DLL4-BINDING MOLECULES

FIELD OF THE INVENTION

The invention relates to the field of human therapy, in particular cancer therapy and agents and compositions useful in such therapy.

BACKGROUND OF THE INVENTION

As summarized in US 2008/0014196, angiogenesis is implicated in the pathogenesis of a number of disorders, including solid tumors and metastasis. In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment for the growth and metastasis of the tumor. Folkman *et al.*, Nature 339 -58 (1989), which allows the tumor cells to acquire a growth advantage compared to the normal cells. Therefore, anti-angiogenesis therapies have become an important treatment option for several types of tumors. These therapies have focused on blocking the VEGF pathway (Ferrara *et al.*, Nat Rev Drug Discov. 2004 May;3(5):391-400.

The Notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic development and in adult organisms. Notch signaling is dysregulated in many cancers, e.g. in T-cell acute lymphoblastic leukemia and in solid tumors (Sharma *et al.* 2007, Cell Cycle 6 (8): 927–30; Shih *et al.*, Cancer Res. 2007 Mar 1;67(5): 1879-82).

Dll4 (or Delta like 4 or delta-like ligand 4) is a member of the Delta family of Notch ligands. The extracellular domain of Dll4 is composed of an N-terminal domain, a Delta/Serrate/Lag-2 (DSL) domain, and a tandem of eight epidermal growth factor (EGF)-like repeats. Generally, the EGF domains are recognized as comprising

amino acid residues 218-251 (EGF-1; domain 1), 252-282 (EGF-2; domain 2), 284-322 (EGF-3; domain 3), 324-360 (EGF-4; domain 4), and 362-400 (EGF-5; domain 5), with the DSL domain at about amino acid residues 173-217 and the N-terminal domain at about amino acid residues 27-172 of hDll4 (WO 2008/076379).

It has been reported that Dll4 exhibits highly selective expression by vascular endothelium, in particular in arterial endothelium (Shutter *et al.* (2000) Genes Develop. 14: 1313-1318). Recent studies in mice have shown that Dll4 is induced by VEGF and is a negative feedback regulator that restrains vascular sprouting and branching. Consistent with this role, the deletion or inhibition of Dll4 results in excessive angiogenesis (Scehnet *et al.*, Blood. 2007 Jun 1; 109(11):4753-60). This unrestrained angiogenesis paradoxically decreases tumor growth due to the formation of non-productive vasculature, even in tumors resistant to anti-VEGF therapies (Thurston *et al.*, Nat Rev Cancer. 2007 May;7(5):327-31; WO 2007/070671; Noguera-Troise *et al.*, Nature. 2006 Dec 21; 444(7122)). Furthermore, the combined inhibition of VEGF and Dll4 is shown to provide superior anti-tumor activity compared to anti-VEGF alone in xenograft models of multiple tumor types (Noguera-Troise *et al.*, Nature. 2006 Dec 21; 444(7122):1032-7; Ridgway *et al.*, Nature. 2006 Dec 21;444(7122):1083-7). Due to these results, Dll4 is being considered a promising target for cancer therapy, and several biological compounds that target Dll4 are in (pre-)clinical development have been described: REGN-421 (= SAR153192; Regeneron, Sanofi-Aventis; WO2008076379) and OPM-21M18 (OncoMed) (Hoey *et al.*, Cell Stem Cell. 2009 Aug 7; 5(2):168-77), both fully human Dll4 antibodies; YW152F (Genentech), a humanized Dll4 antibody (Ridgway *et al.*, Nature. 2006 Dec 21;444(7122):1083-7); Dll4-Fc (Regeneron, Sanofi-Aventis), a recombinant fusion protein composed of the extracellular region of Dll4 and the Fc region of human IgG1 (Noguera-Troise *et al.*, Nature. 2006 Dec 21;444(7122)).

However, the state-of-the art monoclonal antibodies (MAbs) and fusion proteins have several shortcomings in view of their therapeutic application: To prevent their

degradation, they must be stored at near freezing temperatures. Also, since they are quickly digested in the gut, they are not suited for oral administration. Another major restriction of MAbs for cancer therapy is poor transport, which results in low concentrations and a lack of targeting of all cells in a tumor.

In view of the above, it has been an object of the invention to provide improved Dll4-binding molecules for human therapy.

Such Dll4- binding molecules, or Dll4 antagonists, are useful as pharmacologically active agents in compositions in the prevention, treatment, alleviation and/or diagnosis of diseases or conditions associated with Dll4-mediated effects on angiogenesis. Examples for such diseases are cancer and eye diseases including Age-related Macular Degeneration (AMD) and Diabetic Retinopathy (DR). It has been a further object of the invention to provide methods for the prevention, treatment, alleviation and/or diagnosis of such diseases, disorders or conditions, involving the use and/or administration of such agents and compositions.

In particular, it is has been an object of the invention to provide such pharmacologically active agents, compositions and/or methods that provide certain advantages compared to the agents, compositions and/or methods currently used and/or known in the art. These advantages include improved therapeutic and/or pharmacological properties and/or other advantageous properties, e.g. for manufacturing purposes, especially as compared to conventional anti-Dll4 antibodies as those described above, or fragments thereof.

More in particular, it has been an object of the invention to provide novel Dll4-binding molecules and/or polypeptides containing them, and, specifically, Dll4-binding molecules that bind to mammalian and, especially, human Dll4, wherein such molecules or polypeptides are suitable for the therapeutic and diagnostic

purposes as described herein. It has been a further object of the invention to provide immunoglobulin single variable domains that specifically bind to Dll4.

BRIEF SUMMARY OF THE INVENTION

According to a first aspect, there are provided Dll4-binding molecules, preferably Dll4-binding immunoglobulin single variable domains like VHHs and VHs.

In another aspect, the invention relates to nucleic acids encoding Dll4-binding molecules as well as host cells containing same.

The invention further relates to a product or composition containing or comprising at least one Dll4-binding molecule of the invention and optionally one or more further components of such compositions.

The invention further relates to methods for preparing or generating the Dll4-binding molecules, nucleic acids, host cells, products and compositions described herein.

The invention further relates to applications and uses of the Dll4-binding molecules, nucleic acids, host cells, products and compositions described herein, as well as to methods for the prevention and/or treatment for diseases and disorders associated with with Dll4-mediated effects on angiogenesis.

These and other aspects, embodiments, advantages and applications of the invention will become clear from the further description hereinbelow.

DEFINITIONS

Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to

the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989); Lewin, "Genes IV", Oxford University Press, New York, (1990), and Roitt *et al.*, "Immunology" (2nd Ed.), Gower Medical Publishing, London, New York (1989), as well as to the general background art cited herein; Furthermore, unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known *per se*, as will be clear to the skilled person. Reference is for example again made to the standard handbooks, to the general background art referred to above and to the further references cited therein;

Unless indicated otherwise, the terms "immunoglobulin" - whether used herein to refer to a *heavy chain antibody* or to a *conventional 4-chain antibody* – are used as general terms to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof.

Unless indicated otherwise, the term “Dll4-binding molecule” includes anti-Dll4 antibodies, anti-Dll4 antibody fragments, “anti-Dll4 antibody-like molecules” and conjugates with any of these. Antibodies include, but are not limited to, monoclonal and chimerized monoclonal antibodies. The term „antibody“ encompasses complete immunoglobulins, like monoclonal antibodies produced by recombinant expression in host cells, as well as Dll4-binding antibody fragments or “antibody-like molecules”, including single-chain antibodies and linear antibodies, so-called “SMIPs” (“Small Modular Immunopharmaceuticals”), as e.g described in WO 02/056910. Anti-Dll4 antibody-like molecules include immunoglobulin single variable domains, as defined herein. Other examples for antibody-like molecules are immunoglobulin super family antibodies (IgSF), or CDR-grafted molecules.

The term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "(single) variable domain sequence", "VHH sequence" or "protein sequence"), should generally be understood to include both the relevant amino

acid sequence as well as nucleic acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

"*Sequence identity*" between two Dll4-binding molecule sequences indicates the percentage of amino acids that are identical between the sequences. It may be calculated or determined as described in paragraph f) on pages 49 and 50 of WO 08/020079. ("Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions.)

The term "*domain*" (of a polypeptide or protein) as used herein refers to a folded protein structure which has the ability to retain its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

The term "*immunoglobulin domain*" as used herein refers to a globular region of an antibody chain (such as e.g. a chain of a conventional 4-chain antibody or of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a 2-layer sandwich of about 7 antiparallel beta-strands arranged in two beta-sheets, optionally stabilized by a conserved disulphide bond.

The term "*immunoglobulin variable domain*" as used herein means an immunoglobulin domain essentially consisting of four "framework regions" which are referred to in the art and hereinbelow as "framework region 1" or "FR1"; as "framework region 2" or"FR2"; as "framework region 3" or "FR3"; and as "framework region 4" or "FR4", respectively; which framework regions are interrupted by three "complementarity determining" regions or "CDRs", which are referred to in the art and hereinbelow as "Complementarity Determining

Region 1"or "CDR1"; as "Complementarity Determining Region 2" or "CDR2"; and as "Complementarity Determining Region 3" or "CDR3", respectively. Thus, the general structure or sequence of an immunoglobulin variable domain can be indicated as follows: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4.

It is the immunoglobulin variable domain(s) that confer specificity to an antibody for the antigen by carrying the antigen-binding site.

The term "*immunoglobulin single variable domain*" as used herein means an immunoglobulin variable domain which is capable of specifically binding to an epitope of the antigen without pairing with an additional variable immunoglobulin domain. Examples of immunoglobulin single variable domains in the meaning of the present invention are the immunoglobulin single variable domains VH and VL and (VH domains and VL domains) and "*VHH domains*" (or simply "VHHs") from camelides, as defined hereinafter.

In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')2 fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain, as binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e. by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

"*VHH domains*", also known as VHHs, V_HH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of "heavy chain antibodies" (i.e. of "antibodies devoid of light chains"; Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R.: "Naturally

occurring antibodies devoid of light chains"; Nature 363, 446-448 (1993)). The term "VHH domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_H domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_L domains"). As opposed to VH or VL domains, which will normally not bind to an epitope as a single antigen binding domain, VHH domains can specifically bind to an epitope without an additional antigen binding domain. VHH domains are small, robust and efficient antigen recognition units formed by a single immunoglobulin domain.

In the context of the present invention, the terms VHH domain, VHH, V_HH domain, VHH antibody fragment, VHH antibody, as well as "Nanobody®" and "Nanobody® domain" ("Nanobody" being a trademark of the company Ablynx N.V.; Ghent; Belgium) are used interchangeably and are representatives of immunoglobulin single variable domains (having the general structure: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 and specifically binding to an epitope without requiring the presence of a second immunoglobulin variable domain), and which are distinguished from the VHs by the so-called “hallmark residues”, as defined in e.g. WO 2009/109635, Fig.1.

“VH domains” and “VL domains” (or simply “VHs” or VLs”), respectively, which are derived from 4-chain antibodies, in particular from human antibodies are "single domain antibodies", also known as "domain antibodies", "Dab"s, "Domain Antibodies", and "dAbs" (the terms "Domain Antibodies" and "dAbs" being used as trademarks by the GlaxoSmithKline group of companies) have been described in e.g. Ward, E.S., *et al.*: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli"; Nature 341: 544-546 (1989); Holt, L.J. *et al.*: "Domain antibodies: proteins for therapy"; TRENDS in Biotechnology 21(11): 484-490 (2003); and WO 2003/002609.

Single domain antibodies correspond to the variable domains of either the heavy or light chains of non-camelid mammalian, in particular human antibodies. In order to bind an epitope as a single antigen binding domain, i.e. without being paired with a VL or VH domain, respectively, specific selection for such antigen binding properties is required, e.g. by using libraries of human single VH or VL domain sequences.

Single domain antibodies have, like VHHs, a molecular weight of approximately ca. 13 to ca. 16 kDa and, if derived from fully human sequences, do not require humanization for e.g. therapeutic use in humans. As in the case of VHH domains, they are well expressed also in prokaryotic expression systems, providing a significant reduction in overall manufacturing costs.

The amino acid residues of an immunoglobulin single variable heavy domain are numbered according to the general numbering for V_H domains given by Kabat *et al.* ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to VHH domains from Camelids, as shown e.g. in Figure 2 of Riechmann and Muyldermans, J. Immunol. Methods 231, 25-38 (1999). According to this numbering, by way of example

- FR1 comprises the amino acid residues at positions 1-30,
- CDR1 comprises the amino acid residues at positions 31-35,
- FR2 comprises the amino acids at positions 36-49,
- CDR2 comprises the amino acid residues at positions 50-65,
- FR3 comprises the amino acid residues at positions 66-94,
- CDR3 comprises the amino acid residues at positions 95-102, and
- FR4 comprises the amino acid residues at positions 103-113.

As described in detail in e.g. WO 2006/040153 and WO 2006/122786, VHH domains can specifically be classified in three groups, depending on certain combinations of amino acids within the framework regions, i.e. (a) the "GLEW-group", also including "GLEW-like" sequences; (b) the "KERE-group", also

including the KQRE sequence; and (c) the "103 P, R, S-group", and can further be characterized by specific "Hallmark residues".

An "affinity matured Dll4-binding molecule" has one or more alterations in one or more CDRs which result in an improved affinity for Dll4, as compared to the respective parent Dll4-binding molecule. Afffinity-matured Dll4-binding molecules of the invention may be prepared by methods known in the art, for example, as described by Marks *et al.*, 1992, Biotechnology 10:779-783, or Barbas, *et al.*, 1994, Proc. Nat. Acad. Sci, USA 91: 3809-3813.; Shier *et al.*, 1995, Gene 169:147-155; Yelton *et al.*, 1995, Immunol. 155: 1994-2004; Jackson *et al.*, 1995, J. Immunol. 154(7):3310-9; and Hawkins *et al.*, 1992, J. Mol. Biol. 226(3): 889 896; KS Johnson and RE Hawkins, "Affinity maturation of antibodies using phage display", Oxford University Press 1996.

For the present invention, an “amino acid sequences of SEQ ID NO: x”: includes, if not otherwise stated, with respect to the relevant sequence, e.g.a full immunoglobulin single variable domain sequence or a CDR sequence,

a) an amino acid sequence that is 100% identical with the sequence shown in the respective SEQ ID NO: x;
b) amino acid sequences that have at least 80% amino acid identity with the sequence shown in the respective SEQ ID NO: x;
c) amino acid sequences that have 3, 2, or 1 amino acid differences with the sequence shown in the respective SEQ ID NO: x.

The terms "*epitope*" and "*antigenic determinant*", which can be used interchangeably, refer to the part of a macromolecule, such as a polypeptide, that is recognized by antigen-binding molecules, such as conventional antibodies or immunoglobulin single variable domains of the invention, and more particularly by the antigen-binding site of said molecules. Epitopes define the minimum binding site for an immunoglobulin, and thus convey specificity to an immunoglobulin.

The term "*biparatopic Dll4-binding molecule"* or "*biparatopic immunoglobulin single variable domain"* as used herein shall mean a Dll4-binding molecule comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain as herein defined, wherein the molecules are capable of binding to two different epitopes of the Dll4 antigen. The biparatopic polypeptides according to the invention are composed of immunoglobulin single variable domains which have different specificities. The part of an antigen-binding molecule (such as an antibody or a polypeptide of the invention) that recognizes the epitope is called a *paratope*.

A polypeptide (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain of the invention or a polypeptide containing the same, or generally an antigen-binding molecule or a fragment thereof) that can "*bind to*" or "*specifically bind to*", that "*has affinity for*" and/or that "*has specificity for*" a certain epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "*against*" or "*directed against*" said epitope, antigen or protein or is a "*binding*" molecule with respect to such epitope, antigen or protein.

Generally, the term "*specificity*" refers to the number of different types of antigens or epitopes to which a particular antigen-binding molecule or antigen-binding protein (such as an immunoglobulin single variable domain of the invention) molecule can bind. The specificity of an antigen-binding molecule can be determined based on its affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (KD), is a measure for the binding strength between an epitope and an antigen-binding site on the antigen-binding protein: the lesser the value of the KD, the stronger the binding strength between an epitope and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (KA), which is 1/KD). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner

known per se, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain or a polypeptide containing it) and the pertinent antigen. Avidity is related to both the affinity between an epitope and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule.

Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code, as generally known and agreed upon in the art. When comparing two amino acid sequences, the term "*amino acid difference*" refers to insertions, deletions or substitutions of the indicated number of amino acid residues at a position of the reference sequence, compared to a second sequence. In case of substitution(s), such substitution(s) will preferably be conservative amino acid substitution(s), which means that an amino acid residue is replaced by another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 98/49185, wherein conservative amino acid substitutions preferably are substitutions in which one amino acid within the following groups (i) - (v) is substituted by another amino acid residue within the same group: (i) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (ii) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (iii) polar, positively charged residues: His, Arg and Lys; (iv) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (v) aromatic residues: Phe, Tyr and Trp. Particularly preferred conservative amino acid substitutions are as follows:

Ala into Gly or into Ser;

Arg into Lys;

Asn into Gln or into His;

Asp into Glu;

Cys into Ser;
Gln into Asn;
Glu into Asp;
Gly into Ala or into Pro;
His into Asn or into Gln;
Ile into Leu or into Val;
Leu into Ile or into Val;
Lys into Arg, into Gln or into Glu;
Met into Leu, into Tyr or into Ile;
Phe into Met, into Leu or into Tyr;
Ser into Thr;
Thr into Ser;
Trp into Tyr;
Tyr into Trp or into Phe;
Val, into Ile or into Leu.

A nucleic acid or polypeptide molecule is considered to be "*(in) essentially isolated (form)*" - for example, when compared with its native biological source and/or the reaction medium or cultivation medium from which it has been obtained - when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another nucleic acid, another protein/polypeptide, another biological component or macromolecule or at least one contaminant, impurity or minor component. In particular, a nucleic acid or polypeptide molecule is considered "essentially isolated" when it has been purified at least 2-fold, in particular at least 10-fold, more in particular at least 100-fold, and up to 1000-fold or more. A nucleic acid or polypeptide molecule that is "in essentially isolated form" is preferably essentially homogeneous, as determined using a suitable technique, such as a suitable chromatographical technique, such as polyacrylamide gel electrophoresis.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer to be treated with a Dll4-binding molecule of the invention, include but are not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers, as suggested for treatment with Dll4 antagonists in US 2008/0014196, include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both non-neoplastic and neoplastic conditions. Neoplasties include but are not limited those described above. Non-neoplastic disorders include, but are not limited to, as suggested for treatment with Dll4 antagonists in US 2008/0014196, undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, acute lung injury/ ARDS, sepsis, primary pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/ closed head injury/ trauma), synovial inflammation, pannus formation in RA, myositis ossificans, hypertropic bone

formation, osteoarthritis (OA), refractory ascites, polycystic ovarian disease, endometriosis, 3rd spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), hemophilic joints, hypertrophic scars, inhibition of hair growth, Osier-Weber syndrome, pyogenic granuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention relates to a Dll4-binding molecule comprising at least a variable domain with four framework regions and three complementarity determining regions CDR1, CDR2 and CDR3, respectively, wherein said CDR3 has an amino acid sequence selected from amino acid sequences shown in

a) SEQ ID NOs: 1 to 166 and 458,

b) SEQ ID NOs: 333 to 353, or

c) SEQ ID NOs: 375 to 395.

An amino acid sequence a), selected from a first group of SEQ ID NOs: 1 to166 and 458, is contained as partial sequence in a corresponding amino acid sequence selected from a second group of sequences shown in Table 5 and in SEQ ID NO: 167 to 332 and 459.

An amino acid sequence b), selected from a first group of SEQ ID NOs: 333 to 353, is contained as partial sequence in a corresponding sequence selected from

a second group of sequences shown in Table 16-A and in SEQ ID NOs: 354 to 374.

An amino acid sequence c) selected from a first group of SEQ ID NOs: 375 to 395 is contained as partial sequence in a corresponding sequence selected from a second group of sequences shown in Table 16-B and in SEQ ID NOs: 396 to 416.

In a second aspect, said Dll4-binding molecule is an isolated immunoglobulin single variable domain or a polypeptide containing one or more of said immunoglobulin single variable domains, wherein said immunoglobulin single variable domain consists of four framework regions and three complementarity determining regions CDR1, CDR2 and CDR3, respectively, and wherein said CDR3 has an amino acid sequence selected from amino acid sequences shown in

a) SEQ ID NOs: 1 to 166 and 458,
b) SEQ ID NOs: 333 to 353, or
c) SEQ ID NOs: 375 to 395.

In a further aspect, said immunoglobulin single variable domain contains

a) a CDR3 with an amino acid sequence selected from a first group of amino acid sequences shown in SEQ ID NOs: 1 to 166 and 458;
b) a CDR1 and a CDR2 with an amino acid sequence that is contained, as indicated in Table 5, as partial sequence in a sequence selected from a second group of amino acid sequences shown in SEQ ID NOs: 167 to 332 and 459;

wherein a SEQ ID NO: x of said first group, for SEQ ID Nos 1- 166: corresponds to SEQ ID NO: y of said second group in that y = x +166.

In a further aspect said immunoglobulin single variable domain contains

a) a CDR3 with an amino acid sequence selected a said first group of amino acid sequences shown in SEQ ID NOs: 333 to 353;

b) a CDR1 and a CDR2 with an amino acid sequence that is contained, as indicated in Table 16-A, as a partial sequence in a sequence selected from a second group of sequences shown in SEQ ID NOs: 354 to 374;

wherein a SEQ ID NO: x of said first group corresponds to SEQ ID NO: y of said second group in that y = x +21.

In a further aspect said immunoglobulin single variable domain has

a) a CDR3 with an amino acid sequence selected a said first group of amino acid sequences shown in SEQ ID NOs: 375 to 395;

b) a CDR1 and a CDR2 with an amino acid sequence that is contained, as indicated in Table 16-B, as a partial sequence in a sequence selected from a second group of sequences shown in SEQ ID NOs: 396 to 416;

wherein a SEQ ID NO: x of said first group corresponds with SEQ ID NO: y of said second group in that y = x +21.

In a preferred embodiment, the immunoglobulin single variable domain is a VHH.

In a further aspect, the VHH has an amino acid sequence selected from amino acid sequences shown in Table 5 and in SEQ ID NOs: 167 to 332 and 459.

Dll4-binding molecules with improved properties in view of therapeutic application, e.g. enhanced affinity or decreased immunogenicity, may be obtained from individual Dll4-binding molecules of the invention by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, humanizing, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing. Reference is, for example, made to standard handbooks, as well as to the further description and Examples.

Preferably, a Dll4-binding molecule of the invention with increased affinity is obtained by affinity-maturation of another Dll4-binding molecule, the latter representing, with respect to the affinity-matured molecule, the "parent" Dll4-binding molecule.

Thus, in yet another preferred embodiment, a Dll4-binding molecule of the invention is an immunoglobulin single variable domain that has been obtained by affinity maturation of a parent immunoglobulin single variable domain defined above.

