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(54) Title: SCAFFOLD PROTEINS AND THERAPEUTIC NANOCONJUGATES BASED ON NIDOGEN

(57) Abstract: The present invention relates to proteins suitable for being used as scaffolds to which a peptide of interest is bound, or which are comprised within a conjugate to which an agent of interest is attached. It also relates to said conjugates suitable for the selective delivery of their conjugated agents of interest to specific cell and tissue types, wherein said agent 5 can be a therapeutic agent or an imaging agent. It also relates to nanoparticles comprising such conjugates and the therapeutic uses thereof.

SCAFFOLD PROTEINS AND THERAPEUTIC NANOCONJUGATES BASED ON NIDOGEN

FIELD OF THE INVENTION

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The present invention relates to the field of nanostructured protein materials, more specifically to therapeutic agent-carrying polypeptides which can be used for therapy.

BACKGROUND OF THE INVENTION

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The systemic administration of drugs in form of nanoconjugates benefits from enhanced drug stability when compared to free molecules. Valuable additional properties such as cell targeting might be also merged into a given hybrid composite through the chemical incorporation of functional groups in nanoscale vehicles, taking profit from the high surface/volume ratio of nanomaterials. When administered systemically, the resulting drug loaded conjugates sizing between ~8 and 100 nm escape from renal filtration in absence of aggregation in lung or other highly vascularized organs. This fact, combined with appropriate physicochemical properties of the material might result in extended circulation time and prolonged drug exposure to target organs, thus enhancing the therapeutic impact and benefits for the patient.

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Among the diversity of materials under investigation as drug carriers, that includes metals, ceramics, polymers and carbon nanotubes, proteins offer unique properties regarding biocompatibility and degradability that, in the context of rising nanotoxicological concerns, make them especially appealing. As the engineering of protein self-assembling into nanostructured materials is rapidly progressing and the control over the final geometry and physicochemical properties becomes tighter, protein materials are gaining functional and structural versatility as vehicles from chemically coupled drugs.

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In fact, the attachment of a cytotoxic “payload” to an antibody to form an antibody–drug conjugate (ADC) has been shown to provide a mechanism for selective delivery of the cytotoxic agent to cancer cells via the specific binding of the antibody to cancer-selective

cell surface molecules. Multiple examples of this strategy have been proved to be effective, like gemtuzumab ozogamicin, which comprises an anti-CD33 antibody conjugated to a highly potent DNA-targeting antibiotic, calicheamicin, which was used against acute myeloid leukemia. Also, maytansinoids, a highly potent microtubule-
5 disrupting agents, have been tested as payloads for ADCs, resulting in the formulation ado-trastuzumab emtansine for treating HER2-positive breast cancer.

Nonetheless, the structural complexity of antibodies may become a cumbersome hindrance in terms of cost and synthesis. The inventors previously probed into the field
10 of nanomedicine by applying a nanoarchitectonic principle based on the addition, to a core protein, of a cationic N-terminal domain plus a C-terminal poly-histidine. [Serna, N. *et al.* 2016. *Nanomedicine*, 12:1241-51] It has been described in the art that these end-terminal tags and the resulting charge balance in the whole fusion promote self-assembling and oligomerization of monomeric proteins as robust toroid nanoparticles,
15 stable in plasma [Cespedes, M. V. *et al.* 2014. *ACS Nano.*, 8:4166-4176] and with high cellular penetrability if empowered with cell-targeting peptides. [Xu, Z. K. *et al.* 2015. *Materials Letters*, 154:140-3] The building blocks of these protein structures might also contain functional peptides such as cell-targeting agents, endosomolytic agents or nuclear localization signals, in form of fused stretches with modular organization.

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Since current therapy methods still show a margin of failure, mostly due to tumor resistance phenomena which may result from intra-tumor clonal selection of those cells most resistant to the chemotherapy, for instance, there is still in the art a need for the development of more specific therapeutic approaches which can be targeted to the
25 concrete tumor cells responsible for therapy failure and tumor progression while reducing the side and off-target effects of the therapeutic agents.

