyde for histological analysis. Table 3 presents the percentage of mice displaying various pre-cancerous or cancerous colon lesions.

Colon lesion	Treatment	
	PBS	4C11
Focal hyperplasia	0%	86%
Low grade adenoma	50%	0%
High-grade adenoma	16.7%	0%
Early ADK	33.3%	42.8%
ADK	66.7%	14.3%

Example 11: Netrin-1 protein quantification in human cancer cells:

For immunoblot analysis, cells are lysed by sonication in modified RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor cocktail and 5mM DTT) and incubated 1h at 4°C. Cellular debris are pelletted by centrifugation (10.000g 15' at 4°C) and protein extracts (200 µg per lane) are loaded onto 10% SDS-polyacrylamide gels and blotted onto PVDF sheets (Millipore Corporation, Billerica, MA, U.S.A.). Filters are blocked with 10% non-fat dried milk and 5% BSA in PBS/0.1% Tween 20 (PBS-T) over-night and then incubated for 2h with rabbit polyclonal α-netrin-1 (dilution 1:500, clone H104, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal β-actin (Santa Cruz Biotechnologies) antibodies. After three washes with PBS-T, filters are incubated with the appropriate HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch, Suffolk, UK) for 1h. Detection is performed using West Dura Chemiluminescence System (Pierce, Rockford, IL, U.S.A.).

For immunofluorescence study, cells are detached, centrifuged on cover slips with a cytospiner (Shandon Cytospin 3, Thermo Scientific) and fixed for 30 minutes with 4% (v/v) paraformaldehyde. Cells are then permeabilized for 30 minutes in 0.2% Triton X-100/PBS and blocked in PBS containing 2% BSA and 2% normal donkey serum. Endogenous netrin-1 is stained using rat monoclonal α-netrin-1 antibody (R&D systems) and Alexa-488 Donkey anti-rat IgG (Molecular probes). Nuclei are counterstained using Hoescht staining (Sigma).

Example 12: Example of cancers over-expressing netrin-1 and expressing DCC and/or UNC5A and/or B and/or C and/or D to be candidate for the treatment with a UNC5-TRAP or humanized 4C11 antibody.

The percentage of netrin-1 overexpressing cases is given for each type of cancers for which expression of netrin-1 and its receptors have been quantified.

- 60 % of metastatic breast cancer (Fitamant et al., PNAS 2008),
- 47 % of non-small cell lung cancer (Delloye-Bourgeois et al., JNCI 2009),
- 38 % of aggressive neuroblastoma (Delloye-Bourgeois et al., J. Exp. Med. 2009),

- 61 % of pancreatic adenocarcinoma (Link et al., Annals of Chir. Onco. 2007; Dumartin et al., Gastro 2010),
- 100 % of primary melanoma (n=7), melanoma metastasis (n=6) (Kaufmann et al., Cellular Oncology 2009),
- 76 % of ovarian cancers (Panastasiou et al0., Oncotarget 2011),
- 65% of glioblastoma,
- 60 % of acute myeloid leukemia and chronic lymphocytic leukemia
- 50 % of aggressive B-cell lymphoma,
- 30 % of sarcoma,
- 40 % of renal adenocarcinoma,
- 22 % of head and neck cancers,
- Testicular cancers (36 % of embryonal carcinoma, 50 % of teratoma, 100 % of yolk sac tumors)
- 50 % of kidney cancers,
- 26 % of stomach cancers,
- 19 % of uterus cancers.

Example 13. Quantitative RT-PCR allowing to assess netrin-1 expression or overexpression in accordance with PCT/EP2013/068937:

Total RNA is extracted using NucleoSpin® RNA II Kit (Macherey Nagel, Düren, Germany) according to manufacturer's protocol. RT-PCR reactions are performed with iScript® cDNA Synthesis Kit (Biorad). One µg total RNA is reverse-transcribed using the following program: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. For expression studies, the target transcripts are amplified in LightCycler® 2.0 apparatus (Roche Applied Science), using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Applied Science). Expression of target genes is normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) genes, used as housekeeping genes. The amount of target transcripts, normalized to the housekeeping gene, is calculated using the comparative C_T method. A validation experiment is performed, in order to demonstrate that efficiencies of target and housekeeping genes are approximately equal. The sequences of the primers are as follows:

Forward primer: aaaagtactgcaagaaggactatgc SEQ ID NO:33.

Reverse primer: ccctgcttatacacggagatg SEQ ID NO:34.

Example 14. Netrin-1 protein quantification in human cancer cells in accordance with PCT/EP2013/068937:

For immunoblot analysis, cells are lysed by sonication in modified RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor cocktail and 5mM DTT) and incubated 1h at 4°C. Cellular debris are

pelletted by centrifugation (10.000g 15' at 4°C) and protein extracts (200 µg per lane) are loaded onto 10% SDS-polyacrylamide gels and blotted onto PVDF sheets (Millipore Corporation, Billerica, MA, U.S.A.). Filters are blocked with 10% non-fat dried milk and 5% BSA in PBS/0.1% Tween 20 (PBS-T) over-night and then incubated for 2h with rabbit polyclonal α-netrin-1 (dilution 1:500, clone H104, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal β-actin (Santa Cruz Biotechnologies) antibodies. After three washes with PBS-T, filters are incubated with the appropriate HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch, Suffolk, UK) for 1h. Detection is performed using West Dura Chemiluminescence System (Pierce, Rockford, IL, U.S.A.).