In yet another preferred embodiment, the invention relates to an immunoglobulin single variable obtained by affinity-maturation of a VHH.

Suitable parent Dll4-binding molecules for affinity maturation are, by way of example, the above-described VHHs with amino acid sequences shown in SEQ ID NOs: 167 to 332 and 459.

In yet another preferred embodiment, the invention relates to an immunoglobulin single variable domain that has been obtained by affinity maturation of a VHH with an amino acid sequence shown in SEQ ID NO: 197.

In yet another embodiment, said immunoglobulin single variable domain that is derived from a VHH with the amino acid sequence shown in SEQ ID NO: 197 is selected from immunoglobulin single variable domains with amino acid sequences shown in SEQ ID NOs: 354 to 374.

In a preferred embodiment, the immunoglobulin single variable domain is a VHH with an amino acid sequence shown in SEQ ID NO: 358.

In an even more preferred embodiment, the immunoglobulin single variable domain has been obtained by humanization of a VHH with an amino acid sequence shown in SEQ ID NO: 358.

In another preferred embodiment, the immunoglobulin single variable domain is a VHH with an amino acid sequence shown in SEQ ID NO: 356.

In an even more preferred embodiment, the invention relates to an immunoglobulin single variable domain that has been obtained by humanization of a VHH with an amino acid sequence shown in SEQ ID NO: 356.

In yet another preferred embodiment, the invention relates to an immunoglobulin single variable domain that has been obtained by affinity maturation of a VHH with an amino acid sequence shown in SEQ ID NO: 224.

In yet another embodiment, said immunoglobulin single variable domain derived from a VHH with the amino acid sequence shown in SEQ ID NO: 224 is selected from immunoglobulin single variable domains with amino acid sequences shown in SEQ ID NOs: 396 to 416.

In another preferred embodiment, the immunoglobulin single variable domain is a VHH with an amino acid sequence shown in SEQ ID NO: 402.

In an even more preferred embodiment, the immunoglobulin single variable domain has been obtained by humanization of the VHH with the amino acid sequence shown in SEQ ID NO: 402.

In another preferred embodiment, the immunoglobulin single variable domain is a VHH with an amino acid sequence shown in SEQ ID NO: 416.

In an even more preferred embodiment, the immunoglobulin single variable domain has been obtained by humanization of the immunoglobulin single variable domain with the amino acid sequence shown in SEQ ID NO: 416

In another preferred embodiment, the immunoglobulin single variable domain is a VHH with an amino acid sequence shown in SEQ ID NO:407.

In an even more preferred embodiment, the immunoglobulin single variable domain has been obtained by humanization of the immunoglobulin single variable domain with the amino acid sequence shown in SEQ ID NO: 413.

Immunoglobulin single variable domains, e.g. VHs and VHHs, according to the preferred embodiments of the invention, have a number of unique structural characteristics and functional properties which makes them highly advantageous for use in therapy as functional antigen-binding molecules. In particular, and without being limited thereto, VHH domains (which have been "designed" by nature to functionally bind to an antigen without pairing with a light chain variable domain) can function as single, relatively small, functional antigen-binding structural units.

Due to their unique properties, immunoglobulin single variable domains, as defined herein, like VHHs or VHs (or VLs) - either alone or as part of a larger polypeptide, e.g. a biparatopic molecule - offer a number of significant advantages:

- only a single domain is required to bind an antigen with high affinity and with high selectivity, so that there is no need to have two separate domains present, nor to assure that these two domains are present in the right spacial conformation and configuration (i.e. through the use of especially designed linkers, as with scFv's);
- immunoglobulin single variable domains can be expressed from a single nucleic acid molecule and do not require any post-translational modification (like glycosylation;
- immunoglobulin single variable domains can easily be engineered into multivalent and multispecific formats (as further discussed herein);
- immunoglobulin single variable domains have high specificity and affinity for their target, low inherent toxicity and can be administered via alternative routes than infusion or injection;
- immunoglobulin single variable domains are highly stable to heat, pH, proteases and other denaturing agents or conditions and, thus, may be prepared, stored or transported without the use of refrigeration equipments;
- immunoglobulin single variable domains are easy and relatively inexpensive to prepare, both on small scale and on a manufacturing scale. For example, immunoglobulin single variable domains and polypeptides containing the same can be produced using microbial fermentation (e.g. as further described below) and do not require the use of mammalian expression systems, as with for example conventional antibodies;
- immunoglobulin single variable domains are relatively small (approximately 15 kDa, or 10 times smaller than a conventional IgG) compared to conventional 4-chain antibodies and antigen-binding fragments thereof, and therefore show high(er) penetration into tissues (including but not limited to

solid tumors and other dense tissues) and can be administered in higher doses than such conventional 4-chain antibodies and antigen-binding fragments thereof;

- VHHs have specific so-called “cavity-binding properties” (inter alia due to their extended CDR3 loop, compared to VH domains from 4-chain antibodies) and can therefore also access targets and epitopes not accessible to conventional 4-chain antibodies and antigen-binding fragments thereof;
- VHHs have the particular advantage that they are highly soluble and very stable and do not have a tendency to aggregate (as with the mouse-derived antigen-binding domains described by Ward *et al.*, Nature 341: 544-546 (1989)).

The immunoglobulin single variable domains of the invention are not limited with respect to a specific biological source from which they have been obtained or to a specific method of preparation. For example, obtaining VHHs may include the following steps:

(1) isolating the VHH domain of a naturally occurring heavy chain antibody; or screening a library comprising heavy chain antibodies or VHHs and isolating VHHs therefrom;

(2) expressing a nucleic acid molecule encoding a VHH with the naturally occurring sequence;

(3) "humanizing" (as described herein) a VHH, optionally after affinity maturation, with a naturally occurring sequence or expressing a nucleic acid encoding such humanized VHH;

(4) "camelizing" (as described below) a immunoglobulin single variable heavy domain from a naturally occurring antibody from an animal species, in particular a species of mammal, such as from a human being, or expressing a nucleic acid molecule encoding such camelized domain;

(5) "camelizing" a VH, or expressing a nucleic acid molecule encoding such a camelized VH;

(6) using techniques for preparing synthetically or semi-synthetically proteins, polypeptides or other amino acid sequences;
(7) preparing a nucleic acid molecule encoding a VHH domain using techniques for nucleic acid synthesis, followed by expression of the nucleic acid thus obtained;
(8) subjecting heavy chain antibodies or VHHs to affinity maturation, to mutagenesis (e.g. random mutagenesis or site-directed mutagenesis) and/or any other technique(s) in order to increase the affinity and/or specificity of the VHH; and/or
(9) combinations or selections of the foregoing steps.

Suitable methods and techniques for performing the above-described steps are known in the art and will be clear to the skilled person.

According to a specific embodiment, the immunoglobulin single variable domains of the invention or present in the polypeptides of the invention are VHH domains with an amino acid sequence that essentially corresponds to the amino acid sequence of a naturally occurring VHH domain, but that has been "humanized" (optionally after affinity-maturation), i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring VHH sequence by one or more of the amino acid residues that occur at the corresponding position(s) in a variable heavy domain of a conventional 4-chain antibody from a human being. This can be performed using methods known in the art, which can by routinely used by the skilled person.

A humanized VHH domain may contain one or more fully human framework region sequences, and, in an even more specific embodiment, may contain human framework region sequences derived from the human germline Vh3 sequences DP-29, DP-47, DP-51, or parts thereof, or be highly homologous thereto. Thus, a humanization protocol may comprise the replacement of any of the VHH residues with the corresponding framework 1, 2 and 3 (FRl, FR2 and FR3) residues of

germline VH genes such as DP 47, DP 29 and DP 51) either alone or in combination. Suitable framework regions (FR) of the immunoglobulin single variable domains of the invention can be selected from those as set out e.g. in WO 2006/004678 and specifically, include the so-called "KERE" and "GLEW" classes. Particularly preferred are immunoglobulin single variable domains having the amino acid sequence G-L-E-W at about positions 44 to 47, and their respective humanized counterparts.

A preferred, but non-limiting humanizing substitution for VHH domains belonging to the 103 P,R,S-group and/or the GLEW-group (as defined below) is 108Q to 108L. Methods for humanizing immunoglobulin single variable domains are known in the art.

According to another embodiment, the immunoglobulin single variable domain is a VH domain, as defined herein.

In yet another embodiment, the representatives of the class of Dll4-binding immunoglobulin single variable domains of the invention or present in the polypeptides of the invention have amino acid sequences that correspond to the amino acid sequence of a naturally occurring VH domain that has been "camelized", i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring variable heavy chain from a conventional 4-chain antibody by one or more amino acid residues that occur at the corresponding position(s) in a VHH domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, and reference is additionally be made to WO 94/04678. Such camelization may preferentially occur at amino acid positions which are present at the VH-VL interface and at the so-called Camelidae Hallmark residues (see for example also WO 94/04678). A detailled description of such "humanization" and "camelization" techniques and preferred framework region sequences consistent therewith can

additionally be taken from e.g. pp. 46 and pp. 98 of WO 2006/040153 and pp. 107 of WO 2006/122786.

The Dll4-binding molecules of the invention, e.g. immunoglobulin single variable domains and or polypeptides containing them, have specificity for Dll4 in that they comprise one or more immunoglobulin single variable domains specifically binding to one or more epitopes within the Dll4 molecule.

Specific binding of an Dll4-binding molecule to its antigen Dll4 can be determined in any suitable manner known per se, including, for example, the assays described herein, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA and ELISA) and sandwich competition assays, and the different variants thereof known per se in the art.

With regard to the antigen Dll4, a Dll4-binding molecule of the invention, e.g. an immunoglobulin single variable domain, is not limited with regard to the species. Thus, the immunoglobulin single variable domains of the invention or polypeptides containing them preferably bind to human Dll4, if intended for therapeutic purposes in humans. However, immunoglobulin single variable domains that bind to Dll4 from another mammalian species, or polypeptides containing them, are also within the scope of the invention. An immunoglobulin single variable domain of the invention binding to one species form of Dll4 may cross-react with Dll4 from one or more other species. For example, immunoglobulin single variable domains of the invention binding to human Dll4 may exhibit cross reactivity with Dll4 from one or more other species of primates and/or with Dll4 from one or more species of animals that are used in animal models for diseases, for example monkey (in particular Cynomolgus or Rhesus), mouse, rat, rabbit, pig, dog or) and in particular in animal models for diseases and disorders associated with Dll4-mediated effects on angiogenesis (such as the species and animal models mentioned herein). Immunoglobulin single variable domains of the invention that show such cross-reactivity are advantageous in a research and/or drug development, since it

allows the immunoglobulin single variable domains of the invention to be tested in acknowledged disease models such as monkeys, in particular Cynomolgus or Rhesus, or mice and rats.

Also, the Dll4-binding molecules of the invention are not limited to or defined by a specific domain or an antigenic determinant of Dll4 against which they are directed. Preferably, in view of cross-reactivity with one or more Dll4 molecules from species other than human that is/are intended for use as an animal model during development of a therapeutic Dll4 antagonist, a Dll4-binding molecule recognizes an epitope in a region of the Dll4 of interest that has a high degree of identity with human Dll4. By way of example, in view of using a mouse model, an immunoglobulin single variable domain of the invention recognizes an epitope which is, totally or in part, located within the EGF-2 domain, which shows a high identity between human and mouse.

Therefore, according to a preferred embodiment, the invention relates to a Dll4-binding molecule, in particular an immunoglobulin single variable domain or a polypeptide containing same, wherein said immunoglobulin single variable domain is selected from the group that binds to an epitope that is totally or partially contained within the EGF-2 domain that corresponds to amino acid residues 252-282 of SEQ ID NO: 417.

If a polypeptide of the invention is a biparatopic molecule as defined herein, which contains more than one immunoglobulin single variable domain of the invention, at least one of the immunoglobulin single variable domain components binds to the epitope within the EGF-2 domain, as defined above.

Preferably, an immunoglobulin single variable domain of the invention binds to Dll4 with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM (as determined by Surface Plasmon Resonance analysis, as described in Example 5.7).

Preferably, the immunoglobulin single variable domains of the invention have IC_{50} values, as measured in a competition ELISA assay as described in Example 5.1. in the range of 10^{-6} to 10^{-10} moles/litre or less, more preferably in the range of 10^{-8} to 10^{-10} moles/litre or less and even more preferably in the range of 10^{-9} to 10^{-10} moles/litre or less.

According to a non-limiting but preferred embodiment of the invention, Dll4-binding immunoglobulin single variable domains of the invention or polypeptides containing them bind to Dll4 with an dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter (M) or less, and preferably 10^{-7} to 10^{-12} moles/liter (M) or less and more preferably 10^{-8} to 10^{-12} moles/liter (M), and/or with an association constant (K_A) of at least 10^7 M^{-1}, preferably at least 10^8 M^{-1}, more preferably at least 10^9 M^{-1}, such as at least 10^{12} M^{-1}; and in particular with a K_D less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. The K_D and K_A values of the immunoglobulin single variable domain of the invention against Dll4 can be determined.

In a further embodiment, the invention relates to Dll4-binding molecules comprising two or more immunoglobulin single variable domains that bind to the antigen Dll4 at different non-overlapping epitopes. More specifically, such polypeptide of the invention essentially consists of or comprises (i) a first immunoglobulin single variable domain specifically binding to a first epitope of Dll4 and (ii) a second immunoglobulin single variable domain specifically binding to a second epitope of Dll4, wherein the first epitope of Dll4 and the second epitope of Dll4 are not identical epitopes. In other words, such polypeptide of the invention comprises or essentially consist of two or more immunoglobulin single variable domains that are directed against at least two different epitopes present in Dll4, wherein said immunoglobulin single variable domains are linked to each other in such a way that they are capable of simultaneously binding Dll4. In this sense, the polypeptide of the invention can also be regarded as a “bivalent” or "multivalent" immunoglobulin construct, and especially as a "multivalent immunoglobulin single

variable domain construct", in that the polypeptide contains at least two binding sites for Dll4.

Such Dll4-binding molecule of the invention includes (at least) two anti-Dll4 immunoglobulin single variable domains, wherein (the) two immunoglobulin single variable domains are directed against different epitopes within the Dll4 molecule. Thus, these two immunoglobulin single variable domains will have a different antigen specificity and therefore different CDR sequences. For this reason, such polypeptides of the invention will herein also be named "biparatopic polypeptides", or "biparatopic single domain antibody constructs" (if the immunoglobulin single variable domains consist or essentially consist of single domain antibodies), or "biparatopic VHH constructs" (if the immunoglobulin single variable domains consist or essentially consist of VHHs), respectively, as the two immunoglobulin single variable domains will include two different paratopes.

According to a specific embodiment of the invention, in case that the polypeptide of the invention includes more than two anti-Dll4 immunoglobulin single variable domains, i.e. three, four or even more anti-Dll4 immunoglobulin single variable domains, at least two of the anti-Dll4 immunoglobulin single variable domains are directed against different epitopes within the Dll4 molecule, wherein any further immunoglobulin single variable domain may bind to any of these two different epitopes and/or a further epitope present in the Dll4 molecule.

According to the invention, the two or more immunoglobulin single variable domains can be, independently of each other, VHs or VHHs, and/or any other sort of immunoglobulin single variable domains, such as VL domains, as defined herein, provided that these immunoglobulin single variable domains will bind the antigen, i.e. Dll4.

According to a preferred embodiment, the first and the second immunoglobulin single variable domains essentially consist of either VH sequences or VHH

sequences, as defined herein. According to a particularly preferred embodiment, the first and the second immunoglobulin single variable domains essentially consist of VHH sequences.

According to certain embodiments of the invention, the at least two immunoglobulin single variable domains present in a Dll4-binding molecule of the invention can be connected with each other directly (i.e. without use of a linker) or via a linker. The linker is preferably a linker peptide and will be selected so as to allow binding of the at least two different immunoglobulin single variable domains to each of their at least two different epitopes of Dll4, either within one and the same Dll4 molecule, or within two different molecules.

Selection of linkers will *inter alia* depend on the epitopes and, specifically, the distance between the epitopes on Dll4 to which the immunoglobulin single variable domains bind, and will be clear to the skilled person based on the disclosure herein, optionally after some limited degree of routine experimentation. As a starting point for such experimentation, it can generally be assumed that the distance between the N-terminus and the C-terminus of the two immunoglobulin single variable domains present in such a polypeptide of the invention will preferably at least 50 Angstroms, and more preferably in the region of 55-200 Angstroms, and in particular in the region of 65-150 Angstroms, with the upper limit being less critical, and being chosen for reasons of convenience, e.g. with a view to expression/production of the protein.

Also, when the two or more immunoglobulin single variable domains that bind to Dll4 are VHs or VHHs, they may be linked to each other via a third VH or VHH, respectively (in such Dll4-binding molecules, the two or more immunoglobulin single variable domains may be linked directly to said third immunoglobulin single variable domain or via suitable linkers). Such a third VH or VHH may for example be a VH or VHH that provides for an increased half-life. For example, the latter VH

or VHH may be a VH or VHH that is capable of binding to a (human) serum protein such as (human) serum albumin or (human) transferrin.

Alternatively, the two or more immunoglobulin single variable domains that bind to Dll4 may be linked in series (either directly or via a suitable linker) and the third VH or VHH (which may provide for increased half-life) may be connected directly or via a linker to one of these two or more aforementioned immunoglobulin sequences.

Suitable linkers may - for example and without limitation - comprise an amino acid sequence, which preferably has a length of nine or more amino acids, more preferably more than 17 amino acids, e.g. about 20 - 40 amino acid residues. However, the upper limit is not critical but is chosen for reasons of convenience regarding e.g. biopharmaceutical production of such polypeptides.

The linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. If used for therapeutical purposes, the linker is preferably non-immunogenic in the subject to which the polypeptide of the invention is administered.

One useful group of linker sequences are linkers derived from the hinge region of heavy chain antibodies as described in WO 96/34103 and WO 94/04678.

Other examples are poly-alanine linker sequences such as Ala- Ala- Ala.

If the polypeptide of the invention is modified by the attachment of a polymer, for example of a polyethylene glycol PEG (polyethylene glycol) moiety, the linker sequence preferably includes an amino acid residue, such as a cysteine or a lysine, allowing such modification, e.g. PEGylation, in the linker region.

Furthermore, the linker may also be a poly(ethylene glycol) moiety, as shown in e.g. WO 04/081026.

In another embodiment, the at least two Dll4-binding immunoglobulin single variable domains of the polypeptide of the invention are linked to each other via another moiety (optionally via one or two linkers), such as another polypeptide which, in a preferred but non-limiting embodiment, may be a further immunoglobulin single variable domain as described above. Such moiety may either be essentially inactive or may have a biological effect such as improving the desired properties of the polypeptide or may confer one or more additional desired properties to the polypeptide. For example, and without limitation, the moiety may improve the half-life of the protein or polypeptide, and/or may reduce its immunogenicity or improve any other desired property.

According to a preferred embodiment of the invention, a Dll4-binding molecule of the invention includes, in view of its use as a therapeutic agent, a moiety which extends the half-life of the polypeptide of the invention in serum or other body fluids of a patient. The term "half-life" is defined as the time it takes for the serum concentration of the (modified) polypeptide to reduce by 50%, in vivo, for example due to degradation of the polypeptide and/or clearance and/or sequestration by natural mechanisms.

More specifically, such half-life extending moiety can be covalently linked or fused to said polypeptide and may be, without limitation, an Fc portion, an albumin moiety, a fragment of an albumin moiety, an albumin binding moiety, such as an anti-albumin immunoglobulin single variable domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin single variable domain, a polyoxyalkylene molecule, such as a polyethylene glycol molecule, an albumin binding peptide or a hydroxyethyl starch (HES) derivative.

In another preferred embodiment, the polypeptide of the invention comprises a moiety which binds to an antigen found in blood, such as serum albumin, serum immunoglobulins, thyroxine-binding protein, fibrinogen or transferrin, thereby conferring an increased half-life in vivo to the resulting polypeptide of the invention. According to a specifically preferred embodiment, such moiety is an albumin-binding immunoglobulin and, especially preferred, an albumin-binding immunoglobulin single variable domain such as an albumin-binding VHH domain.

If intended for use in humans, such albumin-binding immunoglobulin single variable domain will preferably bind to human serum albumin and will preferably be a humanized albumin-binding VHH domain.

Immunoglobulin single variable domains binding to human serum albumin are known in the art and are described in further detail in e.g. WO 2006/122786. Specifically, useful albumin binding VHHs are ALB 1 and its humanized counterpart, ALB 8 (WO 2009/095489). Other albumin binding VHH domains mentioned in the above patent publication may, however, be used as well.

According to a further embodiment of the invention, the immunoglobulin single variable domain may be fused to a serum albumin molecule, such as described e.g. in WO01/79271 and WO03/59934. As e.g. described in WO01/79271, the fusion protein may be obtained by conventional recombinant technology: a DNA molecule coding for serum albumin, or a fragment thereof, is joined to the DNA coding for the Dll4-binding molecule, the obtained construct is inserted into a plasmid suitbale for expression in the selected host cell, e.g. a yeast cell like Pichia pastoris or a bacterial cell, and the host cell is then transfected with the fused nucleotide sequence and grown under suitable conditions.

According to another embodiment, a half-life extending modification of a polypeptide of the invention (such modification also reducing immunogenicity of the polypeptide) comprises attachment of a suitable pharmacologically acceptable

polymer, such as straight or branched chain poly(ethylene glycol) (PEG) or derivatives thereof (such as methoxypoly(ethylene glycol) or mPEG). Generally, any suitable form of PEGylation can be used, such as the PEGylation used in the art for antibodies and antibody fragments (including but not limited to single domain antibodies and scFv's); reference is made, for example, to: Chapman, Nat. Biotechnol., 54, 531-545 (2002); Veronese and Harris, Adv. Drug Deliv. Rev. 54, 453-456 (2003); Harris and Chess, Nat. Rev. Drug. Discov. 2 (2003); and WO 04/060965. Various reagents for PEGylation of polypeptides are also commercially available, for example from Nektar Therapeutics, USA, or NOF Corporation, Japan, such as the Sunbright® EA Series, SH Series, MA Series, CA Series, and ME Series, such as Sunbright® ME-100MA, Sunbright® ME-200MA, and Sunbright® ME-400MA.

Preferably, site-directed PEGylation is used, in particular via a cysteine-residue (see for example Yang *et al.*, Protein Engineering 16, 761-770 (2003)). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a polypeptide of the invention, a polypeptide of the invention may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the N- and/or C-terminus of a polypeptide of the invention, all using techniques of protein engineering known per se to the skilled person.