SUMMARY OF THE INVENTION

30 In a first aspect, the invention relates to a polypeptide comprising:

- (i) eleven beta strand domains designated A, B, C, D, E, F, G, H, I, J and K
and

- (ii) ten loop regions designated as AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK loops wherein each loop region connects each two consecutive beta strand domains;

wherein at least one of the loop regions is a variant of the cognate loop region in SEQ ID NO: 62, wherein the cognate loop regions in SEQ ID NO:62 are as defined in SEQ ID NO:1 (loop region AB), SEQ ID NO:2 (loop region BC), SEQ ID NO:3 (loop region CD), SEQ ID NO:4 (loop region DE), SEQ ID NO:5 (loop region EF), SEQ ID NO:6 (loop region FG), amino acids 149 to 150 in SEQ ID NO:62 (loop region GH), SEQ ID NO:7 (loop region HI), SEQ ID NO:8 (loop region IJ) and SEQ ID NO:9 (loop region JK), and

wherein at least one beta strand domain is a variant of the cognate beta strand in SEQ ID NO: 62 and has at least 50% sequence identity with said cognate beta strand domain, wherein the cognate beta strand domains in SEQ ID NO:62 are as defined in SEQ ID NO:9 (beta strand domain A), SEQ ID NO:11 (beta strand domain B), SEQ ID NO:12 (beta strand domain C), SEQ ID NO:13 (beta strand domain D), SEQ ID NO:14 (beta strand domain E), SEQ ID NO:15 (beta strand domain F), SEQ ID NO:16 (beta strand domain G), SEQ ID NO:17 (beta strand domain H), SEQ ID NO:18 (beta strand domain I), SEQ ID NO:19 (beta strand domain J) and SEQ ID NO:20 (beta strand domain K).

In a second aspect, the invention relates to a polypeptide display library comprising a plurality of polypeptides according to the first aspect of the invention, wherein the plurality of polypeptides is formed by polypeptides that differ in the sequence of one or more loop regions.

In a third aspect, the invention relates to a polynucleotide encoding the polypeptide according to the first aspect of the invention, or a polypeptide of the polypeptide display library according to the second aspect of the invention.

In a fourth aspect, the invention relates to a vector comprising the polynucleotide according to the third aspect of the invention.

In a fifth aspect, the invention relates to a host cell comprising the polynucleotide according to the third aspect of the invention, or the vector according to the fourth aspect of the invention.

- 5 In a sixth aspect, the invention relates to a conjugate comprising
- (i) a first polypeptide region comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
 - (ii) an agent of interest.

- 10 In a seventh aspect, the invention relates to a method for preparing a conjugate according to the sixth aspect of the invention, comprising

- (i) providing the polypeptide of the conjugate according to the sixth aspect of the invention comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof wherein the polypeptide is provided in an
15 activated form and
- (ii) contacting said polypeptide with the agent of interest which is capable of reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a
20 bond between the reactive group in the polypeptide and the group in the agent of interest.

In an eighth aspect, the invention relates to a polypeptide comprising

- (i) a first region comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- 25 (ii) a second region which comprises an antagonistic CXCR4 ligand.

In a ninth aspect, the invention relates to a method for preparing a conjugate according to the sixth aspect of the invention, comprising

- (i) providing the polypeptide of the conjugate according to the sixth aspect of the
30 invention comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and

- (ii) contacting said polypeptide with an activated form of the agent of interest of the conjugate according to the sixth aspect of the invention which is capable of reacting with at least one group in the polypeptide and wherein the contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the agent of interest and the group in polypeptide.

In a tenth aspect, the invention relates to a method for preparing a conjugate according to the sixth aspect of the invention, comprising

- (i) providing the polypeptide of the conjugate according to the sixth aspect of the invention comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof wherein the polypeptide is provided in an activated form and
- (ii) contacting said polypeptide with the agent of interest which is capable of reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptide and the group in the agent of interest.

In an eleventh aspect, the invention relates to a polypeptide comprising a first region comprising

- (i) the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- (ii) a second region which comprises an antagonistic CXCR4 ligand.