For immunofluorescence study, cells are detached, centrifuged on cover slips with a cytospiner (Shandon Cytospin 3, Thermo Scientific) and fixed for 30 minutes with 4% (v/v) paraformaldehyde. Cells are then permeabilized for 30 minutes in 0.2% Triton X-100/PBS and blocked in PBS containing 2% BSA and 2% normal donkey serum. Endogenous netrin-1 is stained using rat monoclonal α-netrin-1 antibody (R&D systems) and Alexa-488 Donkey anti-rat IgG (Molecular probes). Nuclei are counterstained using Hoescht staining (Sigma).

Example 15: *In vivo* xenograft models.

Different human cell lines (lung adenocarcinoma epithelial cell lines H358 and A549, and mantle cell lymphoma cell lines GRANTA-519 and diffuse large B-Cell lymphoma oci-ly3) in exponential growth were harvested from culture, washed twice with sterile PBS, counted and 5.10^6 cells were resuspended in PBS before s.c. implantation on the right flank of 5 week old female Swiss/nude mice. Tumor volumes (V) were determined by the formula V= 0.5 (length x $width^2$) with a caliper. 100+/- $20mm^3$ tumors were established before randomization into groups (10 mice each). Anti-netrine 1 antibody or an isotypic control were i.v. (H358, A549, oci-ly3) or i.p. (GRANTA-519) injected into mice at 10mg/kg twice a week. Percent of Tumor Growth Inhibition (%TGI) was determined for each cell line grafted at the day (d) indicated in the following table and calculated by the formula TGI (%)=(1-T/C)x100 where T indicates the mean tumor volume of test group (treated with the anti-netrine 1 antibodies) at day d, and C indicates the mean volume of the isotype control-treated group. Tumor growth inhibition <50% is considered meaningful.

Human cell line xenograft	Antibody	% TGI	Day d after treatment
GRANTA-519	Murine 4C11	63	40
H358	Humanized 4C11 HUM03	56	40
A549	Humanized 4C11 HUM03	40	47
OCYl3	Humanized 4C11 HUM03	33	20

The murine and humanized antibodies of the invention had efficient tumor growth inhibiting effects.

CLAIMS:

1. An isolated or purified polypeptide:

- consisting of sequence SEQ ID NO: 3 or SEQ ID NO: 35 or a variant consisting of a fragment of sequence SEQ ID NO: 3 comprising SEQ ID NO: 35, or

- encoded by the cDNA of sequence SEQ ID NO: 4 or SEQ ID NO: 36, or a variant thereof by virtue of the degeneracy of the genetic code.

2. Use of a polypeptide according to claim 1 for the preparation of a monoclonal antibody.

3. A netrin-1 binding antibody which comprises a CDR1-H of sequence SEQ ID NO: 5, a CDR2-H of sequence SEQ ID NO: 6, a CDR3-H of sequence SEQ ID NO: 7, and a CDR1-L of sequence SEQ ID NO: 8, a CDR2-L of sequence YAS and a CDR3-L of sequence SEQ ID NO: 9, wherein the antibody has the property of binding to netrin-1 and inducing cell death or apoptosis of a tumor cell via an UNC5 receptor or DCC receptor.

4. A netrin-1 binding antibody which comprises a CDR1-H of sequence SEQ ID NO: 28, a CDR2-H of sequence SEQ ID NO: 29, a CDR3-H of sequence SEQ ID NO: 30, and a CDR1-L of sequence SEQ ID NO: 31, a CDR2-L of sequence SEQ ID NO: 32 and a CDR3-L of sequence SEQ ID NO: 9, wherein the antibody has the property of binding to netrin-1 and inducing cell death or apoptosis of a tumor cell via an UNC5 receptor or DCC receptor.

5. The netrin-1 binding antibody of claim 3 or 4, which is an epitope binding fragment thereof.

6. The netrin-1 binding antibody of any one of claims 3 to 5, which comprises an amino acid sequence SEQ ID NO: 10, 11, 12 or 13, preferably both sequences SEQ ID NO: 10 and 11, or SEQ ID NO: 12 and 13.

7. The netrin-1 binding antibody of any one of claims 3 to 6, which comprises an amino acid sequence selected from the group of SEQ ID NO:14 to 19 and/or from the group of SEQ ID NO: 20 to 27, preferably it comprises an amino acid sequence selected from the group of SEQ ID NO: 14 to 19 and an amino acid sequence selected from the group of SEQ ID NO: 20 to 27.

8. The netrin-1 binding antibody of claim 7, which comprises a pair of amino acid sequences selected from the pairs SEQ ID NO: 14 and 20; 15 and 21; 16 and 22; 17 and 23; 17 and 24; 16 and 25; 17 and 26; 17 and 22; 18 and 25; and 16 and 21.

9. A pharmaceutical composition comprising an antibody according to any one of claims 3 to 8, and a pharmaceutically acceptable vehicle, carrier or diluent.

10. Use of a pharmaceutical composition of claim 9 in the manufacture of a medicament for treating cancer, wherein the cancer has tumoral cells expressing a netrin-1 receptor, in the presence of netrin-1.

11. Use of a pharmaceutical composition of claim 9 in the manufacture of a medicament for treating cancer, wherein the cancer has tumoral cells expressing a netrin-1 receptor and netrin-1 or a tumoral cells expressing netrin-1 within stroma cells expressing netrin-1.

12. Use of a pharmaceutical composition according to claim 9 in the manufacture of a medicament for treating cancer.

13. Use of the netrin-1 binding antibody of any one of claims 3 to 8 in the manufacture of a medicament for treating cancer.