Preferably, for the polypeptides of the invention, a PEG is used with a molecular weight of more than 5 kDa, such as more than 10 kDa and less than 200 kDa, such as less than 100 kDa; for example in the range of 20 kDa to 80 kDa.
With regard to PEGylation, its should be noted that generally, the invention also encompasses any biparatopic Dll4-binding molecule that has been PEGylated at one or more amino acid positions, preferably in such a way that said PEGylation either (1) increases the half-life in vivo; (2) reduces immunogenicity; (3) provides one or more further beneficial properties known per se for PEGylation; (4) does

not essentially affect the affinity of the polypeptide for Dll4 (e.g. does not reduce said affinity by more than 50 %, and more preferably not by more than 10%, as determined by a suitable assay described in the art); and/or (4) does not affect any of the other desired properties of the Dll4-binding molecules of the invention. Suitable PEG-groups and methods for attaching them, either specifically or non-specifically, will be clear to the skilled person. Suitable kits and reagents for such pegylation can for example be obtained from Nektar (CA, USA).

In another aspect, the invention relates to nucleic acid molecules that encode Dll4-binding molecules of the invention. Such nucleic acid molecules will also be referred to herein as "nucleic acids of the invention" and may also be in the form of a genetic construct, as defined herein. A nucleic acid of the invention may be genomic DNA, cDNA or synthetic DNA (such as DNA with a codon usage that has been specifically adapted for expression in the intended host cell or host organism). According to one embodiment of the invention, the nucleic acid of the invention is in essentially isolated form, as defined hereabove.

The nucleic acid of the invention may also be in the form of, may be present in and/or may be part of a vector, such as for example a plasmid, cosmid or YAC. The vector may especially be an expression vector, i.e. a vector that can provide for expression of the Dll4-binding molecule *in vitro* and/or *in vivo* (i.e. in a suitable host cell, host organism and/or expression system). Such expression vector generally comprises at least one nucleic acid of the invention that is operably linked to one or more suitable regulatory elements, such as promoter(s), enhancer(s), terminator(s), and the like. Such elements and their selection in view of expression of a specific sequence in a specific host are common knowledge of the skilled person. Specific examples of regulatory elements and other elements useful or necessary for expressing Dll4-binding molecules of the invention, such as promoters, enhancers, terminators, integration factors, selection markers, leader sequences, reporter genes, and the like, are disclosed e.g. on pp. 131 to 133 of WO 2006/040153.

The nucleic acids of the invention may be prepared or obtained in a manner known *per se* (e.g. by automated DNA synthesis and/or recombinant DNA technology), based on the information on the amino acid sequences for the polypeptides of the invention given herein, and/or can be isolated from a suitable natural source.

In another aspect, the invention relates to host cells that express or that are capable of expressing one or more a Dll4-binding molecule of the invention; and/or that contain a nucleic acid of the invention. According to a particularly preferred embodiment, said host cells are bacterial cells; other useful cells are yeast cells, fungal cells or mammalian cells.

Suitable bacterial cells include cells from gram-negative bacterial strains such as strains of *Escherichia coli*, *Proteus*, and *Pseudomonas*, and gram-positive bacterial strains such as strains of *Bacillus*, *Streptomyces*, *Staphylococcus*, and *Lactococcus.* Suitable fungal cell include cells from species of *Trichoderma*, *Neurospora*, and *Aspergillus*. Suitable yeast cells include cells from species of *Saccharomyces (*for example *Saccharomyces cerevisiae), Schizosaccharomyces* (for example *Schizosaccharomyces pombe*), *Pichia* (for example *Pichia pastoris* and *Pichia methanolica*), and *Hansenula*.

Suitable mammalian cells include for example CHO cells, BHK cells, HeLa cells, COS cells, and the like. However, amphibian cells, insect cells, plant cells, and any other cells used in the art for the expression of heterologous proteins can be used as well.

The invention further provides methods of manufacturing a Dll4-binding molecule of the invention, such methods generally comprising the steps of:

- culturing host cells comprising a nucleic acid capable of encoding a Dll4-binding molecule under conditions that allow expression of the Dll4-binding molecule of the invention; and
- recovering or isolating the polypeptide expressed by the host cells from the culture; and
- optionally further purifying and/or modifying and/or formulating the Dll4-binding molecule of the invention.

For production on an industrial scale, preferred host organisms include strains of *E. coli*, *Pichia pastoris*, and *S. cerevisiae* that are suitable for large scale expression, production and fermentation, and in particular for large scale pharmaceutical expression, production and fermentation.

The choice of the specific expression system depends in part on the requirement for certain post-translational modifications, more specifically glycosylation. The production of a Dll4-binding molecule of the invention for which glycosylation is desired or required would necessitate the use of mammalian expression hosts that have the ability to glycosylate the expressed protein. In this respect, it will be clear to the skilled person that the glycosylation pattern obtained (i.e. the kind, number and position of residues attached) will depend on the cell or cell line that is used for the expression.

Dll4-binding molecules of the invention produced in a cell as set out above can be produced either intracellullarly (e.g. in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or they can be produced extracellularly (e.g. in the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified.

Methods and reagents used for the recombinant production of polypeptides, such as specific suitable expression vectors, transformation or transfection methods, selection markers, methods of induction of protein expression, culture conditions,

and the like, are known in the art. Similarly, protein isolation and purification techniques useful in a method of manufacture of a polypeptide of the invention are well known to the skilled person.

In a further aspect, the invention relates to a peptide with an amino acid sequence selected from amino acid sequences shown in SEQ ID NOs: 1 to 166 and 458, SEQ ID NOs: 333 to 353, or SEQ ID NOs: 375 to 395, respectively, and a nucleic acid molecule encoding same.

These peptides correspond to CDR3s derived from the VHHs of the invention. They, in particular the nucleic acid molecules encoding them, are useful for CDR grafting in order to replace a CDR3 in an immunoglobulin chain, or for insertion into a non-immunoglobulin scaffold, e.g. a protease inhibitor, DNA-binding protein, cytochrome b562, a helix-bundle protein, a disulfide-bridged peptide, a lipocalin or an anticalin, thus conferring target-binding properties to such scaffold. The method of CDR-grafting is well known in the art and has been widely used, e.g. for humanizing antibodies (which usually comprises grafting the CDRs from a rodent antibody onto the Fv frameworks of a human antibody).

In order to obtain an immunoglobulin or a non-immunoglobulin scaffold containing a CDR3 of the invention, the DNA encoding such molecule may be obtained according to standard methods of molecular biology, e.g. by gene synthesis, by oligonucleotide annealing or by means of overlapping PCR fragments, as e.g. described by Daugherty *et al.*, 1991, Nucleic Acids Research, Vol. 19, 9, 2471 – 2476. A method for inserting a VHH CDR3 into a non-immunoglobulin scaffold has been described by Nicaise *et al.*, 2004, Protein Science, 13, 1882 – 1891.

The invention further relates to a product or composition containing or comprising at least one Dll4-binding molecule of the invention and optionally one or more further components of such compositions known *per se*, i.e. depending on the intended use of the composition.

For pharmaceutical use, a Dll4-binding molecule of the invention or a polypeptide containing same may be formulated as a pharmaceutical preparation or composition comprising at least one Dll4-binding molecule of the invention and at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally one or more further pharmaceutically active polypeptides and/or compounds. By means of non-limiting examples, such a formulation may be in a form suitable for oral administration, for parenteral administration (such as by intravenous, intramuscular or subcutaneous injection or intravenous infusion), for topical administration, for administration by inhalation, by a skin patch, by an implant, by a suppository, etc. Such suitable administration forms - which may be solid, semi-solid or liquid, depending on the manner of administration - as well as methods and carriers for use in the preparation thereof, will be clear to the skilled person, and are further described herein.

Thus, in a further aspect, the invention relates to a pharmaceutical composition that contains at least one Dll4-binding molecule, in particular one immunoglobulin single variable domain of the invention or a polypeptide containing same and at least one suitable carrier, diluent or excipient (i.e. suitable for pharmaceutical use), and optionally one or more further active substances.

The Dll4-binding molecules of the invention may be formulated and administered in any suitable manner known per se: Reference, in particular for the immunoglobulin single variable domains, is for example made to WO 04/041862, WO 04/041863, WO 04/041865, WO 04/041867 and WO 08/020079, as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990), Remington, the Science and Practice of Pharmacy, 21th Edition, Lippincott Williams and Wilkins (2005); or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley, Weinheim, 2007 (see for example pages 252-255).

For example, an immunoglobulin single variable domain of the invention may be formulated and administered in any manner known per se for conventional antibodies and antibody fragments (including ScFv's and diabodies) and other

pharmaceutically active proteins. Such formulations and methods for preparing the same will be clear to the skilled person, and for example include preparations suitable for parenteral administration (for example intravenous, intraperitoneal, subcutaneous, intramuscular, intraluminal, intra-arterial or intrathecal administration) or for topical (i.e. transdermal or intradermal) administration.

Preparations for parenteral administration may for example be sterile solutions, suspensions, dispersions or emulsions that are suitable for infusion or injection. Suitable carriers or diluents for such preparations for example include, without limitation, sterile water and pharmaceutically acceptable aqueous buffers and solutions such as physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution; water oils; glycerol; ethanol; glycols such as propylene glycol or as well as mineral oils, animal oils and vegetable oils, for example peanut oil, soybean oil, as well as suitable mixtures thereof. Usually, aqueous solutions or suspensions will be preferred.

Thus, the Dll4-binding molecule of the invention may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. For oral therapeutic administration, the Dll4-binding molecule of the invention may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of the Dll4-binding molecule of the invention. Their percentage in the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of the Dll4-binding molecule of the invention in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, pills, capsules, and the like may also contain binders, excipients, disintegrating agents, lubricants and sweetening or flavouring agents, for example those mentioned on pages 143-144 of WO 08/020079. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid

carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the Dll4-binding molecules of the invention, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the Dll4-binding molecules of the invention may be incorporated into sustained-release preparations and devices.

Preparations and formulations for oral administration may also be provided with an enteric coating that will allow the constructs of the invention to resist the gastric environment and pass into the intestines. More generally, preparations and formulations for oral administration may be suitably formulated for delivery into any desired part of the gastrointestinal tract. In addition, suitable suppositories may be used for delivery into the gastrointestinal tract.

The Dll4-binding molecules of the invention may also be administered intravenously or intraperitoneally by infusion or injection, as further described on pages 144 and 145 of WO 08/020079.

For topical administration of the Dll4-binding molecules of the invention, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid, as further described on page 145 of WO 08/020079.

Generally, the concentration of the Dll4-binding molecules of the invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the Dll4-binding molecules of the invention required for use in treatment will vary not only with the particular Dll4-binding molecule selected, but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the

attendant physician or clinician. Also, the dosage of the Dll4-binding molecules of the invention varies depending on the target cell, tumor, tissue, graft, or organ. The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen may include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

According to a further embodiment, the invention relates to the use of Dll4-binding molecules of the invention, e.g. immunoglobulin single variable domains or polypeptides containing them, for therapeutic purposes, such as

- for the prevention, treatment and/or alleviation of a disorder, disease or condition, especially in a human being, that is associated with Dll4-mediated effects on angiogenesis or that can be prevented, treated or alleviated by modulating the Notch signaling pathway with a Dll4-binding molecule,
- in a method of treatment of a patient in need of such therapy, such method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one Dll4-binding molecule of the invention, e.g. an immunoglobulin single variable domain, or a pharmaceutical composition containing same;
- for the preparation of a medicament for the prevention, treatment or alleviation of disorders, diseases or conditions associated with Dll4-mediated effects on angiogenesis;

- as an active ingredient in a pharmaceutical composition or medicament used for the above purposes.

According to a specific aspect, said disorder disorder, disease or condition is a cancer or cancerous disease, as defined herein.

According to another aspect, the disease is an eye disease associated with associated with Dll4-mediated effects on angiogenesis or which can be treated or alleviated by modulating the Notch signaling pathway with a Dll4-binding molecule.

Depending on the cancerous disease to be treated, a Dll4-binding molecule of the invention may be used on its own or in combination with one or more additional therapeutic agents, in particular selected from chemotherapeutic agents like DNA damaging agents or therapeutically active compounds that inhibit angiogenesis, signal transduction pathways or mitotic checkpoints in cancer cells.

The additional therapeutic agent may be administered simultaneously with, optionally as a component of the same pharmaceutical preparation, or before or after administration of the Dll4-binding molecule.

In certain embodiments, the additional therapeutic agent may be, without limitation, one or more inhibitors selected from the group of inhibitors of EGFR, VEGFR, HER2-neu, Her3, AuroraA, AuroraB, PLK and PI3 kinase, FGFR, PDGFR, Raf, KSP, PDK1, PTK2, IGF-R or IR.

Further examples of additional therapeutic agents are inhibitors of CDK, Akt, src/bcr abl, cKit, cMet/HGF, c-Myc, Flt3, HSP90, hedgehog antagonists, inhibitors of JAK/STAT, Mek, mTor, NFkappaB, the proteasome, Rho, an inhibitor of wnt signaling or an inhibitor of the ubiquitination pathway or another inhibitor of the Notch signaling pathway.

Examples for Aurora inhibitors are, without limitation, PHA-739358, AZD-1152, AT 9283, CYC-116, R-763, VX-680, VX-667, MLN-8045, PF-3814735.

An example for a PLK inhibitor is GSK-461364.

Examples for raf inhibitors are BAY-73-4506 (also a VEGFR inhibitor), PLX 4032, RAF-265 (also in addition a VEGFR inhibitor), sorafenib (also in addition a VEGFR inhibitor), and XL 281.

Examples for KSP inhibitors are ispinesib, ARRY-520, AZD-4877, CK-1122697, GSK 246053A, GSK-923295, MK-0731, and SB-743921.

Examples for a src and/or bcr-abl inhibitors are dasatinib, AZD-0530, bosutinib, XL 228 (also an IGF-1R inhibitor), nilotinib (also a PDGFR and cKit inhibitor), imatinib (also a cKit inhibitor), and NS-187.

An example for a PDK1 inhibitor is BX-517.

An example for a Rho inhibitor is BA-210.

Examples for PI3 kinase inhibitors are PX-866, BEZ-235 (also an mTor inhibitor), XL 418 (also an Akt inhibitor), XL-147, and XL 765 (also an mTor inhibitor).

Examples for inhibitors of cMet or HGF are XL-184 (also an inhibitor of VEGFR, cKit, Flt3), PF-2341066, MK-2461, XL-880 (also an inhibitor of VEGFR), MGCD-265 (also an inhibitor of VEGFR, Ron, Tie2), SU-11274, PHA-665752, AMG-102, and AV-299.

An example for a c-Myc inhibitor is CX-3543.

Examples for Flt3 inhibitors are AC-220 (also an inhibitor of cKit and PDGFR), KW 2449, lestaurtinib (also an inhibitor of VEGFR, PDGFR, PKC), TG-101348 (also an inhibitor of JAK2), XL-999 (also an inhibitor of cKit, FGFR, PDGFR and VEGFR), sunitinib (also an inhibitor of PDGFR, VEGFR and cKit), and tandutinib (also an inhibitor of PDGFR, and cKit).

Examples for HSP90 inhibitors are tanespimycin, alvespimycin, IPI-504 and CNF 2024.

Examples for JAK/STAT inhibitors are CYT-997 (also interacting with tubulin), TG 101348 (also an inhibitor of Flt3), and XL-019.

Examples for Mek inhibitors are ARRY-142886, PD-325901, AZD-8330, and XL 518.

Examples for mTor inhibitors are temsirolimus, AP-23573 (which also acts as a VEGF inhibitor), everolimus (a VEGF inhibitor in addition). XL-765 (also a PI3 kinase inhibitor), and BEZ-235 (also a PI3 kinase inhibitor).

Examples for Akt inhibitors are perifosine, GSK-690693, RX-0201, and triciribine.

Examples for cKit inhibitors are AB-1010, OSI-930 (also acts as a VEGFR inhibitor), AC-220 (also an inhibitor of Flt3 and PDGFR), tandutinib (also an inhibitor of Flt3 and PDGFR), axitinib (also an inhibitor of VEGFR and PDGFR), XL-999 (also an inhibitor of Flt3, PDGFR, VEGFR, FGFR), sunitinib (also an inhibitor of Flt3, PDGFR, VEGFR), and XL-820 (also acts as a VEGFR- and PDGFR inhibitor), imatinib (also a bcr-abl inhibitor), nilotinib (also an inhibitor of bcr-abl and PDGFR).

Examples for hedgehog antagonists are IPI-609 and CUR-61414.

Examples for CDK inhibitors are seliciclib, AT-7519, P-276, ZK-CDK (also inhibiting VEGFR2 and PDGFR), PD-332991, R-547, SNS-032, PHA-690509, and AG 024322.

Examples for proteasome inhibitors are bortezomib, carfilzomib, and NPI-0052 (also an inhibitor of NFkappaB).

An example for an NFkappaB pathway inhibitor is NPI-0052.

An example for an ubiquitination pathway inhibitor is HBX-41108.

In preferred embodiments, the additional therapeutic agent is an anti-angiogenic agent.

Examples for anti-angiogenic agents are inhibitors of the FGFR, PDGFR and VEGFR or the respective ligands (e.g VEGF inhibitors like pegaptanib or the anti-VEGF antibody bevacizumab), and thalidomides, such agents being selected from, without limitation, bevacizumab, motesanib, CDP-791, SU-14813, telatinib, KRN-951, ZK-CDK (also an inhibitor of CDK), ABT-869, BMS-690514, RAF-265, IMC-KDR, IMC-18F1, IMiDs (immunomodulatory drugs), thalidomide derivative CC-4047, lenalidomide, ENMD 0995, IMC-D11, Ki 23057, brivanib, cediranib, XL-999 (also an inhibitor of cKit and Flt3), 1B3, CP 868596, IMC 3G3, R-1530

(also an inhibitor of Flt3), sunitinib (also an inhibitor of cKit and Flt3), axitinib (also an inhibitor of cKit), lestaurtinib (also an inhibitor of Flt3 and PKC), vatalanib, tandutinib (also an inhibitor of Flt3 and cKit), pazopanib, GW 786034, PF-337210, IMC-1121B, AVE-0005, AG-13736, E-7080, CHIR 258, sorafenib tosylate (also an inhibitor of Raf), RAF-265 (also an inhibitor of Raf), vandetanib, CP-547632, OSI-930, AEE-788 (also an inhibitor of EGFR and Her2), BAY-57-9352 (also an inhibitor of Raf), BAY-73-4506 (also an inhibitor of Raf), XL 880 (also an inhibitor of cMet), XL-647 (also an inhibitor of EGFR and EphB4), XL 820 (also an inhibitor of cKit), and nilotinib (also an inhibitor of cKit and brc-abl).

The additional therapeutic agent may also be selected from EGFR inhibitors, it may be a small molecule EGFR inhibitor or an anti-EGFR antibody. Examples for anti-EGFR antibodies, without limitation, are cetuximab, panitumumab, matuzumab; an example for a small molecule EGFR inhibitor is gefitinib. Another example for an EGFR modulator is the EGF fusion toxin.

Among the EGFR and Her2 inhibitors useful for combination with the Dll4-binding molecule of the invention are lapatinib, gefitinib, erlotinib, cetuximab, trastuzumab, nimotuzumab, zalutumumab, vandetanib (also an inhibitor of VEGFR), pertuzumab, XL-647, HKI-272, BMS-599626 ARRY-334543, AV 412, mAB-806, BMS-690514, JNJ-26483327, AEE-788 (also an inhibitor of VEGFR), ARRY-333786, IMC-11F8, Zemab.

Other agents that may be advantageously combined in a therapy with the Dll4-binding molecule of the invention are tositumumab and ibritumomab tiuxetan (two radiolabelled anti-CD20 antibodies), alemtuzumab (an anti-CD52 antibody), denosumab, (an osteoclast differentiation factor ligand inhibitor), galiximab (a CD80 antagonist), ofatumumab (a CD20 inhibitor), zanolimumab (a CD4 antagonist), SGN40 (a CD40 ligand receptor modulator), rituximab (a CD20 inhibitor) or mapatumumab (a TRAIL-1 receptor agonist).

Other chemotherapeutic drugs that may be used in combination with the Dll4-binding molecules of the present invention are selected from, but not limited to hormones, hormonal analogues and antihormonals (e.g. tamoxifen, toremifene,

raloxifene, fulvestrant, megestrol acetate, flutamide, nilutamide, bicalutamide, cyproterone acetate, finasteride, buserelin acetate, fludrocortisone, fluoxymesterone, medroxyprogesterone, octreotide, arzoxifene, pasireotide, vapreotide), aromatase inhibitors (e.g. anastrozole, letrozole, liarozole, exemestane, atamestane, formestane), LHRH agonists and antagonists (e.g. goserelin acetate, leuprolide, abarelix, cetrorelix, deslorelin, histrelin, triptorelin), antimetabolites (e.g. antifolates like methotrexate, pemetrexed, pyrimidine analogues like 5 fluorouracil, capecitabine, decitabine, nelarabine, and gemcitabine, purine and adenosine analogues such as mercaptopurine thioguanine, cladribine and pentostatin, cytarabine, fludarabine); antitumor antibiotics (e.g. anthracyclines like doxorubicin, daunorubicin, epirubicin and idarubicin, mitomycin-C, bleomycin dactinomycin, plicamycin, mitoxantrone, pixantrone, streptozocin); platinum derivatives (e.g. cisplatin, oxaliplatin, carboplatin, lobaplatin, satraplatin); alkylating agents (e.g. estramustine, meclorethamine, melphalan, chlorambucil, busulphan, dacarbazine, cyclophosphamide, ifosfamide, hydroxyurea, temozolomide, nitrosoureas such as carmustine and lomustine, thiotepa); antimitotic agents (e.g. vinca alkaloids like vinblastine, vindesine, vinorelbine, vinflunine and vincristine; and taxanes like paclitaxel, docetaxel and their formulations, larotaxel; simotaxel, and epothilones like ixabepilone, patupilone, ZK-EPO); topoisomerase inhibitors (e.g. epipodophyllotoxins like etoposide and etopophos, teniposide, amsacrine, topotecan, irinotecan) and miscellaneous chemotherapeutics such as amifostine, anagrelide, interferone alpha, procarbazine, mitotane, and porfimer, bexarotene, celecoxib.

The efficacy of Dll4-binding molecules of the invention or polypeptides containing them, and of compositions comprising the same, can be tested using any suitable *in vitro* assay, cell- based assay, *in vivo* assay and/or animal model known per se, or any combination thereof, depending on the specific disease or disorder of interest. Suitable assays and animal models will be clear to the skilled person, and

for example include the assays described herein and used in the Examples below, e.g. a proliferation assay.

The data obtained in the experiments of the invention confirm that Dll4-binding molecules of the invention have properties that are superior to those of Dll4-binding molecules of the prior art, as can e.g. be taken from the ELISA data of Figure 10, showing that affinity-matured VHHs block hDLL4/hNotch1-Fc interaction in a complete manner, as well as the IC_{50} (nM) values for affinity matured VHHs in hDLL4/hNotch1-Fc competition ELISA; and the affinity K_D (nM) of purified affinity matured VHHs on recombinant human DLL4 and mouse DLL4. This indicates that Dll4-binding molecules of the invention are promising candidates to have therapeutic efficacy in diseases and disorders associated with Dll4-mediated effects on angiogenesis, such as cancer.

According to another embodiment of the invention, there is provided a method of diagnosing a disease by
a) contacting a sample with a Dll4-binding molecule of the invention as defined above, and
b) detecting binding of said Dll4-binding molecule to said sample, and
c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disease or disorder associated with Dll4-mediated effects on angiogenesis.

For this and other uses, it may be useful to further modify a Dll4-binding molecule of the invention, such as by introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the Dll4-binding molecule of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a Dll4-binding molecule of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or

streptavidin. For example, such a conjugated Dll4-binding molecule of the invention may be used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin.

Brief description of the Figures:

Figure 1: Amino acid sequence alignment of human, rhesus and cynomolgus DLL4.

Figure 2: Human and mouse DLL4 deletion mutants (amino acid domain boundaries in superscript).

Figure 3: Purified VHHs blocking hDLL4/hNotch1-Fc interaction (ELISA).

Figure 4: Purified VHHs blocking hDLL4/hNotch1-Fc interaction (AlphaScreen).

Figure 5: Purified VHHs blocking CHO-hDLL4/hNotch1-Fc and CHO-mDLL4/hNotch1-Fc interaction (FMAT).

Figure 6: Purified VHHs blocking DLL4 mediated Notch1 cleavage (reporter).

Figure 7: Binding of purified VHHs to recombinant human and mouse DLL4 (ELISA).

Figure 8: Binding of purified VHHs to recombinant human DLL1 and human Jagged-1 (ELISA).

Figure 9: Binding of purified VHHs to human/mouse/cynomolgus DLL4 (FACS).

Figure 10: Affinity matured VHHs blocking hDLL4/hNotch1-Fc interaction (ELISA).

Figure 11: Purified affinity matured VHHs blocking CHO-hDLL4/hNotch1-Fc and CHO-mDLL4/hNotch1-Fc interaction (FMAT).

Figure 12: Binding of purified VHHs to human/mouse DLL4 (ELISA).

Figure 13: Binding of purified affinity matured VHHs to recombinant human DLL1 and human Jagged-1 (ELISA).

Figure 14: Binding of purified VHHs to human/mouse/cynomolgus DLL4 (FACS).

Figure 15: Evaluation of VHH effects on Dll4-mediated inhibition of HUVEC proliferation.

Materials and methods

a) Generation CHO and HEK293 cell lines overexpressing human, mouse and cynomolgus Dll4

The cDNAs encoding human (SEQ ID NO: 417; NM_019074.2) and mouse Dll4 (NM_019454.3) are amplified from a Human Adult Normal Tissue Heart cDNA library (BioChain, Hayward, CA, USA) and a Mouse Heart Tissue cDNA library (isolated from C57/Bl6 strain), respectively, using oligonucleotides designed in the 5' and 3' UTR of the corresponding sequence (see Table 1; SEQ ID NO:421 to 426). Amplicons are cloned into the mammalian expression vector pCDNA3.1(+)-neo (Invitrogen, Carlsbad, CA, USA).

Table 1: Oligonucleotide sequences used for amplification of DLL4 gene full length orthologues.

Human DLL4	**Mouse DLL4**	**Cynomolgus DLL4**
>Fwd_hDLL4 GCGAACAGAGCCAGATTGAGG	>Fwd_mDLL4 GAGCGACATCCCTAACAAGC	>Fwd_cDLL4 GCGAACAGAGCCAGATTCAGG
>Rev_hDLL4 GGATGTCCAGGTAGGCTCCTG	>Rev_mDLL4 CCTCAACTCTGTTCCCTTGG	>Rev_cDLL4 CCAGACAGACACCCAAAGGT

Cynomolgus Dll4 cDNA is amplified from a Cynomolgus Normal Tissue Heart cDNA library (BioChain, Hayward, CA, USA), using primers designed on the 5' and 3' UTR of the Dll4 encoding sequence of the closely related species rhesus (Macaca mulatta Dll4, SEQ ID NO:418; XM_001099250.1) (see Table 1). The final amplicon is cloned in the mammalian expression vector pCDNA3.1(+)-neo (Invitrogen, Carlsbad, CA, USA). The amino acid sequence of cynomolgus Dll4 was shown to be 100% identical to rhesus, and 99% identical to human (see Figure 1; differences from the human sequence are indicated as bold-underlined).

To establish Chinese Hamster Ovary (CHO) cells overexpressing human Dll4, mouse Dll4 or cynomolgus Dll4, parental CHO cells are electroporated with pCDNA3.1(+)-neo-hDll4, pcDNA3.1(+)-neo-mDll4 or pcDNA3.1(+)-neo-cDll4, respectively. Human Embyonic Kidney (HEK293) cells overexpressing human Dll4 and mouse Dll4 are generated by lipid-mediated transfection with Fugene (Roche) of pCDNA3.1(+)-neo-hDll4 or mDll4 plasmids, respectively, in the HEK293 parental cell line. For all conditions, transfectants are selected by adding 1 mg/mL geneticin (Invitrogen, Carlsbad, CA, USA).

b) Generation of monoclonal anti-Dll4 IgG and Fab fragment

In US 2008/0014196 (Genentech) a human/mouse cross-reactive Dll4 mAb is described that was used by Ridgway *et al.* (2006) to show additive effects of VEGF mAb and Dll4 mAb on tumor growth in a number of xenograft models. This anti-Dll4 mAb and its corresponding Fab are purified to assess the properties of this antibody (fragment) in biochemical/cellular assays and xenograft models and for specific elutions during phage selections. The published variable heavy and light chain sequences of Dll4 mAb are cloned into a hIgG2aκ framework, transiently expressed in HEK293 cells and purified from supernatants using protein A chromatography. Purified Dll4 mAb shows binding to human Dll4 and mouse Dll4 in ELISA and FACS (using CHO-mDll4 and CHO-hDll4 cells), sub-nanomolar affinities to both growth factor orthologues in Biacore.

The corresponding Dll4 Fab fragment is constructed via gene assembly based on back-translation and codon optimization for expression in E. *coli* using Leto's Gene Optimization software (www.entechelon.com). Oligonucleotide primers for the assembly of the variable light chain (V_L), variable heavy chain (V_H), constant light chain (C_L) and constant domain 1 of the heavy chain (C_{H1}) are designed and an assembly PCR is performed. The cDNA seqments encoding V_L+C_L and V_H+C_{H1} are cloned into a pUC119-derived vector, which contains the LacZ promotor, a resistance gene for kanamycin, a multiple cloning site and a hybrid gIII-pelB leader sequence, using the restriction sites *SfiI* and *AscI* and the restriction sites *KpnI* and *NotI*, respectively. In frame with the Fab coding sequence, the expression

vector encodes a C-terminal HA and His6-tag. The Fab fragment is expressed in E. *coli* as His6-tagged protein and subsequently purified from the culture medium by immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). Relevant amino acid sequences of the variable heavy and variable light chain are depicted (SEQ ID NO: 1 and SEQ ID NO: 2; respectively, of US 2008/0014196); the amino acid sequences of the complete heavy and light chain are shown in SEQ ID NOs: 419 and 420, respectively.

c) Generation of Dll4 mutants for epitope mapping

To identify the region in the extracellular domain (ECD) of Dll4 that comprises the epitope recognized by the anti-Dll4 VHHs, progressive deletion mutants of the Dll4 ECD are generated. The mammalian expression vector pSecTag2/Hygro (Invitrogen, Carlsbad, CA, USA) comprising a CMV promotor upstream of polynucleotides encoding a nested series of deletion fragments of the Dll4 ECD fused to a polyHis-tag are generated using standard recombinant DNA technology (see Figure 2; amino acid domain boundaries in superscript).). These recombinant proteins are expressed in transiently transfected HEK293 cells using the Freestyle 293 Expression System (Invitrogen, Carlsbad, CA, USA) from which conditioned medium is collected and purified via IMAC. Only Dll4 mutants lacking the EGF2-like domain showed impaired binding to the humanized human/mouse cross-reactive anti-Dll4 mAb described above (immobilized via a capturing anti-human IgG coated Biacore sensor chip). This IgG is known to have a specific binding epitope in this Dll4 domain (patent application Genentech, US 2008/0014196A1).

d) Generation of Dll4 reporter assay plasmids

*A repor*ter assay is developed based on the γ-secretase mediated cleavage of Notch1 and nuclear translocation of the intracellular domain of Notch1 (NICD) upon stimulation with Dll4, essentially as described (Struhl and Adachi, Cell. 1998 May 15;93(4):649-60). Gal4/VP16 coding sequences are inserted into the NICD-coding sequence. The potent hybrid transcriptional activator GAL4-VP16, which consists of a DNA binding fragment of yeast GAL4 fused to a Herpes simplex viral

transcriptional activator domain VP16, is inserted carboxy-terminal to the transmembrane domain of Notch1. Cleavage of this construct by γ-secretase results in the release of the Gal4/VP16 NICD fusion protein which will translocate to the nucleus where it will bind to and transcriptionally activate a co-transfected luciferase reporter plasmid, containing a strong GAL4-UAS promoter sequence (Struhl, G. and Adachi, A., Cell, vol. 93, 649-660, 1998). The human Notch1-Gal4/VP16 expression cassette is cloned in pcDNA3.1(+)-neo (Invitrogen, Carlsbad, CA, USA). The pGL4.31[Luc2P/Gal4UAS/Hygro] vector (Promega, Madison, WI, USA) is used as luciferase reporter plasmid.

Example 1

Immunization with Dll4 from different species induces a humoral immune response in llama

1.1. Immunizations

After approval of the Ethical Committee of the faculty of Veterinary Medicine (University Ghent, Belgium), 4 llamas (designated No. 208, 209, 230, 231) are immunized with 6 intramuscular injections (100 or 50 µg/dose at weekly intervals) of recombinant human Dll4 (R&D Systems, Minneapolis, MN, US). The Dll4 antigen is formulated in Stimune (Cedi Diagnostics BV, Lelystad, The Netherlands). Three additional llamas (designated No. 127b, 260, 261) are immunized according to standard protocols with 4 subcutaneous injections of alternating human Dll4 and mouse Dll4 overexpressing CHO cells which are established as described above. Cells are re-suspended in D-PBS and kept on ice prior to injection. Furthermore, three additional llamas (designated No. 282, 283, 284) are immunized according to standard protocols with 4 intramuscular injections (100 or 50 µg/dose at biweekly intervals) of alternating recombinant human Dll4 and mouse Dll4 (R&D Systems, Minneapolis, MN, US). The first injection at day 0 with human Dll4 is formulated in Complete Freund's Adjuvant (Difco, Detroit, MI, USA), while the subsequent injections with human and mouse Dll4 are formulated in Incomplete Freund's Adjuvant (Difco, Detroit, MI, USA).

1.2. Evaluation of induced immune responses in llama

To evaluate the induction of an immune responses in the animals against human Dll4 by ELISA, sera are collected from llamas 208, 209, 230 and 231 at day 0 (pre-immune), day 21 and day 43 (time of peripheral blood lymphocyte [PBL] collection), from llamas 127b, 260 and 261 at day 0 and day 51, and from llamas 282, 283 and 284 at day 0, day 28 and day 50. In short, 2 µg/mL of recombinant human Dll4 or mouse Dll4 (R&D Systems, Minneapolis, MN, USA) are immobilized overnight at 4˚C in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1%). After addition of serum dilutions, specifically bound immunoglobulins are detected using a horseradish peroxidase (HRP)-conjugated goat anti-llama immunoglobulin (Bethyl Laboratories Inc., Montgomery, TX, USA) and a subsequent enzymatic reaction in the presence of the substrate TMB (3,3',5,5'-tetramentylbenzidine) (Pierce, Rockford, IL, USA), showing that a significant antibody-dependend immune response against Dll4 is induced. The antibody response is mounted both by conventional and heavy-chain only antibody expressing B-cell repertoires since specifically bound immunoglobulins can be detected with antibodies specifically recognizing the conventional llama IgG1 antibodies or the heavy chain only llama IgG2 or IgG3 antibodies (Table 2-A). In all llamas injected with mouse Dll4, an antibody response is mounted by conventional and heavy chain only antibody expressing B-cells specifically against mouse Dll4. Additionally, serum titers of cell immunized animals are confirmed by FACS analysis on human and mouse Dll4 overexpressing HEK293 cells (Table 2-B). The Dll4 serum titer responses for each llama are depicted in Table 2.

Table 2: Antibody mediated specific serum response against DLL4.

A) ELISA (recombinant protein solid phase coated)

		Recombinant human DLL4				Recombinant mouse DLL4			
Llama	Immunogen	Total IgG	IgG1	IgG2	IgG3	Total IgG	IgG1	IgG2	IgG3
208	rec. human DLL4	+	+	+/-	+/-	ND	ND	ND	ND
209	rec. human DLL4	+	+	+/-	+/-	ND	ND	ND	ND
230	rec. human DLL4	++	++	+/-	+/-	ND	ND	ND	ND
231	rec. human DLL4	++	++	++	++	ND	ND	ND	ND
127b	CHO-hDLL4 + CHO-mDLL4	++	++	+/-	+/-	+	++	+/-	+/-
260	CHO-hDLL4 + CHO-mDLL4	++	++	+	+	++	++	+	++
261	CHO-hDLL4 + CHO-mDLL4	++	++	+/-	+/-	+	+	+/-	+/-
282	rec. human DLL4 + mouse DLL4	++	++	++	++	++	++	+	+
283	rec. human DLL4 + mouse DLL4	++	++	++	++	++	++	++	++
284	rec. human DLL4 + mouse DLL4	+	+	+	+	+	++	+	++

ND: not determined

B) FACS (natively expressed protein on HEK293 cells)

		human DLL4				mouse DLL4			
Llama	**Immunogen**	**Total IgG**	**IgG1**	**IgG2**	**IgG3**	**Total IgG**	**IgG1**	**IgG2**	**IgG3**
208	rec. human DLL4	ND	ND	ND	ND	ND	ND	ND	ND
209	rec. human DLL4	ND	ND	ND	ND	ND	ND	ND	ND
230	rec. human DLL4	ND	ND	ND	ND	ND	ND	ND	ND
231	rec. human DLL4	ND	ND	ND	ND	ND	ND	ND	ND
127b	CHO-hDLL4 + CHO-mDLL4	+	ND	ND	ND	+	ND	ND	ND
260	CHO-hDLL4 + CHO-mDLL4	++	ND	ND	ND	++	ND	ND	ND
261	CHO-hDLL4 + CHO-mDLL4	+	ND	ND	ND	+	ND	ND	ND
282	rec. human DLL4 + mouse DLL4	ND	ND	ND	ND	ND	ND	ND	ND
283	rec. human DLL4 + mouse DLL4	ND	ND	ND	ND	ND	ND	ND	ND
284	rec. human DLL4 + mouse DLL4	ND	ND	ND	ND	ND	ND	ND	ND

ND: not determined

Example 2

Cloning of the heavy-chain only antibody fragment repertoires and preparation of phage

Following the final immunogen injection, immune tissues as the source of B-cells that produce the heavy-chain antibodies are collected from the immunized llamas. Typically, two 150-ml blood samples, collected 4 and 8 days after the last antigen injection, and one lymph node biopsy, collected 4 days after the last antigen injection are collected per animal. From the blood samples, peripheral blood mononuclear cells (PBMCs) are prepared using Ficoll-Hypaque according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). From the PBMCs and the lymph node biopsy, total RNA is extracted, which is used as starting material for RT-PCR to amplify the VHH encoding DNA segments, as described in WO 05/044858. For each immunized llama, a library is constructed by pooling the total RNA isolated from all collected immune tissues of that animal. In short, the PCR-amplified VHH repertoire is cloned via specific restriction sites into a vector designed to facilitate phage display of the VHH library. The vector is derived from pUC119 and contains the LacZ promoter, a M13 phage gIII protein coding sequence, a resistance gene for ampicillin or carbenicillin, a multiple cloning site and a hybrid gIII-pelB leader sequence (pAX050). In frame with the VHH coding sequence, the vector encodes a C-terminal c-myc tag and a His6 tag. Phage are prepared according to standard protocols and stored after filter sterilization at 4ºC for further use.

Example 3

Selection of Dll4 specific VHHs via phage display

VHH repertoires obtained from all llamas and cloned as phage library are used in different selection strategies, applying a multiplicity of selection conditions. Variables include i) the Dll4 protein format (C-terminally His-tagged recombinantly expressed extracellular domain of human Dll4 (Met1-Pro524) and mouse Dll4 (Met1-Pro525) (R&D Systems, Minneapolis, MN, USA), or full length human Dll4 and mouse Dll4 present on Dll4-overexpressing CHO or HEK293 cells, ii) the

antigen presentation method (plates directly coated with Dll4 or Neutravidin plates coated with Dll4 via a biotin-tag; solution phase: incubation in solution followed by capturing on Neutravidin-coated plates), iii) the antigen concentration and iv) different elution methods (non-specific via trypsin or specfic via cognate receptor Notch1/Fc chimera or anti-Dll4 IgG/Fab). All selections are done in Maxisorp 96-well plates (Nunc, Wiesbaden, Germany).

Selections are performed as follows: Dll4 antigen preparations for solid and solution phase selection formats are presented as described above at multiple concentrations. After 2h incubation with the phage libraries followed by extensive washing, bound phage are eluted with trypsin (1 mg/mL) for 30 minutes. In case trypsin is used for phage elution, the protease activity is immediately neutralized applying 0.8 mM protease inhibitor ABSF. As control, selections w/o antigen are performed in parallel. Phage outputs that show enrichment over background (non-antigen control) are used to infect E. coli. Infected E. coli cells are either used to prepare phage for the next selection round (phage rescue) or plated on agar plates (LB+amp+glucose$^{2\%}$) for analysis of individual VHH clones. In order to screen a selection output for specific binders, single colonies are picked from the agar plates and grown in 1 mL 96-deep-well plates. LacZ-controlled VHH expression is induced by adding IPTG (0.1-1mM final) in the absence of glucose. Periplasmic extracts (in a volume of ~ 80 uL) are prepared according to standard protocols

Example 4

Screening of periplasmic extracts in Dll4-Notch1 AlphaScreen and FMAT competition assay

Periplasmic extracts are screened in a human Dll4/human Notch1 AlphaScreen assay to assess the blocking capacity of the expressed VHHs. Human Dll4 is biotinylated using biotin (Sigma, St Louis, MO, USA) and biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sigma, St Louis, MO, USA). Notch1/Fc chimera (R&D Systems, Minneapolis, MN, USA) is captured using an anti-Fc VHH which is coupled to acceptor beads according to the manufacturer's

instructions (Perkin Elmer, Waltham, MA, US). To evaluate the neutralizing capacity of the VHHs, dilution series of the periplasmic extracts are pre-incubated with biotinylated human Dll4. To this mixture, the acceptor beads and the streptavidin donor beads are added and further incubated for 1 hour at room temperature. Fluorescence is measured by reading plates on the Envision Multilabel Plate reader (Perkin Elmer, Waltham, MA, USA) using an excitation wavelength of 680 nm and an emission wavelength of 520 nm. Decrease in fluorescence signal indicates that the binding of biotinylated human Dll4 to the human Notch1/Fc receptor is blocked by the VHH expressed in the periplasmic extract.

Alternatively, CHO-hDll4 and CHO-mDll4 cells are used in a human Notch1/Fc FMAT (Fluorometric Microvolume Assay Technology) competition assay. Recombinant human Notch1/Fc chimera (R&D Systems, Minneapolis, MN, USA) is randomly labeled with Alexa-647 (Invitrogen, Carlsbad, CA, USA). In brief, 5 µL periplasmic material is added to 100 pM or 175 pM labeled human Notch1/Fc together with 7,500 CHO-hDll4 or CHO-mDll4 overexpressing cells, respectively, and readout is performed after 2 hours of incubation. To set the no-competition baseline, at least 30 replicates of cells with human Notch1/Fc~Alexa647 are included and the percentage of inhibition is calculated from this baseline. All calculations are based on the FL1_total signal which comprises the average of the fluorescence per well times the number of counts per well.

From this screening, inhibiting VHHs are selected and sequenced. Sequence analysis revealed 167 unique VHHs belonging to 40 different B-cell lineages. The total number of variants found for each B-cell lineage is depicted in Table 3. An overview of periplasmic screening data is given in Table 4. The amino acid sequences of all obtained unique VHHs are shown in the Sequence Listing (SEQ ID NO:167 – 332 and 459) and in Table 5 (CDRs and framework regions are indicated).

Table 3: Selection parameters used for the identification of DLL4 specific VHH B-cell lineages.

B-cell lineage	VHH ID	# variants	library	selection format	phage elution	selection rounds
1	DLLBII8A09	31	231	rhDLL4 (3 nM)	trypsin	1
2	DLLBII5B11	1	231	rhDLL4 (3 nM)	trypsin	1
3	DLLBII7B5	21	231	RI: biot-rhDLL4 (3 nM) RII: biot-rhDLL4 (0.03 nM)	trypsin	2
4	DLLBII6B11	13	231	biot-rhDLL4 (3 M)	trypsin	1
5	DLLBII8C11	5	231	RI: biot-rhDLL4 (3 nM) RII: biot-rhDLL4 (3 nM)	trypsin	2
6	DLLBII19D10	1	231	biot-rhDLL4 (3 nM)	trypsin	1
7	DLLBII33C5	2	231	CHO-hDLL4 (2E6/mL)	trypsin	1
8	DLLBII28B6	2	231	rmDLL4 (0.5 ug/mL)	trypsin	1
9	DLLBII17G10	1	231	biot-rhDLL4 (3 nM)	trypsin	1
10	DLLBII17C1	8	231	biot-rhDLL4 (3 nM)	trypsin	1
11	DLLBII19F4	1	231	biot-rhDLL4 (3 nM)	trypsin	1
12	DLLBII17F10	1	231	biot-rhDLL4 (3 nM)	trypsin	1
13	DLLBII17B3	5	231	biot-rhDLL4 (3 nM)	trypsin	1
14	DLLBII19F12	2	231	biot-rhDLL4 (3 nM)	trypsin	1
15	DLLBII42B7	1	231	RI: biot-rhDLL4 (3 nM) RII: biot-rhDLL4 (3 nM)	rhNotch1/ Fc	2

16	DLLBII47D1	1	230	RI: biot-rhDLL4 (3 nM) RII: biot-rhDLL4 (3 nM)	rhNotch1/ Fc	2
17	DLLBII56A09	15	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	rhNotch1/ Fc	2
18	DLLBII95F2	5	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	trypsin	2
19	DLLBII96C3	20	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	trypsin	2
20	DLLBII104G1	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (+rhDLL4)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
21	DLLBII102F8	3	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (0.01 nM)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
22	DLLBII112A3	1	209	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	trypsin	2

23	DLLBII102G4	2	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (0.01 nM)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
24	DLLBII101G8	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (0.1 nM)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
25	DLLBII112A4	1	209	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	trypsin	2
26	DLLBII101H9	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (0.1 nM)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
27	DLLBII101H5	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (1 nM)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
28	DLLBII112E7	1	209	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	trypsin	2

29	DLLBII101F1	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (1 nM)	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
30	DLLBII104A3	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (1 nM) + rhDLL4	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
31	DLLBII104C4	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (1 nM) + rhDLL4	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
32	DLLBII104B5	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (1 nM) + rhDLL4	rhNotch1/ Fc (RI-RII) trypsin (RIII)	3
33	DLLBII107C3	1	208	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL)	rhNotch1/ Fc	2
34	DLLBII58A11	4	260	RI: biot-rhDLL4 (3 nM) RII: biot-rmDLL4 (3 nM)	rhNotch1/ Fc	2

35	DLLBII61F5	1	260	RI: HEK293H-hDLL4 (2E6/mL) RII: HEK293H-hDLL4 (2E6/mL)	trypsin	2
36	DLLBII61F7	1	260	RI: HEK293H-hDLL4 (2E6/mL) RII: HEK293H-hDLL4 (2E6/mL)	trypsin	2
37	DLLBII62C11	1	260	RI: HEK293H-hDLL4 (2E6/mL) RII: HEK293H-mDLL4 (2E6/mL)	trypsin	2
38	DLLBII115A5	1	230	RI: CHO-mDLL4 (2E6/mL) RII: CHO-mDLL4 (2E6/mL) RIII: biot-rhDLL4 (1 nM) RIV:CHO-mDLL4 (2E6/mL)	rhNotch1/Fc (RI-RII) trypsin (RIII) trypsin (RIV)	4
39	DLLBII83G1	4	284	RI: CHO-mDLL4 (2E6/mL) RI: CHO-hDLL4 (2E6/mL)	DLL4 IgG	2
40	DLLBII80E8	1	283	RI: CHO-hDLL4 (2E6/mL) RI: CHO-hDLL4 (2E6/mL)	DLL4 IgG	2

Table 4: Screening of periplasmic extracts containing expressed anti-DLL4 VHH

B-cell line age	Represen-tative VHH ID	# unique sequences	ELISA	Alpha Screen	FMAT	FMAT	Biacore (a)
			hDLL4 % inhibition	hDLL4 % inhibition	hDLL4 % inhibition	mDLL4 % inhibition	k_d (s^{-1})
1	DLLBII8A09	31	96	-	-	-	(1.2^{E-03}-2.4^{E-04})
2	DLLBII5B11	1	98	-	-	-	-
3	DLLBII7B05	21	84	-	-	-	(2.4^{E-04})
4	DLLBII6B11	13	98	-	-	-	(9.4^{E-04}-3.7^{E-04})
5	DLLBII8C11	5	57	-	-	-	(7.3^{E-04}-6.0^{E-04})
6	DLLBII19D10	1	98	85	-	-	1.3^{E-03}
7	DLLBII33C05	2	86	75	-	-	9.2^{E-04} (2.1^{E-03})
8	DLLBII28B06	2	23	54	-	-	7.5^{E-03} (1.6^{E-04})
9	DLLBII17G10	1	93	82	-	-	1.5^{E-03}
10	DLLBII17C01	8	82	84	-	-	5.6^{E-04} (5.6^{E-04}-5.3^{E-04})
11	DLLBII19F04	1	98	95	-	-	1.1^{E-03}
12	DLLBII17F10	1	98	88	-	-	1.1^{E-03} / $3.1^{E-04\ (b)}$
13	DLLBII17B03	5	76	77	-	-	1.2^{E-03} / $2.2^{E-04\ (b)}$
14	DLLBII19F12	2	98	98	-	-	4.9^{E-04} (1.0^{E-03})
15	DLLBII42B07	1	-	-	-	-	-
16	DLLBII47D01	1	-	-	87	-	-
17	DLLBII56A09	15	-	-	-	-	1.1^{E-03} (9.5^{E-03}-1.1^{E-03})
18	DLLBII95F02	5	-	-	81	71	6.7^{E-04}
19	DLLBII96C03	20	-	-	75	83	-
20	DLLBII104G01	1	-	-	94	86	1.2^{E-03} (1.4^{E-03}-9.4^{E-04})
21	DLLBII102F08	3	-	-	85	75	-
22	DLLBII112A03	1	-	-	72	97	-
23	DLLBII102G04	2	-	-	86	82	-
24	DLLBII101G08	1	-	-	91	92	2.1^{E-03}
25	DLLBII112A04	1	-	-	75	90	-
26	DLLBII101H09	1	-	-	87	75	-
27	DLLBII101H05	1	-	-	85	83	-

28	DLLBII112E07	1	-	-	80	85	-
29	DLLBII101F01	1	-	-	85	78	2.0^{E-02}
30	DLLBII104A03	1	-	-	86	83	-
31	DLLBII104C04	1	-	-	87	83	1.0^{E-03}
32	DLLBII104B05	1	-	-	86	78	-
33	DLLBII107C03	1	-	-	75	80	-
34	DLLBII58A11	4	-	-	95	73	1.6^{E-03} (1.7^{E-03} - 1.6^{E-03})
35	DLLBII61F05	1	-	-	74	76	-
36	DLLBII61F07	1	-	-	79	77	-
37	DLLBII62C11	1	-	-	74	71	-
38	DLLBII115A05	1	-	-	74	84	3.1^{E-03}
39	DLLBII83G01	4	-	-	87	93	4.1^{E-04}
40	DLLBII80E08	1	-	-	71	82	-

[a] if multiple unique variants within a B-cell lineage are identified, the range (max-min) in off-rate or the off-rate of a lineage member is given between brackets in italics).

[b] heterogeneous fit: fast and slow off-rate determined.

Table 5

VHH ID **SEQ ID NO**	**Framework 1**	**CDR 1**	**Framework 2**	**CDR 2**	**Framework 3**	**CDR 3**	**Framework 4**
DLLBII 05B06 **167**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	 TYNIG	 WFRQAPGKEREW VS	CISSSD GSTNYA DSVKG	RFTISRDNAKNTVYL QMNNLKPEDTAVYYC AA	 PFAYYSDLCGV NGVDY	 WGQGTQVTVSS
DLLBII 05B08 **168**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	 YYNIG	 WFRQAPGKEREW VS	CINSSD GSTYYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAIYYC AA	 PFSYYSHLCGV NGYDY	 WGQGTQVTVSS
DLLBII 05B09 **169**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	 YYNIG	 WFRQAPGKEREW VS	CISSSD GSTAYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	 PFSYYSSLCGV NEYDY	 WGQGTQVTVSS
DLLBII 05B11 **170**	EVQLVESGGGLV QPGGSLRLSCAI SGFTLD	 LHVIG	 WLRQAPGKEREW VS	CISSSD GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	 PWDSWYCGIGN DYDY	 WGQGTQVTVSS
DLLBII 05D11 **171**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	 YYNIG	 WFRQAPGKEREW VS	CIRGSN GSTGYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	 PFIHYSDLCGV NGYDY	 WGQGTQVTVSS
DLLBII 06A02 **172**	EVQLVESGGGLV QAGGSLRLSCAA SGFTLD	 KYAIG	 WFRQAPGKEREG VS	CISSRG GSTYYV DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	 DPIHNCYSGSS YYYSPEAVYDY	 WGQGTQVTVSS
DLLBII 06A05 **173**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	 YYNIG	 WFRQAPGKEREW VS	CITSSN GSTYYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	 PFAHYSDLCGV NGYDY	 WGQGTQVTVSS
DLLBII 06B11 **174**	EVQLVESEGGLV QAGGSLRLSCAA SGSTFS	 SYAMG	 WYRQAPGKQREL VA	VISNGG ITNYPN SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC FY	 SGSYYYPTDVH EYDY	 WGQGTQVTVSS

DLLBII 06E02 **175**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CINSSD GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFEYYSDLCGV NGYDY	WGQGTQVTVSS
DLLBII 06E04 **176**	EVQLVESGGGLV QAGGSLRLSCAA SGFTLD	YYAIG	WFRQAPGKEREG IS	CISSRG GSTFYV DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYSGRY YYSPEAVYEY	WGQGTQVTVSS
DLLBII 06E12 **177**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YHNIG	WFRQAPGKEREW VS	CISSSG GSTAYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFSHYSDLCGV NAIDY	WGQGTQVTVSS
DLLBII 06G09 **178**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CINSSD GSTYYA DSVKG	RFTVSRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFEYYSDLCGV NGYDY	WGQGTQVTVSS
DLLBII 07A02 **179**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFN	SYAMG	WYRQAPGKQREW VA	AFSTGG STNYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC FY	SGSYYYPTDVF EYDY	WGQGTQVTVSS
DLLBII 07B05 **180**	EVQLVESGGGLV QAGGSLRLSCAA SGFALD	YYAVG	WFRQAPGKEREG VS	CISSRG GSTFYA DSVKG	RFTTSRNNAKNTVYL QMNSLKPEDTAVYYC AA	HPLQNCCGGSA YASPEAVYEY	WGQGTQVTVSS
DLLBII 08A09 **181**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CINSSD GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFAYYSNLCGV NGYDY	WGQGTQVTVSS
DLLBII 08B05 **182**	EVQLVESGGGLV QAGGSLRLSCAA SGFALD	YYAVG	WFRQAPGKEREG VS	CISSRG GSTYYV DSVKG	RFTTSRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYSGNY YASPEAVYDY	WGQGTQVTVSS
DLLBII 08C11 **183**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSHD RTTYYA DSVKG	RFTISSDNAKNTVYL QMNSLKPEDTAVYYC AA	DPLVCGYNDPR LADY	WGQGTQVTVSS
DLLBII 08H06 **184**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CITSSY GSTYYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFAHYSDLCGV NGYDY	WGQGTQVTVSS

DLLBII 09C01 **185**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YHNIG	WFRQAPGKEREW VS	CISSSD GRTAYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFTHYSDLCGV NEYDY	WGQGTQVTVSS
DLLBII 100G01 **186**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEG IG	CISSSG GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY	WGQGTQVTVSS
DLLBII 101B12 **187**	KVQLVESGGGLV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEG IS	CISSSG GITYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY	TGQGTQVTVSS
DLLBII 101E04 **188**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRQAPGKGPEW VS	AINSGG GTTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 101F01 **189**	EVQLVESGGGLV QAGGSLRLSCAA SGRTFS	NYAMG	WFRQAPGKEREF VA	AISWSG GDTYYA DSVKG	RFTISRDNAKNTVCL QMNSLKPEDTAVYYC AA	SFQSGAAPGAN FYDY	WGQGTQVTVSS
DLLBII 101F03 **190**	EVQLVESEGGSV QAGGSLRLSCAA SGRTFS	SYAMG	WFRQAPGKEREF VA	AINWSG GYTYYA DSVRG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PAPGSSGYEYD Y	WGQGTQVTVSS
DLLBII 101F06 **191**	EVQLVESGGGLV QAGGSLRLSCAA SGRTFS	SYAMG	WFRQAPGKEREF VA	AIFWSG GSTYYA DSVRG	RFTISRDIAKNTVYL QMNSLKPEDTAVYYC AA	PSPGSSGYEYD Y	WGQGTQVTVSS
DLLBII 101F08 **192**	EVQLVESGGGLV QTGDSLRLSCAA SGSTFS	NYRMG	WFRQGPGKEREF VA	AIGRNG QNTYYT DSVKG	RFTVTRDNAKNMMYL QMNSLKPEDSAVYTC AA	SLRGWDTTRID YEY	WGQGTQVTVSS
DLLBII 101F10 **193**	EVQLVESGGGLV QPGGSLRLSCTA SGFTFD	VYAIG	WFRQAPGKEPEG IS	CISSSG SITYYA DSVKG	RFTTSRDSAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY	WGQGTQVTVSS
DLLBII 101G02 **194**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRQAPGKGPEW VS	AINSGG DTTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS

DLLBII 101G03 **195**	EVQLVESRGGLV QAGGSLRLSCAA SGRTFN	SYAMG	WFRQAPGKEREF VA	TINWSG GSTYYA DSVKG	RFTISRDNAKNTAYL QMNSLKPEDTAVYYC AA	PAPGSSGYEYD Y	WGQGTQVTVSS
DLLBII 101G05 **196**	EVQLVESGGGSV QAGGSLRLSCAA SGRTFS	SYAMG	WFRQAPGKEREF VA	AVYWSG GSTYYA DSVRG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PSPGSSGYEYD Y	WGQGTQVTVSS
DLLBII 101G08 **197**	EVQLVESGGGLV QAGGSLRLSCAA SGRTFS	SYAMA	WFRQAPGKEREF VA	AIRWSG GTAYYA DSVQG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AN	RAADTRLGPYE YDY	WGQGTQVTVSS
DLLBII 101H02 **198**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRQAPGKGPEW VS	AINSGG GITYYA DFVKG	RFTISRDNAKNTLYL QMSSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 101H03 **199**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEG IS	CISSSG GITYYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY	TGQGTQVTVSS
DLLBII 101H05 **200**	EVQLVESGGGSA QAGGSLRLSCAA SGRTSS	TYAMG	WFRQAPGKEHEF VS	AIGRGT GATSYG DSVKG	RFTISRDNAKNTVYL QMNSLQLEDTGDYYC VA	GRGFYHDYSSY EY	RGQGTQVTVSS
DLLBII 101H09 **201**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLG	YYTIV	WFRQAPGKERKG VS	CISSRD GSRYYA DSVKG	RFTISRDNAKNTVYL RMNSLKPEDTAVYYC AA	GPDCSSYDY	WGQGTQVTVSS
DLLBII 102F08 **202**	EVQLVESGGGLV QTGDSLRLSCAA SGSTFS	NYRMG	WFRQGPGKEREF VA	AIGRNG QNTYYT DSVKG	RFTVTRDNAKNMVYL QMNSLKPEDSAVYTC AA	SLRGWDTTRID YEY	WGQGTQVTVSS
DLLBII 102G04 **203**	EVQLMESGGGLV QPGGSLRLSCAA SGFTFS	SYAMS	WVRQAPGKGLEW VS	RITSGG RTTYRD SVKG	RFTISRDNSKNTLYL QMNSLKPEDTALYYC AK	ARGDIDVYTLS DS	RGQGTQVTVSS
DLLBII 102H07 **204**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFS	SYAMS	WVRQAPGKGLEW VS	RITSGG RATYRD SVKG	RFTISRDNSKNTLYL QMNSLKPEDTALYYC AK	ARGDIDVYTLS DS	RGQGTQVTVSS

DLLBII 102H09 **205**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRRPPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 103A04 **206**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSD GSTYYA DSVKG	RFTVSSNNADDTVYL QMNSLKPEDTAVYYC AV	RLFSGGCAVVA GTSWADFGS	SGQGTQVTVSS
DLLBII 103B05 **207**	AVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSD GSTHYA DSVKG	RFTISSDKVKNTVYL QMNSLKPEDTAVYYC AV	RLFKGGCAVVA GTSWADFGS	TGQGTQVTVSS
DLLBII 104A03 **208**	EVQLVESGGGLV QAGGSLRLSCAA SGDIPR	IAAMG	WYRQAPGKQREL VA	TVSNAA TTRYAD SAKG	RFTISRDNAKTVSLQ MDNLKPEDTGVYYCY S	LATTVTPSWVN Y	WGQGTQVTVSS
DLLBII 104A05 **209**	EVQLVESGGGLV QPGGSLRLSCAA SGFAFG	YYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNMLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YDY	WGQGTQVTVSS
DLLBII 104B02 **210**	EVQLVESGGGLV QAGGSLRLSCDA SGRGFS	YYRMG	WFRQAPGKEREF VA	AIGKSG RNTYYG DYVKG	RFTVSRDNAKNTVYL QMTSLKPEDTAVYTC AA	SLRGWDTTWID YEY	WGQGTQVTVSS
DLLBII 104B05 **211**	EVQLVESGGGSV QAGGSLRLSCAA SGSISR	IDVMA	WYRQAPGKEREL VA	SISSGG STNYAD SVKG	RFTISREYFKNMMYL QMNSLKFEDTAVYYC NA	DSRRGGVGNFF RS	WGQGTQVTVSS
DLLBII 104B08 **212**	EVQLVESRGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKSTLYL QMNSLKPEDTAVYYC AI	PRGWGPTGPIE YAY	WGQGTQVTVSS
DLLBII 104C04 **213**	EVQLVESGGGLV QAGGSLRLSCAA AGSTFS	SYVMG	WYRQAPGKQREL VA	HISTRG ITYYAD SVKG	RFTISRDNAKNTMYL QMNSLKPEDTAVYYC NT	RRNFLSNY	WGQGTQVTVSS
DLLBII 104C12 **214**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEG IS	CISSSG GITYYA DSVKG	RFTISRDNAKSTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY	TGQGTQVTVSS

DLLBII 104G01 **215**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSD GSTYYA DSVKG	RFTISSDNAKNTVYL QMNSLKPEDTAVYYC AT	AWCDSSWYRSF VGY	WGQGTQVTVSS
DLLBII 105G01 **216**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSD DSTYYA DSVKG	RFTISSNNAKNTAYL QMNSLKPEDTAVYYC AV	RLFSGGCAVVA RTSWADFGS	SGQGTQVTVSS
DLLBII 106A01 **217**	EVQLVESGGGFV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEE IS	CISSSG GITYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY	WGQGTQVTVSS
DLLBII 106F01 **218**	EVQLVESGGGLV QSGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GITYYA DSVNG	RFTISRDNTKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 106H01 **219**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	NYAMG	WYRQAPGKQREL VV	GISSDG STHYAD SAKG	RFTISRDDAKNTVYL QMNSLKTEDTAVYYC YV	PVKVAGLEYAY	WGQGTQVTVSS
DLLBII 107C03 **220**	EVQLVESGGGLV QPGGSLRLSCEV SGSIGS	VSDMR	WYRQAPGLQYEL VA	RITSGS ITDYSD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC NA	DVQHSAWLKPL TY	WGQGTQVTVSS
DLLBII 112A03 **221**	EVQLVESGGGLV QPGGSLRLSCAA SGIRFS	INGMG	WYRQAPGKQREA VA	TITRGG IRDYTD SVKG	RFTISRDIARNTVYL QMNNLKPEDSAVYYC NI	DIY	WGRGTQVTVSS
DLLBII 112A04 **222**	EVQLVESGGGLV QAGGSLRLSCAA FGRTPY	GMG	WFRQAPGKEREF VA	AITSDG STNYAD SVKG	RFTISRDNAKNAVSL QMNSLKPEDTAVYYC TA	PYYSDFEGTTT EYDY	WGQGTQVTVSS
DLLBII 112E07 **223**	EVQLVESGGGLV QAGGSLRLSCAA SGRTVR	SYATG	WFRQAPGKEREF VA	ALRWSI GSIASV YYDDSV KG	RFTISGDNAENTVYL QMNALKPEDTAIYYC AS	TTRGRYSALSA SAYDY	WGQGTQVTVSS
DLLBII 115A05 **224**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRRSPGKGPEW VS	SINSGG GSTYYA DFVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AA	DRYIRARQGDY WGAYEYDY	WGQGTQVTVSS

DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSRG	RFTISRDNAKNTVYL	DPIHNCYSGRY	
16A03	QAGGSLRLSCAA	YYAIG	VS	GSTYYE	QMNSLKPEDTAVYYC	YASPDAVYDY	WGQGTQVTVSS
225	SGFTLD			DSVKG	AA		
DLLBII	KVQLVESGGGLV		WFRQAPGKEREG	CISSHD	RFTISSDNAKNTVYL	DPLVCGYNDPR	
16A07	QAGGSLRLSCAA	DYAIG	VS	GTTYYA	QMNSLKPEDTAVYYC	LADY	WGQGTQVTVSS
226	SGFTFD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSG	RFTISRDNAKNTVYL	PFNHYSDLCGV	
16A09	QPGGSLRLSCAA	YHNIG	VS	GSTAYA	QMNSLKPEDTAVYYC	NAIDY	WGQGTQVTVSS
227	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSD	RFTISRDNGKNTVYL	PFSYYSSLCGV	
16C11	QPGGSLRLSCAA	YYNIG	VS	GSTAYA	QMNSLKPEDTAVYYC	NEYDY	WGQGTQVTVSS
228	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSTN	RFSISRDNARNTVYL	PFSYYNNLCGV	
16D11	QPGGSLRLSCAA	YYNIG	VS	GNTYYA	QMNSLKPEDTAVYYC	NGVDY	WGQGTQVTVSS
229	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSSD	RFTISSNNAKNTVYL	RLFSGGCAVVA	
16E02	QAGGSLRLSCAA	DYAIG	VS	DSTYYA	QMNSLKPEDTAVYYC	GTSWADFGS	SGQGTQVTVSS
230	SGFTFD			DSVKG	AV		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CITSSN	RFTISRDNAKNTVYL	PFAHYSDLCGV	
16E08	QPGGSLRLSCAA	YYNIG	VS	GSTYYT	QMNSLKPEDTAVYYC	NGYDY	WGQGTQVTVSS
231	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSD	RFTISRDNAKNTVYL	PFAYYSDLCGV	
16H02	QPGGSLRLSCAA	YYNIG	VS	GSTGYA	QMNSLKPEDTAVYYC	NEYDY	WGQGTQVTVSS
232	SGFALD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WYRQAPGKQREL	AISSDD	RFTISRDYAKNTVYL	PHSDYDEEAPS	
16H09	QAGGSLRLSCAA	SYAMG	VA	STYYAD	QMNSLKPEDTAVYYC	DFGS	WGQGTQVTVSS
233	SGSTFT			CVKG	NA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSSD	RFTISRNNAKNTVYL	RLFSGGCAVVV	
17A12	QAGGSLRLSCAA	DYAIG	VS	DSTYYA	QMNSLKPEDTAVYYC	GTSWADFGS	SGQGTQVTVSS
234	SGFTFD			DSVKG	AV		

DLLBII 17B03 **235**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFE	NYALG	WFRQAPGKEREW VS	CISSSD GTTYYA DSVKG	RFTISRDNVKNTVYL QMNRLKPEDTAIYYC AL	SLGSSWCAYDY WGQGTQVTVSS
DLLBII 17B09 **236**	EVQLMESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSYD GTTYYA DSVKG	RFTISSDNAKNTVYL QMNSLKPEDTAVYYC AA	DPLVCGYNDPR LADY WGQGTQVTVSS
DLLBII 17C01 **237**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYPIG	WLRQAPGKEREG VS	CISSSD DSTYYA DSVKG	RFTISSNNAKNTVYL QMNSLKPEDTAVYYC AV	RLFSGGCAVVA GTSWADFGS SGQGTQVTVSS
DLLBII 17C08 **238**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	SYAMG	WYRQAPGKQREL VA	VISSGD RTNYLD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC FY	SGSYYYPTDVH EYAY WGQGTQVTVSS
DLLBII 17E04 **239**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CISSGD GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFEYYSAYCGV NRYDY WGQGTQVTVSS
DLLBII 17F10 **240**	EVQLVESGGGLV QAGGSLRLSCAS SGRTLL	NYAMG	WFRQAPGKEREF VS	GINWSG GSTYYA DSVKG	RFTISRDNAENTVYL HMNSLKPEDTAVYYC AA	AHDNYWFTDDS LGRGLKY WGQGTQVTVSS
DLLBII 17G10 **241**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	SYAMG	WYRHQAPGKQRE LV	AAISSD GSTHYA DSVKG	RFTISRDNAKNTMYL QMNSLKPEDTAVYYC NT	KTFGSNWYDDY WGQGTQVTVSS
DLLBII 18D02 **242**	EVQLVESGGGLV QAGGSLRLSCAA SGFTLD	YYAIG	WFRQAPGKEREG VS	CISSRG GSTYYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTGVYYC AA	DPIHNCYSGRY YASPEAVYDY WGQGTQVTVSS
DLLBII 18F05 **243**	EVQLVESGGGLV QAGGSLRLSCAA SAFTLD	YYAVG	WFRQAPGKEREG VS	CISSSG GSTYYE DSVKG	RFTISRDNAKNTVYL QMNNLKPEDTAVYYC AA	DPFHNCYSGSH YSSPEAVYEY WGQGTQVTVSS
DLLBII 18H08 **244**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKGREW VS	CINSSD GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFEYYSDLCGV NGYDY WGQGTQVTVSS

DLLBII 19B09 **245**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CISSSD GRTNYV DSVKG	RFTMSRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFNYYSDLCGV NGVDY	WGQGTQVTVSS
DLLBII 19D04 **246**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	SYAMG	WYRQAPGNQREL VA	VISSGG STNYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYCC FY	SGSYYYPTDVH EYAY	WGQGTQVTVSS
DLLBII 19D07 **247**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	NYNIG	WFRQAPGKEREW VS	CITSSN GSTYYT DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFAHYSDLCGV NGYDY	WGQGTQVTVSS
DLLBII 19D10 **248**	EVQLVESGGGLV QAGDSLRLSCAA SGRTVG	SYDMS	WVRQGPGKEREW VS	SINSGV GKTYYA DSVKG	RFTIFRDNAKNMVYL QMNNLKPEDTAVYYC AT	EMDGSRYV	EGQGTQVTVSS
DLLBII 19F04 **249**	EVQLVESGGGLV QAEGSLRLSCAA SGSTFS	TYAMA	WYRQAPGKQREL VA	GISFDG STHYAE SVKG	RFTISRDDAKNTVSL QMNSLKPEDAAVYYC YS	VHPSTGFGS	WGQGTQVTVSS
DLLBII 19F12 **250**	EVQLVESGGGLV QAGGSLRLSCTA SGSTFT	SYAMG	WYRQAPGKQREL VA	AISSDD STYYAD CVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC NA	PHSDYDEEAPS DFGS	WGQGTQVTVSS
DLLBII 24C07 **251**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSD DSTYYA DSVKG	RFTISSNNAKNTVYL TMNSLKPEDTAVYYC AV	RLFSGGCAVVA STSWADFGS	SGQGTQVTVSS
DLLBII 24D07 **252**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSD GSTYYA DSVKG	RFTISSNNAKNRAYL QMNSLKPEDTAVYYC AV	RLFRGGCAVVA GTSWADFGS	SGQGTQVTVSS
DLLBII 25G05 **253**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSYD GTTYYA DSVKG	RFTISSDNAKNTVYL QMNSLKPEDTAVYYC AA	DPLVCGYNDPR LADY	WGQGTQVTVSS
DLLBII 25H07 **254**	EVQLVESGGGLV QAGGSLRLSCAA SGFTLD	YYAIG	WFRQAPGKEREG VS	CISSRG GSTYYE DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYSGRY YASPDAVYEY	WGQGTQVTVSS

DLLBII 26C02 **255**	EVQLVESGGGLV QAGGSLRLSCAA SGFTLD	YYAIG	WFRQAPGKEREG VS	CISSRG SSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYSGNG YDSPEAVYDY	WGQGTQVTVSS
DLLBII 26H11 **256**	EVQLVESGGGLV QAGGSLRLSCTA SGFTLD	YYAIG	WFRQAPGKEREG VS	CISSSG GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPFHNCYSGSA YSSPEAVYEY	WGQGTQVTVSS
DLLBII 28B06 **257**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	TYAMG	WYRQDPGNQREL VA	AISSDG STHYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC YA	PVKVAGLEYDY	WGQGTQVTVSS
DLLBII 33A06 **258**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	NYAIG	WFRQAPGKEREW VS	CISGFD GSTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	SVGSSWCAYDY	WGQGTQVTVSS
DLLBII 33A10 **259**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFE	NYALG	WFRQAPGKEREW VS	CISSSD GTTYYA DSVRG	RFTISRDNAKNTVYL QMNRLKPEDTAIYYC AL	SLGSSWCAYDY	WGQGTQVTVSS
DLLBII 33C05 **260**	EVQLVESGGGLV QAGGSLRLSCAA SGFTLD	NYVIG	WFRQAPGKEREE VS	CISSSG GSTDYL DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DSLPCYYDKMV YDY	WGQGTQVTVSS
DLLBII 33C09 **261**	EVQLVESGGGLV QAGGSLRLSCTA SGFKLD	YYVIG	WFRQAPGKEREG VS	CTSSSG GSTYYA DSVKG	RFTISRDNAKNTVYL QMHSLKPEDTAVYYC AA	DSFACDYGKMI YDY	WGQGTQVTVSS
DLLBII 33C10 **262**	EVQLVESGGGLV QPGGSLRLSCAA SGFGFD	NYAMG	WFRQAPGKEREW VS	CISGSD GSTYYA DSVKG	RFTISRDNAKNTVYL QMHSLKPEDTAVYYC AA	SLGSSWCAYDY	WGQGTQVTVSS
DLLBII 33D02 **263**	EVQLVESGGGLV QAGGSLRLSCSA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSHD GTTYYA DSVKG	RFTISSDNAKNTVYL QMNSLKPEDTAVYYC AA	DPLVCGYNDPR LADY	WGQGTQVTVSS
DLLBII 33E01 **264**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	SYAMG	WYRQAPGKQREL VA	AISNGG STNYVD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC FY	SGSYYYPTDVH EYDY	WGQGTQVTVSS

DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSD	RFTMSRDNAKNTVYL	PFNYYSNLCGV	WGQGTQVTVSS
33E03	QPGGSLRLSCAA	YYNIG	VS	GRTNYV	QMNSLKPEDTAVYYC	NGVDY	
265	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISGRG	RFTISRDNAKNTVYL	DPIHNCYSGSH	WGQGTQVTVSS
33F01	QAGGSLRLSCAA	YYAIG	IS	GSTYYI	QMNSLKPEDTAVYYC	YYSPEAVYEY	
266	SGFSLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSRG	RFTTSRDNAKNTVYL	DPIHNCYSGSD	WGQGTQVTVSS
33F04	QAGDSLRLACAA	YYAVG	VS	GSTFYA	QMNSLKPEDTAVYYC	YASPEAVYEY	
267	SGFALD			DSLKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CIRSSD	RFTISRNNAKNTVYL	PFTHYSDLCGV	WGQGTQVTVSS
33H04	QPGDSLRLSCAA	YYNIG	VA	GSTYYT	QMNSLKPEDTAVYYC	NGNDY	
268	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WYRQAPGKQREL	VISSGS	RFTISRDNAKNTVSL	SGSYYYPTDVH	WGQGTQVTVSS
42A08	QAGGSLRLSCAA	SYAMG	VA	VTNYAD	QMNSLKPEDTAVYYC	EYDY	
269	SGSTFN			SVKG	FY		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CMGSSV	RFTISRDNAKNTVYL	APIFFECPSGEI	WGQGTQVTVSS
42B07	QPGGSLRLSCAA	YYAIG	VS	RSTYYA	QMNSLKPEDTAVYYC	YDY	
270	SGFRLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSRG	RFTISRDNAKNTVYL	DPIHNCYSGTY	WGQGTQVTVSS
42B10	QAGGSLRLSCAA	YYAIG	VS	GSTYYT	QMNSLKPEDTGVYYC	YASPEAVYEY	
271	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSRG	RFTTSRDNAKNTVYL	DPIHNCYSGSY	WGQGTQVTVSS
42F08	QAGGSLRLSCAA	YYAVG	VS	GSTYYV	EMNSLKPEDTAVYYC	YASPEAVYDY	
272	SGFALD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WYRQAPGKQREL	VISSGD	RFTISRDNAKNTVYL	SGSYYYPTDVH	WGQGTQVTVSS
42G04	QAGGSLRLSCAA	SYAMG	VA	STNYSD	QMNSLKPEDTAVYYC	EYAY	
273	SGSTFS			SVKG	FY		
DLLBII	EVQLVESGGGLV		WYRQAPGKQREL	VISSGD	RFTISRDNAKNTVYL	SGSYYYPTDVH	WGQGTQVTVSS
43A05	QAGGSLRLSCAA	SYAMG	VA	RTNYLD	QMNSLKPEDTAVYYC	EYAY	
274	SGSSFS			SVKG	FY		

DLLBII 43A10 **275**	EVQLVESGGGLV QPGGSLRLSCAA SGFALD	YYNIG	WFRQAPGKEREW VS	CISGSD GSTGYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFAYYSDLCGV NEYDY	WGQGTQVTVSS
DLLBII 43A12 **276**	EVQLVESGGGLV QPGGSLRLSCAA SGFALD	GHNIG	WFRQAPGKEREW VS	CINSGD GSTGYA DSVKG	RFTISRDNAKNTVYL QMNRLKPEDTAVYYC AA	PFNHYSFLCGV NEYDY	WGQGTQVTVSS
DLLBII 43B04 **277**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	SYAMG	WYRQAPGKQREL AA	VISTGD NTNYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYHC FY	SGSYYYPTEVY EYDY	WGQGTQVTVSS
DLLBII 43B11 **278**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFR	SYAMG	WYRQVPGNQREL VA	VISSGD SANYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC FY	SGSYYYPTDVH EYDY	WGQGTQVTVSS
DLLBII 43C12 **279**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYNIG	WFRQAPGKEREW VS	CINSSD GTTYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PFEYYSDLCGV NGYDY	WGQGTQVTVSS
DLLBII 47A05 **280**	EVQLVESGGGLV QAGGSLRLSCGA SGFSLD	YYAIG	WFRQAPGKEREG VS	CISGRG SNTYYL DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYGGSY YASPEAVYEY	WGQGTQVTVSS
DLLBII 47D01 **281**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEREG VS	CISSSG STYYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AI	AGASSWCFPPG Y	WGQGTQVTVSS
DLLBII 47E03 **282**	EVQLVESGGGLV QAGGSLRLSCAA SGFALD	YYAVG	WFRQAPGKEREG VS	CISSRG GSTYYA DSVKG	RFTTSRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYSGIY YASPEAVYDY	WGQGTQVTVSS
DLLBII 47E12 **283**	EVQLVESGGGLV QAGGSLRLSCAA SGSTFS	SYAMG	WYRQAPVKQREL VA	VISNGG STNYAD SVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC FY	SGSYYYPTDVH EYDY	WGQGTQVTVSS
DLLBII 47F06 **284**	EVQLVESGGGLV QPGGSLRLSCAA SGFTLD	YYAIG	WFRQAPGKEREG VS	CISSRG GSTYYE DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	DPIHNCYSGRY YASPDAVYDY	WGQGTQVTVSS

DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSD	RFTISRDKAKNTVYL	PFSYYSDLCGV	
47G11	QPGGSLRLSCAA		VS	GSTGYA	QMNSLKPEDTAVYYC	NGYDY	WGQGTQVTVSS
285	SEFTLD	HYNIG		DSVKG	AA		
DLLBII	EVQLVESGGGLV		WYRQAPGKQREL	VISSGD	RFTMSRDNAKNTVYL	SGSYYYPSDVH	
47H02	QAGGSLRLSCAA			STNYAD	QMNSLRPEDTAVYYC		
286	SGSTFS	SYAMG	VA	SVKG	FY	EYDY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSD	RFTISRDNAKNTVYL	PFSYYSGLCGV	
48A01	QPGGSLRLSCAA			GSTDYA	QMNSLKPEDTAVYYC		
287	SGFTLD	YYNIG	VS	DSVKG	AA	NGVDY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISGSD	RFTISRDNAKNTVYL		
48A08	QPGGSLRLSCAA			GSTWYA	QMNSPKSEDTAVYYC		
288	SGFTLG	VYATG	VS	DSVKG	AL	SLGSSWCAYDY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISGRG	RFTISRDNAKNTVYL		
48E03	QAGGSLRLSCAA			GSTYYT	QMNSLKPEDTGVYYC	DPVHNCYSGRY	
289	SGFTLD	YYAIG	VS	DSVKG	AA	YASPDAVYEY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WYRQAPGKQREL	VISNGG	RFTISRDNAKNTVYL		
48F05	QPGGSLRLSCAA			STNYAD	QMNSLKPEDTAVYIC	SGSYYYPTDVH	
290	SGSTFS	SYAMG	VA	SVKG	FY	EYAY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CISSSG	RFTISRDNAKNTVYL		
49A12	QPGGSLRLSCAA			GSTAYA	QMNSLKPEDTAVYYC	PFSHYNDLCGV	
291	SGFTLD	YHNIG	VS	DSVKG	AA	NAIDY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSRG	RFTISRDNAKNTVYL		
49B05	QAGGSLRLSCAA			ASTYYA	QMNSLKPEDTAVYYC	DPIHNCYSGNG	
292	SGFTLD	YYAIG	VS	DSVKG	AA	YDSPEAVYDY	WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CINSSD	RFTISRDNAKNTVYL		
49E01	QPGGSLRLSCAA			GSTHYA	QMNSLKPEDTAVYYC	PFEYYSDLCGV	
293	SGFTLH	YYNIG	VS	DSVKG	AA	NGYDY	WGQGTQVTVSS
DLLBII	KVQLVESGGGLV		WFRQAPGKEREW	CINSSD	RFTISRDNAKNTVYL		
49F05	QPGGSLRLSCAA			GSTYYA	QMNSLKPEDTAVYYC	PFEYYSNLCGV	
294	SGFTLD	YYNIG	VS	DSVKG	AA	NGYDY	WGQGTQVTVSS

DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSSG	RFTISRDNAKNTVYL	DPLHNCYSGRG
49G02	QAGGSLRLSCAA	KYSIG	VS	GSTYYV	QMNNLKPEDTAVYYC	YYSPEAVYEY
295	SGFTLD			DSVKG	AA		WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREG	CISSRG	RFTISRDNAKNTVYL	DPIHNCYSGSY
49G05	QAGGSLRLSCAA	YYAIG	VS	GSTYYT	QMNSLKPEDTGVYYC	YASPEAVYEY
296	SGFTLD			DSVKG	AA		WGQGTQVTVSS
DLLBII	EVQLVESGGGLV		WFRQAPGKEREW	CINSSD	RFTISRDNAKNTVYL	PFEYYSNLCGV
49H05	QPGGSLRLSCAA	YYNIG	VS	GSTYYA	QMNSLKPEDTAVYYC	NGYDY	WGQGTQVTVSS
297	SGFTLD			DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEPEG	CISSSG	RFTISRDNAKNTVYL	PGIAACRGIHY
55A07	QPGGSLRLSCTA	DYAIG	IS	SITYDA	QMNSLKPEDTAVYYC		WGQGTQVTVSS
298	SGFTFD			DSVKG	AT		
DLLBII	EVQLVESGGGLV		WFRQAPGKEPEG	CISSSG	RFTISRDNAKNTVYL	PGIAACRGIHY
55D12	QPGGSLRLSCAA	DYAIG	IS	GITYYA	QMNSLKPEDTAVYYC		TGQGTQVTVSS
299	SGFTFD			DSVKG	AT		
DLLBII	EVQLVESGGGLV		WFRQAPGKEPEG	CISSSG	RFTTSRDNAKNTVYL	PGIAACRGIHY
56A09	QPGGSLRLSCAA	DYAIG	IS	GITYYA	QMNSLKPEDTAVYYC		TGQGTQVTVSS
300	SGFTFD			DSVKG	AT		
DLLBII	EVQLVESGGGLV		WFRQAPGKEPEG	CISSSG	RFTTSRDSAKNTVYL	PGIAACRGIHY
56C04	QPGGSLRLSCTA	VYAIG	IS	SITYYA	QMNSLKPEDTAVYYC		WGQGTQVTVSS
301	SGFTFD			DSVKG	AT		
DLLBII	EVQLVESGGGLV		WFRQAPGKEPEE	CISSSG	RFTISRDNAKNTVYL	PGIAACRGIHY
56H08	QPGGSLRLSCAA	DYAIG	IS	GITYYA	QMNSLKPEDTAVYYC		TGQGTQVTVSS
302	SGFTFD			DSVKG	AT		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREA	TISWSG	RFTISRDNTKNTLYL	KPNLKYGSYWP
58A11	QAGGSLRLSCTT	RYSMG	VA	DSTYYA	QIDSLKPEDTAVYYC	PRGYDY	WGQGTQVTVSS
303	SERTVS			DSVKG	VA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREA	TISWSG	RFTISRDNTKNMLYL	KPNLKYGSTWP
58B01	QAGGSLRLSCTT	RYSMG	VA	DSTYYA	QMNSLKPEDTAVYYC	PRGYDY	WGQGTQVTVSS
304	SERTVS			DSVKG	VA		

DLLBII	EVQLVESGGGLV		WLRQAPGKEREA	TISWSG	RFTISRDNTKNTLYL	KPNLKYGSYWP	WGQGTQVTVSS
59B01	QAGGSLRLSCTT	RYTMG	VA	DSTYYA	QMNSLKPEDTADYYC	PRGYDY	
305	SERAVS		VA	DSVKG	AA		
DLLBII	EVQLVKSGGGLV		WFRQAPGKEREA	TISWSG	RFTISRDNTKNTLYL	KPNLKYGSDWP	WGQGTQVTVSS
59B11	QAGGSLRLSCTT	RYGMG	VA	DSTYYA	QMNSLKPEDTAVYYC	PRGYDY	
306	SERTVS		VA	DSVKG	AA		
DLLBII	EVQLVESGGGLV		WVRQAPGKGLEW	FINKDG	RFTISRDNAKNTMYL	RTSRSPRP	RGQGTQVTVSS
61F05	QPGGSLRLSCTA	SYAMS	VS	SDTGYA	QMNSLKPEDTAVYFC		
307	SGFTFS		VS	DSVKG	ET		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREF	AINWSG	RFTISRDNAKNTVYL	SNYYSVYDDRP	WGQGTQVTVSS
61F07	QAGGSLRLSCAA	RYAMG	VA	GSTYYA	QMNSLKPEDTAVYDC	VMDY	
308	SGRTFS		VA	DSVKG	AA		
DLLBII	EVQLMESGGGLV		WVRQAPGKGLEW	VISPDG	RFTISRGNAKNTLFL	GSGSWGV	HGQGTQVTVSS
62C11	QPGGSLRLSCVA	NYYMS	VS	SNTYYA	QMTGLKSEDAAVYYC		
309	AGFTFS		VS	DTVKG	AR		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREF	GISRYG	RFTISRDNVKNTVYL	NEGYCSGYGCY	WGQGTQVTVSS
78B03	QAGGSLRLSCAA	NYIMG	VA	DYTAYA	RMNSLKPDDTAVYYC	EDSGQYDY	
310	SGRTFS		VA	DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREF	GISRYG	RFTISRDNVKNTVYL	NEGYCSGYGCY	WGQGTQVTVSS
78B04	QAGGSLRLSCAA	NYIMG	VA	DYTYYA	RMNSLKPDDTAVYYC	EDSGQYDY	
311	SGRTFS		VA	DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREF	AVSRFG	RFTISRDNTANTLKL	GGRSFLPFVPA	WGQGTQVTVSS
80E08	QAGGSLTLSCAA	TYAMG	VA	VSWDYA	RMNSLKADDTAVYYC	Y	
312	SGGTFT		VA	DSVKG	AA		
DLLBII	EVQLVESGGGLV		WFRQAPGKEREF	GISRYA	RFTISRDNVKNTVYL	NEGYCSGYGCY	WGQGTQVTVSS
83G01	QAGGSLRLSCAA	NYIMG	VA	DYTGYA	RMNSLKPDDTAVYYC	EDSGQYDY	
313	SGRTFS		VA	DSVKG	AA		
DLLBII	EVQLVESGGGLV		WYRQAPGKEREF	GISRYG	RFTISRDSVKNTVYL	NGGYCSGYGCY	WGQGTQVTVSS
83G04	KPGGSLRLSCAA	IMG	VA	DITYAA	RMNSLKPDDTAVYYC	EDSGQYDY	
314	SGRTLY		VA	DSVKG	AA		

DLLBII 87B06 **315**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEG IS	CISSSG GITYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY TGQGTQVTVSS
DLLBII 89B04 **316**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFD	DYAIG	WFRQAPGKEPEE IS	CISSSG GITYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY TGQGTQVTVSS
DLLBII 90E10 **317**	EVQLVESGGGLV QAGGSLRLSCAV SGFSFD	DYAIG	WFRQAPGKEPEG IS	CISSSG GITYYA DSVKG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY TGQGTQVTVSS
DLLBII 95A01 **318**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AI	PRGWGPTGPIE YAY WGQGTQVTVSS
DLLBII 95B03 **319**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AI	PRGWGPTGPIE YGY WGQGTQVTVSS
DLLBII 95C03 **320**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTGVYSC AI	PRGWGPTGPHE YGY WGQGTQVTVSS
DLLBII 95D02 **321**	EVQLVESGGGLV QSGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AI	PRGWGPTGPHE YAY WGQGTQVTVSS
DLLBII 95F02 **322**	EVQLVESGGGSV QAGGSLRLSCAA SGRTFS	SYAMG	WFRQAPGKEREF VA	AINWSG GYTYYA DSVRG	RFTISRDNAKNTVYL QMNSLKPEDTAVYYC AA	PAPGSSGYEYD Y WGQGTQVTVSS
DLLBII 95F03 **323**	EVQLVESGGGLV QPGGSLRLSCTA SGFTFD	VYAIG	WFRQAPGKEPEG IS	CISSSG SITYYA DSVKG	RFTISRDSAKNTVYL QMNSLKPEDTAVYYC AT	PGIAACRGIHY WGQGTQVTVSS
DLLBII 95H02 **324**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRHAPGKGPEW VS	AINSGG GSTYYT DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AI	PRGWGPTGPHE YAY WGQGTQVTVSS

DLLBII 96C02 **325**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GTTYYA DSVKG	RFTISRDNAKNTLFL QMNSLKPEDTAVYYC AI	PRGWGPTGPLE YGY	WGQGTQVTVSS
DLLBII 96C03 **326**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRQAPGKGPEW VS	AINSGG GDTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 96F02 **327**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRQAPGKGPEW VS	AINSGG GITYYA DSVKG	RFAISRDNAKTTLYL QMNNLQPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 96H02 **328**	EVQLVESGGGLV QAGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GITYYA DLVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AI	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 97B02 **329**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	NYDMS	WVRQAPGKGPEW VS	AINSGG GITYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 97D01 **330**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLTPEDTAVYYC AI	PRGWGPTGPHE YAY	WGQGTQVTVSS
DLLBII 97E01 **331**	EVQLVESGGGLV QPGGSLRLSCTA SGFTFG	NYDMS	WVRRPPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNSLKPEDTAVYYC AT	PRGWGPTGPHE YGY	WGQGTQVTVSS
DLLBII 97E02 **332**	EVQLVESGGGLV QPGGSLRLSCAA SGFTFG	SYDMS	WVRQAPGKGPEW VS	AINSGG GSTYYA DSVKG	RFTISRDNAKNTLYL QMNNLKPEDTAVYSC AI	PRGWGPTGPHE YAY	WGQGTQVTVSS

Example 5

Characterization of purified VHHs

Inhibitory anti-Dll4 VHHs selected from the screening described in Example 4 are further purified and characterized. Selected VHHs are expressed in *E. coli* TG1 as c-myc, His6-tagged proteins. Expression is induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37˚C. After spinning the cell cultures, periplasmic extracts are prepared by freeze-thawing the pellets. These extracts are used as starting material and VHHs are purified via IMAC and size exclusion chromatography (SEC) resulting in 95% purity as assessed via SDS-PAGE.

5.1. Evaluation of Dll4 blocking VHHs in ELISA

The blocking capacity of the VHHs is evaluated in a human Dll4 – human Notch1/Fc blocking ELISA. In brief, 1 µg/mL of human Notch1/Fc chimera (R&D Systems, Minneapolis, MN, USA) is coated in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). A fixed concentration of 15 nM biotinylated human Dll4 is preincubated with a dilution series of the VHH for 1 hour, after which the mixture is incubated on the coated Notch1 receptor for an additional hour. Residual binding of biotinylated human Dll4 is detected using horseradish peroxidase (HRP) conjugated extravidin (Sigma, St. Louis, MO, USA) (Figure 3). Human Dll4 is biotinylated as described above. The IC_{50} values for VHHs blocking the human Dll4 – human Notch1/Fc interaction are depicted in Table 6.

Table 6: IC_{50} (nM) values for VHHs in hDLL4/hNotch1-Fc competition ELISA

VHH ID	IC_{50} (nM)
6B11	1.5
55D12	12.3
56A09	4.9
56C04	33.9
56H08	6.9
57C11	17.3
62C11	72.0
96C03	38.4
101G08	9.5
104G01	1.1
115A05	9.1
antiDLL4 Fab	0.7

5.2. Evaluation of Dll4 blocking VHHs in AlphaScreen

In brief, 1 nM biotinylated human Dll4 is captured on streptavidin-coated donor beads (20 µg/mL), while 0.4 nM of the receptor human Notch1 (as a Fc fusion protein) is captured on anti-human Fc VHH-coated acceptor beads (20 µg/mL). Both loaded beads are incubated together with a dilution range of the competing VHH (Figure 4). The IC_{50} values for VHHs blocking the human Dll4 – human Notch1/Fc interaction are depicted in Table 7.

Table 7: IC_{50} (nM) values for VHHs in hDLL4/hNotch1 competition AlphaScreen

VHH ID	IC_{50} (nM)
5B11	0.7
6B11	0.3
7A02	0.4
7B05	1.1
8A09	0.4
8C11	0.7 [(a)]
19F04	0.05 [(a)]
55D12	2.3
56A09	1.2
56C04	5.4
56H08	1.6
57C11	2.2
62C11	24.1
115A05	5.0
antiDLL4 Fab	0.3

[(a)] partial inhibitor

5.3. Inhibition by anti-Dll4 VHHs of human Notch1/Fc binding to human or mouse Dll4 expressed on the CHO cells

The blocking capacity of the VHHs is evaluated in a human and mouse Dll4 – human Notch1/Fc competitive FMAT assay (Figure 5) as outlined in Example 4. The IC_{50} values for VHHs blocking the interaction of human Notch1/Fc to human or mouse Dll4 expressed on CHO cells are depicted in Table 8.

Table 8: (Mean) IC_{50} values (nM) of purified VHHs blocking the interaction of human Notch1/Fc to human or mouse DLL4 expressed on CHO cells (FMAT)

	hDLL4	**mDLL4**
VHH ID	**IC_{50} (nM)**	**IC_{50} (nM)**
6B11	8.9	-
8A09	5.5	-
19F04	33.0	-
55D12	39.1	41.0
56A09	10.6	15.0
56C04	28.7	49.6
56H08	22.0	33.7
57C11	53.9	49.5
62C11	172.2	106.3
96C03	160.8	28.8
101G08	24.6	92.1
104G01	2.5	-
115A05	22.0	43.0
antiDLL4 Fab	5.4	2.3

5.4. Evaluation of Dll4 blocking VHHs in reporter assay

To evaluate the potency of the selected VHHs, a reporter assay is set up which is based on the γ-secretase mediated cleavage of Notch1 and release of the intracellular domain of Notch1 (NICD) upon stimulation with Dll4. The Notch1-GAL4/VP16 construct is cotransfected with the pGL4.31[Luc2P/Gal4UAS/Hygro] reporter plasmid in HEK cells resulting in a transient expression of the fusion protein. These transiently transfected cells are stimulated for 24 hours by co-culture with a HEK293-hDll4 stable cell line. Forty-eight hours post-transfection, the readout is performed. The VHHs are preincubated with the HEK293-hDll4 cells 1 hour before the start of the co-culture and are included during the co-culture (Figure 6). The IC_{50} values of the VHHs for blocking the Dll4-mediated cleavage of

Notch1 and subsequent translocation of its NICD to the nucleus of the receptor cell are depicted in Table 9.

Table 9: (Mean) IC_{50} values (nM) of purified VHHs in a DLL4/Notch1 reporter assay

VHH ID	IC_{50}
56A09	540
62C11	4663
96C03	5156
101G08	2760
104G01	964
115A05	1740
anti-DLL4 Fab	133

5.5. Epitope binning

In order to determine whether VHHs can bind simultaneously to Dll4 when e.g. a benchmark antibody is bound, epitope binning experiments are carried out (via Surface Plasmon Resonance (SPR) on a Biacore T100 instrument). Anti-Dll4 Fab fragment is irreversibly immobilized on the reference and on the active flow cell of a CM5 sensor chip. For each sample (cycle), human Dll4 is injected on the active and reference flow cell and reversibly captured by anti-Dll4 Fab. Additional binding of VHHs is evaluated by injection over the immobilized surface. All VHHs and anti-Dll4 Fab are injected at 100 nM with a surface contact time of 120 seconds and a flow rate of 10 uL/minute. Surface is regenerated using 10 mM glycine (pH1.5). Processed curves are evaluated with Biacore T100 Evaluation software. Table 10-A represents the sequential injection/regeneration path of analysed VHHs and controls. VHHs DLLBII56A09 (SEQ ID NO: 300), DLLBII96C03 (SEQ ID NO: 326), DLLBII101G08 (SEQ ID NO: 197) and DLLBII115A05 (SEQ ID NO: 224) are

shown not to additionally bind to human Dll4 captured by Dll4 Fab. Injection of Dll4 Fab also failed to additionally bind human Dll4 indicating that all epitopes are saturated. Therefore, it can be concluded that these VHHs recognize an epitope overlapping with Dll4 Fab for binding human Dll4. Human-only VHHs DLLBII6B11 (SEQ ID NO: 174) and DLLBII104G01 (SEQ ID NO: 215) show additional binding on Dll4 Fab captured human Dll4, indicating that these VHHs that are specific for human Dll4 recognize a different epitope than the human/mouse cross-reactive VHHs.

Table 10-A: Epitope binning of anti-DLL4 VHHs – simultaneous binding with DLL4 Fab

Injection step	**Binding/** *Regeneration*	**[sample]**	**Binding level (RU)**
1	hDLL4	100 nM	1727
2	DLL4 Fab	100 nM	no binding
3	59A9	100 nM	no binding
4	6B11	100 nM	405
5	*Glycine pH1.5*	10 mM	90
6	hDLL4	100 nM	1349
7	104G1	100 nM	276
8	*Glycine pH1.5*	10 mM	87
9	hDLL4	100 nM	1336
10	*Glycine pH1.5*	10 mM	70
11	hDLL4	100 nM	1333
12	96C3	100 nM	no binding
13	101G8	100 nM	no binding
14	115A05	100 nM	no binding
15	*Glycine pH1.5*	10 mM	70

5.6. Epitope mapping using Dll4 deletion mutants

Binding of the VHHs to these Dll4 mutants is assessed in Biacore. In brief, VHHs DLLBII101G08 (SEQ ID NO:197) and DLLBII115A5 (SEQ ID NO: 224) are coated on a CM4 Sensorchip and 200 nM of each deletion mutant is injected across the chip. Binding is qualitatively assessed. No binding of DLLBII56A09 (SEQ ID NO: 300), DLLBII101G08 (SEQ ID NO: 197) and DLLBII115A05 (SEQ ID NO: 224) is observed to human and mouse Dll4 mutants hDll4.1 and mDll4.8, respectively, lacking EGF-like 2 domain (Table 10-B). Indirect evidence using a hDll4/Dll4 IgG competitive ELISA already pointed to this observation. In brief, 1 µg/mL of Dll4 IgG is coated in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). A fixed concentration of 6 nM biotinylated human Dll4 is preincubated with a dilution series of the VHH for 1 hour, after which the mixture is incubated on the coated IgG for an additional hour. Residual binding of biotinylated human Dll4 is detected using horseradish peroxidase conjugated extravidin (Sigma, St. Louis, MO, USA) (data not shown). Human Dll4 is biotinylated as described above. It is known from patent literature that the monoclonal anti-Dll4 IgG (Genentech, US 2008/0014196A1) binds to an epitope within the EGF-like 2 domain of Dll4.

Table 10-B: Epitope mapping of anti-DLL4 VHHs – binding to DLL4 deletion mutants

	DLLBII56A9		**DLLBII101G8**		**DLLBII115A5**	
sample	**Binding (RU)**	**kd (1/s)**	**Binding (RU)**	**kd (1/s)**	**Binding (RU)**	**kd (1/s)**
hDLL4	281	9.5E-04	373	2.0E-03	324	3.5E-03
mDLL4	389	1.9E-03	502	6.0E-03	344	6.5E-03
hDLL4.1	no binding		no binding		no binding	
hDLL4.3	125	7.4E-04	198	4.65E-03	137	3.5E-03
hDLL4.5	143	1.2E-03	266	2.19E-03	162	4.2E-03
hDLL4.6	136	1.1E-03	229	2.20E-03	152	4.1E-03
mDLL4.8	no binding		no binding		no binding	
mDLL4.10	141	1.1E-03	189	5.14E-03	121	3.8E-03
mDLL4.11	132	1.6E-03	210	6.16E-03	121	6.6E-03
mDLL4.12	161	1.3E-03	244	4.52E-03	152	3.1E-03

5.7. Determining the affinity of the hDll4 – VHH interaction

Kinetic analysis to determine the affinity of the Dll4 – VHH interaction is performed by Surface Plasmon Resonance (SPR) on a Biacore T100 instrument. Recombinant human Dll4 is immobilized onto a CM5 chip via amine coupling using EDC and NHS) or biotinylated human Dll4 is captured on a SA chip (streptavidin surface). Purified VHHs or Fab fragment are injected for 2 minutes at different concentrations (between 10 and 300 nM) and allowed to dissociate for 20 min at a flow rate of 45 µl/min. Between sample injections, the surfaces are regenerated with 10 mM glycine pH1.5 and 100 mM HCl. HBS-N (Hepes buffer pH7.4) is used as running buffer. If possible, data are evaluated by fitting a 1:1 interaction model (Langmuir binding) onto the binding curves. The affinity constant K_D is calculated from resulting association and dissociation rate constants (k_a) and (k_d). The affinities of the anti-Dll4 VHHs are depicted in Table 11.

Table 11: Affinity K_D (nM) of purified VHHs for recombinant human DLL4

	rhDLL4		
VHH ID	**k_a ($M^{-1}.s^{-1}$)**	**k_d (s^{-1})**	**K_D (nM)**
56A09	1.7E+05	9.3E-04	5.6
56C04	1.1E+05	4.9E-03	45
56H08	1.2E+05	1.1E-03	9.4
62C11	1.2E+06	1.3E-01	120
96C03	1.6E+05	4.8E-02	310
101G08	4.3E+04	2.2E-03	52
104G01 [(a)]	1.2E+05 - 1.5E+05	3E-03 - 6E-04	4-24
115A05	1.5E+05	3.9E-03	25
antiDLL4 Fab	2.3E+05	3.4E-04	1.5

[(a)] heterogeneous binding curve resulting in no 1:1 fit

5.8. Binding to orthologues (mDll4, cDll4) and family members (hJagged-1,hDLL1)

In order to determine cross-reactivity to mouse Dll4 a binding ELISA is performed. In brief, recombinant mouse Dll4 (R&D Systems, Minneapolis, MS, USA) is coated overnight at 4˚C at 1 µg/mL in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1% in PBS). VHHs are applied as dilution series and binding is detected using a mouse anti-myc (Roche) and an anti-mouse-AP conjugate (Sigma, St Louis, MO, USA) (Figure 7). As reference, binding to human Dll4 is measured. EC_{50} values are summarized in Table 12.

Table 12: EC_{50} (nM) values for VHHs in a recombinant human DLL4 and mouse DLL4 binding ELISA

	rhDLL4	**rmDLL4**
VHH ID	**EC_{50} (nM)**	**EC_{50} (nM)**
5B11	1.8	-
6B11	1.4	-
7A02	1.4	-
7B05	7.2	-
8A09	0.9	-
8C11	1.1	-
17F10	0.9	-
19F04	0.9	0.8
55D12	13.1	30.0
56A09	3.6	6.3
56C04	44.3	244.0
56H08	4.1	8.7
57C11	7.9	83.4
62C11	137.0	13.1
96C03	86.5	8.7
101G08	8.9	53.9
104G01	8.4	-
115A05	5.0	33.4
antiDLL4 Fab	3.0	3.0

In order to determine the cynomologus cross-reactivity of the VHHs, a FACS binding experiment is performed. Cynomolgus Dll4 expressing HEK293 cells (transient or stable transfection) are used for a titration binding experiment of the VHHs. After a 30 minutes incubation on ice, all samples are washed and detection is performed by applying anti-c-myc~Alexa647 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Human and mouse Dll4 overexpressing HEK293 cells are taken

as reference. The mean MCF value is determined on the FACS Array and used for calculation of the EC_{50} value (see Figure 9).

Absence of binding to homologous ligands human DLL1 and human Jagged-1 is assessed via solid phase binding assay (ELISA). In brief, human DLL1 (Alexis, San Diego, CA, USA) and human Jagged-1 (Alexis, San Diego, CA, USA) are coated overnight at 4˚C at 1 µg/mL in a 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). Wells are blocked with a casein solution (1% in PBS). VHHs are applied as dilution series and binding is detected using a mouse anti-myc (Roche) and an anti-mouse-AP conjugate (Sigma, St. Louis, MO, USA). All anti-Dll4 VHHs are considered as being non-cross reactive to these homologous ligands (Figure 8).

5.9. Evaluation of VHHs in blocking Dll4- mediated HUVEC proliferation

The potency of the selected VHHs is evaluated in a proliferation assay, as described by Ridgway *et al.*, Nature. 2006 Dec 21;444(7122):1083-7), in modified form. In brief, 96-well tissue culture plates are coated with purified Dll4-His (RnD Systems; C-terminal His-tagged human Dll4, amino acid 27-524, 0.75ml/well, 10 ng/ml) in coating buffer (PBS, 0.1% BSA). Wells are washed in PBS before 4000 HUVE cells/well are seeded in quadruplicate. Cell proliferation is measured by [^{3}H]-Thymidine incorporation on day 4. The results, shown in Figure 15, demonstrate that the DLL4 VHHs DLLBII101G08, DLLBII104G01, DLLBII115A05, DLLBII56A09 and the DLL4 Fab inhibit the DLL4-dependent effect on HUVEC proliferation in a dose-dependent manner, the IC_{50} values are summarized in Table 13. The tested VHHs achieve a complete inhibition of the DLL4-dependent effect at 10µM.

Table 13 IC_{50} values obtained in the DLL4 proliferation assay

VHH/ Fab	Fab	56A9	104G1	101G8	115A5
IC_{50} (nM) (experiment 1)	4.9	11.0	103	401	10002
IC_{50} (nM) (experiment 2)	5.6	6.8	32	112	N.D.
n	2	2	2	2	1

Example 6

Affinity maturation of selected VHHs

VHHs DLLBII101G08 and DLLBII115A05 are subjected to two cycles of affinity maturation.

In a first cycle, amino acid substitutions are introduced randomly in both framework (FW) and complementary determining regions (CDR) using the error-prone PCR method. Mutagenesis is performed in a two-round PCR-based approach (Genemorph II Random Mutagenesis kit obtained from Stratagene, La Jolla, CA, USA) using 1 ng of the DLLBII101G08 or DLLBII115A05 cDNA template, followed by a second error-prone PCR using 0.1 ng of product of round 1. After a polish step, PCR products are inserted via unique restriction sites into a vector designed to facilitate phage display of the VHH library. Consecutive rounds of in-solution selections are performed using decreasing concentrations of biotinylated recombinant human DLL4 (biot-rhDLL4) and trypsin elutions. Affinity-driven selections in a third round using cold rhDLL4 (at least 100x excess over biot-rhDLL4) are also performed. No selections on murine DLL4 are included as (conservation of) cross-reactivity is assessed at the screening level. Individual mutants are produced as recombinant protein using an expression vector derived from pUC119, which contains the LacZ promoter, a resistance gene for ampicillin, a multiple cloning site and an ompA leader sequence (pAX50). *E. coli* TG1 cells

are transformed with the expression vector library and plated on agar plates (LB + Amp + 2% glucose). Single colonies are picked from the agar plates and grown in 1 mL 96-deep-well plates. VHH expression is induced by adding IPTG (1mM). Periplasmic extracts (in a volume of ~ 80 uL) are prepared according to standard methods and screened for binding to recombinant human and mouse Dll4 in a ProteOn (BioRad, Hercules, CA, USA) off-rate assay. In brief, a GLC ProteOn Sensor chip is coated with recombinant human Dll4 on the "ligand channels" L2 and L4 (with L1/L3 as reference channel), while "ligand channels" L3 and L6 is coated with mouse Dll4. Periplasmic extract of affinity matured clones is diluted 1/10 and injected across the "analyte channels" A1-A6. An average off-rate is calculated of the wild type clones present in the plate and served as a reference to calculate off-rate improvements.

In a second cycle, a combinatorial library is created by simultaneously randomising the susceptible positions identified in cycle one. For this, the full length DLLBII101G8 or DLLBII115A05 cDNA is synthesized by overlap PCR using oligonucleotides degenerated (NNS) at the randomisation positions and a rescue PCR is performed. A list of the primers used for generating the combinatorial library can be found in Table 14 and SEQ ID NOs: 427 to 457. The randomised VHH genes are inserted into a phage display vector (pAX50) using specific restriction sites as described above (Example 2). Preparation of periplasmic extracts of individual VHH clones is performed as described before.

Table 14: Oligonucleotides affinity maturation libraries

101G08 combinatorial library oligonucleotides	**115A5 combinatorial library oligonucleotides**
>101G08CL_fwd1-bis	>115A05CL_fwd_1
gaggtgcaattggtggagtctgggG	gaggtgcaattggtggagtctgggGGTGG
GTGGTCTGGTTCAGGCTGGT	TCTGGTTCAGCCAGGT
>101G08CL_fwd_2	>115A5CL_rev1-bis

TCCTGCGCAGCTTCTGGTCGTACCT	TGAGGAGACGGTGACCTGGGTCCCCTGAC
TCTCCAGCTACGCGATGGCT	CCC
>101G08CL_fwd_3	>115A05CL_fwd_2
CCAGGCAAAGAACGCGAGTWCGTAG	GTGCAGCTTCCGGCTTTACGWTCGGCTCC
CCGCAATCCGTTGGAGCGGT	TACGACATGTCTTGGG
>101G08CL_fwd_4	>115A05CL1_rev_2
CTGATTCCGTTCAGGGTCGTTTCAC	ACGCACCCCAGTATTCACCCTGACGCGCC
CATCTCTCGTGACAACGCG	CAAATGTAGCGATCTGCAGC
>101G08CL_fwd_5	>115A05CL_fwd_3
CTGCAGATGAACTCTCTGAAACCGG	AGGTCCGGAATGGGTGTCCKCTATCAACT
AAGATACGGCAGTCTACTAC	CTGGTGGTGGTAGCAC
>101G08CL_fwd_6-4	>115A05CL_rev_3
GACACTCGTCTGcgtCCGTACctgT	TCTTCCGGTTTCAGGCTGTTCATCTGCAG
ACGACYATTGGGGTCAGGGTA	GTACAGCGTGTTTTTG
>101G08CL_fwd_6-3	>115A05CL_fwd_4
GACACTCGTCTGGvACCGTACctgT	AAAGGTCGTTTCACCATCTCTCGTGACAA
ACGACYATTGGGGTCAGGGTA	CGCCAAAAACACGCTG
>101G08CL_fwd_6-2	>115A05CL_rev_4
GACACTCGTCTGcgtCCGTACGAGT	TGAAACGACCTTTTWCGWAGTCGGYGTAG
ACGACYATTGGGGTCAGGGTA	WAGGTGCTACCACCAC
>101G08CL_fwd_6-1	>115A05CL_fwd_5
GACACTCGTCTGGVACCGTACGAGT	TGAAACCGGAAGATACCGCGGTATACTAC
ACGACYATTGGGGTCAGGGTA	TGCGCTGCAGATCGCT
>101G08CL_rev_2-2	>115A05CL_rev_5
CAGACGAGTGTCcggCGCACGGTTT	CCATTCCGGACCTTTACCCGGAGAACGAC
GCACAGTAGTAGACTGCCGT	GAACCCAAGACATGTC
>101G08CL_rev_2-1	>115A05CL_fwd_6-1
CAGACGAGTGTCTRCCGCACGGTTT	TACTGGGGTGCGTACGHATACGACTACTG
GCACAGTAGTAGACTGCCGT	GGGTCAGGGTAC
>101G08CL_rev_3	>115A05CL_fwd_6-2
AGAGTTCATCTGCAGATAGACGGTG	TACTGGGGTGCGTACcagTACGACTACTG

TTTTTCGCGTTGTCACGAGA	GGGTCAGGGTAC
>101G08CL_rev_4	>115A05CL_rev_6
CTGAACGGAATCAGSGTAATACGCA	CCGGAAGCTGCACAGCTCAGACGCAGAGA
GTTYCACCGCTCCAACGGAT	ACCACCTGGCTGAACC
>101G08CL_rev_5	>115A05CL2_rev_2-2
GCGTTCTTTGCCTGGAGCCTGACGA	ACGCACCCCAGTAGTAACCCTGACGCGCC
WACCAAGCCATCGCGTAGCT	CRAATGTAGCGATCTGCAGC
>101G08CL_rev_6	>115A05CL2_rev_2-1
AGAAGCTGCGCAGGACAGACGGAGA	ACGCACCCCAGTAKTCACCCTGACGCGCC
GAGCCACCAGCCTGAACCAG	CRAATGTAGCGATCTGCAGC
>101G08CL_rev1-bis	
TGAGGAGACGGTGACCTGGGTCCCC	
TGACCCCAAT	

Screening for binding to recombinant human Dll4 in a ProteOn off-rate assay identifies clones with up to 38-fold (DLLBII101G08) and 11-fold (DLLBII115A05) improved off-rates (Table 15).

Table 15: Off-rate screening of DLLBII101G08 and DLLBII115A05 affinity-matured clones.

	hDLL4 k_d (s^{-1})	fold	mDLL4 k_d (s^{-1})	fold
DLLBII101G08	*2.2E-03*	*1*	*6.7E-03*	*1*
DLLBII129D08	5.9E-05	38	1.9E-04	35
DLLBII129H04	6.8E-05	33	2.5E-04	27
DLLBII129G10	7.3E-05	31	2.6E-04	26
DLLBII129H07	7.4E-05	30	2.5E-04	27
DLLBII129B02	7.6E-05	30	2.6E-04	26
DLLBII129E11	8.0E-05	28	2.5E-04	26
DLLBII130F06	6.5E-05	27	2.6E-04	19
DLLBII130B03	6.7E-05	27	2.4E-04	20
DLLBII129D01	8.5E-05	26	2.6E-04	26
DLLBII130D06	6.9E-05	26	3.1E-04	16
DLLBII129G09	8.8E-05	26	3.4E-04	20
DLLBII129B05	9.3E-05	24	3.4E-04	20
DLLBII130E03	7.5E-05	24	2.7E-04	18
DLLBII129H05	9.4E-05	24	3.5E-04	19
DLLBII130A05	7.5E-05	24	3.0E-04	17
DLLBII130B02	7.8E-05	23	2.9E-04	17
DLLBII129H02	9.9E-05	23	3.4E-04	19
DLLBII130B04	8.3E-05	22	2.9E-04	17
DLLBII129E07	1.1E-04	21	2.8E-04	24
DLLBII129E03	1.1E-04	20	3.6E-04	18
DLLBII129A03	1.2E-04	19	3.8E-04	18

The best top DLLBII101G08 variants and DLLBII115A05 variants are cloned into expression vector pAX100 in frame with a C-terminal c-myc tag and a (His)6 tag. Off-rates on recombinant mouse Dll4 are also improved. VHHs are produced in

E. coli as His6-tagged proteins and purified by IMAC and SEC. Sequences are represented in Tables 16-A (LLBII101G08) and 16-B (DLLBII11A05), respectively.

Table 16-A

VHH ID **SEQ ID NO**	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
DLLBII129 A03 **354**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WFRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLRPEDTAVY YCAN	RAPDTRLRPYL YDY	WGQGTQVTVSS
DLLBII129 B02 **355**	EVQLVESGGGLVQAGG SLSLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYE YDH	WGQGTQVTVSS
DLLBII129 B05 **356**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLAPYE YDH	WGQGTQVTVSS
DLLBII129 D01 **357**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WFRQAPGKEREY VA	AIRWSGGTAYY ADSVQS	RFTITRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYL YDH	WGQGTQVTVSS
DLLBII129 D08 **358**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYL YDY	WGQGTQVTVSS
DLLBII129 E03 **359**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WFRQAPGKDREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLAPYL YDY	WGQGTQVTVSS
DLLBII129 E07 **360**	EVQLVESGGGLVQAGG SLRLSCSASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY PDSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLAPYE YDH	WGQGTQVTVSS
DLLBII129 E11 **361**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLRPYL YDY	WGQGTQVTVSS
DLLBII129 G09 **362**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGETAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYL YDH	WGQGTQVTVSS
DLLBII129 G10 **363**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQS	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYE YDH	WGQGTQVTVSS

DLLBII129 H02 **364**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLRPYE YDY	WGQGTQVTVSS
DLLBII129 H04 **365**	EVQLVESRGGLVQAGG SLRLSCAASGRTFS	SYAMA	WFRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYL YDH	WGQGTQVTVSS
DLLBII129 H05 **366**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRLAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLGPYL YDY	WGQGTQVTVSS
DLLBII129 H07 **367**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYE YDY	WGQGTQVTVSS
DLLBII130 A05 **368**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLGPYL YDH	WGQGTQVTVSS
DLLBII130 B02 **369**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WFRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMYSLKPEDTAVY YCAN	RAPDTRLAPYL YDH	WGQGTQVTVSS
DLLBII130 B03 **370**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLAPYL YDY	WGQGTQVTVSS
DLLBII130 B04 **371**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WFRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLRPYL YDH	WGQGTQVTVSS
DLLBII130 D06 **372**	EVQLVESGGGLVQAGG SLRLSCSASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGETAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYL YDH	WGQGTQVTVSS
DLLBII130 E03 **373**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLEPYE YDH	WGQGTQVTVSS
DLLBII130 F06 **374**	EVQLVESGGGLVQAGG SLRLSCAASGRTFS	SYAMA	WYRQAPGKEREY VA	AIRWSGGTAYY ADSVQG	RFTISRDNAKNTVY LQMNSLKPEDTAVY YCAN	RAPDTRLAPYE YDY	WGQGTQVTVSS

Table 16-B

VHH ID **SEQ ID NO**	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
DLLBII133A05 **396**	EVQLVESGGGLVQPGGSLRLSCAASGFTFG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTFYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYQYDY	WGQGTQVTVSS
DLLBII133A09 **397**	EVQLVESGGGLVQPGGSLRLSCAASGFTFG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYADYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYAYDY	WGQGTQVTVSS
DLLBII133A12 **398**	EVQLVESGGGLVQPGGSLRLSCAASGFTFG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYVYDY	WGQGTQVTVSS
DLLBII133D06 **399**	EVQLVESGGGLVQPGGSLRLSCAASGFTIG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYADYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYQYDY	WGQGTQVTVSS
DLLBII133F01 **400**	EVQLVESGGGLVQPGGSLRLSCAASGFTIG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYVYDY	WGQGTQVTVSS
DLLBII133F06 **401**	EVQLVESGGGLVQPGGSLRLSCAASGFTFG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYQYDY	WGQGTQVTVSS
DLLBII133G05 **402**	EVQLVESGGGLVQPGGSLRLSCAASGFTFG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYAYDY	WGQGTQVTVSS
DLLBII133H03 **403**	EVQLVESGGGLVQPGGSLRLSCAASGFTIG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYAYDY	WGQGTQVTVSS
DLLBII134B11 **404**	EVQLVESGGGLVQPGGSLRLSCAASGFTIG	SYDMS	WVRRSPGKGPEWVS	AINSGGGSTYYTDFVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYAYDY	WGQGTQVTVSS
DLLBII134D10 **405**	EVQLVESGGGLVQPGGSLRLSCAASGFTIG	SYDMS	WVRRSPGKGPEWVS	SINSGGGSTYYTDYVKG	RFTISRDNAKNTLYLQMNSLKPEDTAVYYCAA	DRYIWARQGEYWGAYAYDY	WGQGTQVTVSS

DLLBII1 35H04 **406**	EVQLVESGGGLVQPGGS LRLSCAASGFTIG	SYDMS	WVRRSPGKGPE WVS	AINSGGGSTYYA DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYQYDY	WGQGTQVTVS S
DLLBII1 36C07 **407**	EVQLVESGGGLVQPGGS LRLSCAASGFTFG	SYDMS	WVRRSPGKGPE WVS	SINSGGGSTYYA DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYEYDY	WGQGTQVTVS S
DLLBII1 36D01 **408**	EVQLVESGGGLVQPGGS LRLSCAASGFTIG	SYDMS	WVRRSPGKGPE WVS	AINSGGDSTFYA DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYAYDY	WGQGTQVTVS S
DLLBII1 36H03 **409**	EVQLVESGGGLVQPGGS LRLSCAASGFTFG	SYDMS	WLRRSPGKGPE WVS	AINSGGGSTYYA DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGDY WGAYVYDY	WGQGTQVTVS S
DLLBII1 37A04 **410**	EVQLVESGGGLVQPGGS LRLSCAASGFTFG	SYDMS	WVRRSPGKGPE WVS	AINSGGGSTYYT DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGDY WGAYAYDY	WGQGTQVTVS S
DLLBII1 37A06 **411**	EVQLVESGGGLVQPGGS LRLSCAASGFTFG	SYDMS	WVRRSPGKGPE WVS	AINSGGGSTYYT DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIRARQGEY WGAYAYDY	WGQGTQVTVS S
DLLBII1 37B06 **412**	EVQLVESGGGLVQPGGS LRLSCAASGFTIG	SYDMS	WVRRSPGKGPE WVS	SINSGGGSTYYT DFVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYAYDY	WGQGTQVTVS S
DLLBII1 37C04 **413**	EVQLVESGGGLVQPGGS LRLSCAASGFTIG	SYDMS	WVRRSPGKGPE WVS	AINSGGGSTYYT DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYEYDY	WGQGTQVTVS S
DLLBII1 37F04 **414**	EVQLVESGGGLVQPGGS LRLSCAASGFTIG	SYDMS	WVRRSPGKGPE WVS	SINSGGGSTFYT DFVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYAYDY	WGQGTQVTVS S
DLLBII1 38F12 **415**	EVQLVESGGGLVQPGGS LRLSCAASGFTFG	SYDMS	WVRRSPGKGPE WVS	AINSGGGSTYYT DYVKG	RFTISRNNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYQYDY	WGQGTQVTVS S
DLLBII0 15 **416**	EVQLVESGGGLVQPGGS LRLSCAASGFTIG	SYDMS	WVRRSPGKGPE WVS	AINSGGGSTYYA DYVKG	RFTISRDNAKNTLY LQMNSLKPEDTAVY YCAA	DRYIWARQGEY WGAYAYDY	WGQGTQVTVS S

Example 7

Characterization of affinity matured purified VHHs

Affinity-matured variants of VHHs DLLBII101G08 and DLLBII115A05 are expressed and purified as described above (Example 6). VHHs are characterized in the rhDLL1/ rhJAG1 binding ELISA and hDll4/ mDll4/ cynoDll4 FACS (Example 5.8; Table 20; Figure 12 and 13), the rhDll4 - rhNotch1 competition ELISA (Example 5.1; Table 17; Figure 10), the competition rhNotch1 - CHO-hDll4 FMAT (Example 5.3; Table 18; Figure 11).

Characterization data are summarized in Table 21. Overall, the affinity matured VHHs show clear improvements in affinity and potency, while their binding to mDll4 and cyno Dll4 is maintained and no binding to hDLL1 or hJAG1 is observed

Table 17: IC_{50} (nM) values for affinity matured VHHs in hDLL4/hNotch1-Fc competition ELISA

VHH ID	IC_{50} (nM)
101G08	*10.0*
129A03	1.8
129B05	0.9
129D08	1.2
129E11	1.3
129H07	1.0
130B03	1.5
130F06	1.3
anti-DLL4 Fab	1.5

VHH ID	IC_{50} (nM)
115A05	*7.5*
133A05	2.1
133A09	1.5
133G05	2.0
134D10	1.3
136C07	1.4
015	0.9
anti-DLL4 Fab	1.2

Table 18: IC_{50} values (nM) of purified affinity matured VHHs blocking the interaction of human Notch1/Fc to human or mouse DLL4 expressed on CHO cells (FMAT)

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	hDLL4	mDLL4
VHH ID	IC_{50} (nM)	IC_{50} (nM)
101G08	*69.3*	*140.5*
129B05	7.4	14.4
129D08	7.8	11.0
129E11	8.1	12.3
anti-DLL4 Fab	5.5	3.0

	hDLL4	mDLL4
VHH ID	IC_{50} (nM)	IC_{50} (nM)
115A05	*106.7*	*348.9*
133A09	6.6	18.6
133G05	5.9	12.0
136C07	8.0	31.2
015	5.7	21.2
anti-DLL4 Fab	3.4	1.6

Table 19: Affinity K_D (nM) of purified affinity matured VHHs on recombinant human DLL4 and mouse DLL4

	rhDLL4			rmDLL4		
VHH ID	**k_a ($M^{-1}s^{-1}$)**	**k_d (s^{-1})**	**K_D (nM)**	**k_a ($M^{-1}s^{-1}$)**	**k_d (s^{-1})**	**K_D (nM)**
101G08 (wt)	*4.8E+04*	*2.3E-03*	*48.0*	*9.4E+04*	*5.6E-03*	*60.0*
129A03	2.1E+05	1.2E-04	0.5			
129B05	2.3E+05	7.9E-05	0.3	2.7E+05	3.1E-04	1.1
129D08	1.8E+05	6.4E-05	0.4	2.7E+05	2.0E-04	0.8
129E11	1.9E+05	9.0E-05	0.5	2.5E+05	2.9E-04	1.2
129H07	1.6E+05	7.3E-05	0.5			
130B03	2.2E+05	6.8E-05	0.3			
130F06	2.0E+05	8.0E-05	0.4			
anti-DLL4 Fab	2.3E+05	3.4E-04	1.5			

	rhDLL4			rmDLL4		
VHH ID	**k_a ($M^{-1}s^{-1}$)**	**k_d (s^{-1})**	**K_D (nM)**	**k_a ($M^{-1}s^{-1}$)**	**k_d (s^{-1})**	**K_D (nM)**
115A05 (wt)	*2.5E+05*	*4.0E-03*	*16.0*	*1.7E+05*	*9.1E-03*	*53.0*
133A09	4.4E+05	9.0E-04	2.1	3.5E+05	2.7E-03	7.8
133G05	5.9E+05	4.7E-04	0.8	4.7E+05	1.6E-03	3.4
136C07	6.2E+05	3.9E-04	0.6	5.0E+05	1.3E-03	2.6
015	4.5E+05	4.7E-04	1.0	3.5E+05	1.5E-03	4.3
anti-DLL4 Fab	2.3E+05	3.4E-04	1.5			

Table 20: EC_{50} (nM) values of affinity matured VHHs for binding on CHO-hDLL4, CHO-mDLL4 and CHO-cDLL4 (FACS)

	hDLL4	**mDLL4**	**cDLL4**
VHH ID	**EC_{50} (nM)**	**EC_{50} (nM)**	**EC_{50} (nM)**
101G08(wt)	*17.5*		*11.2*
129B05	9.7	3.9	3.9
129D08	9.6	3.7	3.8
129E11	1.4	4.1	4.2
anti-DLL4 Fab	5.6	2.1	2.5

	hDLL4	**mDLL4**	**cDLL4**
VHH ID	**EC_{50} (nM)**	**EC_{50} (nM)**	**EC_{50} (nM)**
115A05(wt)	*11.3*		*13.8*
133A09	7.2	1.7	2.3
133G05	8.5	2.8	2.7
136C07	10.9	8.3	3.5
015	14.8	7.0	5.1
anti-DLL4 Fab	5.6	2.1	2.5

Table 21: Characteristics of affinity-matured VHHs derived from DLLBII101G08 and DLLBII115A05

			ELISA	FMAT hDLL4	FMAT mDLL4	FACS	FACS	FACS	ELISA	ELISA
	K_D (nM) hDLL4	K_D (nM) mDLL4	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	EC_{50} (nM)	EC_{50} (nM)	hDLL1	hJag-1
101G08	*48.0*	*60.0*	*10.0*	*69.3*	*140.5*	*17.5*	*NF*	*11.2*	*nb*	*nb*
129A03	0.5		1.8							
129B05	0.3	1.1	0.9	7.4	14.4	9.7	3.9	3.9	nb	nb
129D08	0.4	0.8	1.2	7.8	11.0	9.6	3.7	3.8	nb	nb
129E11	0.5	1.2	1.3	8.1	12.3	10.4	4.1	4.2	nb	nb
129H07	0.5		1.0							
130B03	0.3		1.5							
130F06	0.4		1.3							
DLL4 Fab	1.5		1.5	5.5	3.0	5.6	2.1	2.5		

			ELISA	FMAT hDLL4	FMAT mDLL4	FACS	FACS	FACS	ELISA	ELISA
	K_D (nM) hDLL4	K_D (nM) mDLL4	IC_{50} (nM)	IC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	EC_{50} (nM)	EC_{50} (nM)	hDLL1	hJag-1
115A05	*16.0*	*53.0*	*7.5*	*106.7*	*348.9*	*11.3*	*NF*	*13.8*	*nb*	*nb*
133A05			2.1							
133A09	2.1	7.8	1.5	6.6	18.6	7.2	1.7	2.3	nb	nb
133G05	0.8	3.4	2.0	5.9	12.0	8.5	2.8	2.7	nb	nb
134D10			1.3							
136C07	0.6	2.6	1.4	8.0	31.2	10.9	8.3	3.5	nb	nb
015	1.0	4.3	0.9	5.7	21.2	14.8	7.0	5.1	nb	nb
DLL4 Fab	1.5		1.2	3.4	1.6	5.6	2.1	2.5		

nb: no binding

Claims

1. A Dll4-binding molecule comprising at least a variable domain with four framework regions and three complementarity determining regions CDR1, CDR2 and CDR3, respectively, wherein said CDR3 has an amino acid sequence selected from amino acid sequences shown in
   a. SEQ IDs NOs: 1 to 166 and 458,
   b. SEQ ID NOs: 333 to 353, or
   c. SEQ ID NOs: 375 to 395.

2. A Dll4-binding molecule of claim 1, which is an isolated immunoglobulin single variable domain or a polypeptide containing one or more of said immunoglobulin single variable domains, wherein said immunoglobulin single variable domain consists of four framework regions and three complementarity determining regions CDR1, CDR2 and CDR3, respectively, and wherein said CDR3 has an amino acid sequence selected from amino acid sequences shown in
   a. SEQ ID NOs: 1 to 166 and 458,
   b. SEQ ID NOs: 333 to 353, or
   c. SEQ ID NOs: 375 to 395.

3. A Dll4-binding molecule of claim 2, wherein said one or more immunoglobulin single variable domain contain
   a. a CDR3 with an amino acid sequence selected from a first group of amino acid sequences shown in SEQ ID NOs: 1 to 166;
   b. a CDR1 and a CDR2 with an amino acid sequences that is contained, as indicated in Table 5, as partial sequence in a sequence selected from a second group of amino acid sequences shown SEQ ID NOs: 167 to 332 and 459;

   wherein a SEQ ID NO: x of said first group, for SEQ ID NOs: 1 – 166 corresponds to SEQ ID NO: y of said second group in that y = x +166.

4. A Dll4-binding molecule of claim 2, wherein said one or more immunoglobulin single variable domains contain
   a. a CDR3 with an amino acid sequence selected a said first group of amino acid sequences shown in SEQ ID NOs: 333 to 353;
   b. a CDR1 and a CDR2 with an amino acid sequence that is contained, as indicated in Table 16-A , as a partial sequence in a sequence selected from a second group of sequences shown SEQ ID NO: 354 to 374;

   wherein a SEQ ID NO: x of said first group corresponds with SEQ ID NO: y of said second group in that y = x +21.

5. A Dll4-binding molecule of claim 2, wherein said one or more immunoglobulin single variable domains contain
   a. a CDR3 with an amino acid sequence selected a said first group of amino acid sequences shown in SEQ ID NOs:375 to 395;
   b. a CDR1 and a CDR2 with an amino acid sequence that is contained, as indicated in Table 16-B, as a partial sequence in a sequence selected from a second group of sequences shown in SEQ ID NOs: 396 to 416;

   wherein a SEQ ID NO: x of said first group corresponds to SEQ ID NO: y of said second group in that y = x +21.

6. A Dll4-binding molecule of any one of claims 2 to 5, wherein said one or more immunoglobulin single variable domains are VHHs.

7. A Dll4-binding molecule of claim 6, wherein said one or more VHHs have an amino acid sequence selected from amino acid sequences shown in SEQ ID NOs: 167 to 332 and 459.

8. A Dll4-binding molecule of claim 6, said one or more VHHs have an amino acid sequence selected from amino acid sequences shown in SEQ ID NOs: 354 to 374.

9. A Dll4-binding molecule of claim 6, wherein said one or more VHHs have an amino acid sequence selected from amino acid sequences shown in SEQ ID NOs:396 to 416.

10. An immunoglobulin single variable domain which has been obtained by affinity maturation of an immunoglobulin single variable domain as defined in claim 3.

11. A VHH which has been obtained by affinity maturation of a VHH as defined in claim 7.

12. A VHH with an amino acid sequence selected from acid sequences shown in SEQ ID NOs: 356 and 358.

13. An immunoglobulin single variable domain which has been obtained by humanization of a VHH defined in claim 12.

14. A VHH with an amino acid sequence selected from sequences shown in SEQ ID NOs: 402, 407 and 416.

15. An immunoglobulin single variable domain which has been obtained by humanization of a VHH defined in claim 14.

16. An immunoglobulin single variable domain which has been obtained by humanization of an immunoglobulin single variable domain as defined in claim 3.

17. An immunoglobulin single variable domain which has been obtained by humanization of an immunoglobulin single variable domain as defined in claim 10.

18. A Dll4-binding molecule of claim 1, which binds to an epitope of Dll4 that is totally or partially contained within the EGF-2 domain that corresponds to amino acid residues 252-282 of SEQ ID NO: 417.

19. The Dll4-binding molecule of claim 18, which is a immunoglobulin single variable domain or a polypeptide containing same.

20. A nucleic acid molecule encoding a Dll4-binding molecule of any one of claims 1 to 19 or a vector containing same.

21. A host cell containing a nucleic acid molecule of claim 20.

22. A pharmaceutical composition containing at least one Dll4-binding molecule of any one of claims 1 to 19 as the active ingredient.

23. The pharmaceutical composition of claim 22 for the treatment of a disease that is associated with Dll4-mediated effects on angiogenesis.

24. The pharmaceutical composition of claim 22 for the treatment of cancer and cancerous diseases.

25. The pharmaceutical composition of claim 22 for the treatment of eye diseases.

26. A peptide comprising a sequence with an amino acid sequence selected from amino acid sequences shown in
    a. SEQ ID NOs: 1 to 166 and 458,
    b. SEQ ID NOs: 333 to 353, or
    c. SEQ ID NOs: 375 to 395.

27. A nucleic acid molecule encoding a peptide of claim 26.