In a twelfth aspect, the invention relates to a method for preparing a nanoparticle comprising multiple copies of the polypeptide according to the eleventh aspect of the invention, said method comprising placing a preparation of said polypeptide under conditions adequate for the assembly of a plurality of copies of the polypeptide into a nanoparticle.

In a thirteenth aspect, the invention relates to a method for preparing a nanoparticle comprising multiple copies of the conjugate according to the sixth aspect of the invention

or multiple copies of the polypeptide according to the eleventh aspect of the invention selected from:

- (i) A method which comprises placing a preparation of said conjugate or said polypeptide under conditions adequate for the assembly of a plurality of copies of the conjugate or of the polypeptide into a nanoparticle or
- (ii) A method which comprises
 - i. placing a plurality of polypeptides each comprising
 - 1. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,
 - 2. a second polypeptide region which is capable of specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide and
 - 3. a third polypeptide region which is a positively charged amino acid-rich region, wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptide and wherein the polypeptide is provided in an activated form, wherein said activated form of the polypeptide contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of the polypeptide and
 - ii. contacting the nanoparticle obtained in step I with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptide and the group in the agent of interest.

In a fourteenth aspect, the invention relates to a method for preparing a biparatopic nanoparticle comprising multiple copies of a first type of conjugate and multiple copies of a second type of conjugate, wherein the first and second types of conjugates are as defined in the sixth aspect of the invention and wherein the first and the second type of conjugates differ in the sequence of the polycationic peptide, said method being selected from

- (i) A method which comprises contacting a preparation of said first type of conjugate with a preparation of said second type of conjugate under

conditions adequate for the assembly of a plurality of copies of the two types of conjugates into a nanoparticle or

(ii) A method which comprises

i. contacting a preparation of a first polypeptide with a preparation of a second polypeptide wherein the first and second types of polypeptides comprise

a. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,

b. a second polypeptide region which is capable of specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide, and/or comprises an additional positively charged peptide sequence located at its N- or C-terminal end and the sequence of the polycationic peptide of one polypeptide is different from the sequence of the polycationic peptide of the other polypeptide,

c. a third polypeptide region which is a positively charged amino acid-rich region,

d. optionally, a positively charged peptide sequence located at the N- or C-terminal end of the polycationic peptide,

wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptides,

wherein the first and second polypeptides differ in their polycationic peptide,

wherein the first and/or second polypeptides are as the conjugates defined in the sixth aspect of the invention,

wherein the first and/or the second polypeptides are provided in an activated form, wherein said activated form of the polypeptides contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of polypeptides,

- ii. contacting the nanoparticle obtained in step i with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in each polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptides and the group in the agent of interest.

In a fifteenth aspect, the invention relates to a method for preparing a biparatopic nanoparticle comprising multiple copies at least one conjugate according to the sixth aspect of the invention and multiple copies of at least one polypeptide according to the eleventh aspect of the invention wherein the polycationic peptide of the first type of conjugate is different from the sequence of the second region of the at least one polypeptide, said method being selected from

- (i) A method which comprises placing a preparation of multiple copies of said at least one conjugate and multiple copies of said at least one polypeptide under conditions adequate for the assembly of a plurality of copies of the two conjugates into a nanoparticle or

- (ii) A method which comprises
- i. contacting a preparation of a first polypeptide with a preparation of a second polypeptide wherein the first and second type of polypeptides comprise
 - a. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,
 - b. a second polypeptide region which is capable of specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide, and/or comprises an additional positively charged peptide sequence located at its N- or C-terminal end and the sequence of the polycationic peptide of one polypeptide is different from the sequence of the polycationic peptide of the other polypeptide,

- c. a third polypeptide region which is a positively charged amino acid-rich region,
wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptides,
wherein the first polypeptide is as the conjugated defined in the sixth aspect of the invention and the second polypeptide is as defined in the eleventh aspect of the invention,
wherein the polycationic peptide of the first polypeptide is different from the polycationic peptide of the second polypeptide,
wherein the first and/or the second polypeptides are provided in an activated form, wherein said activated form of the polypeptides contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of polypeptides,
- ii. contacting the nanoparticle obtained in step i with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in each polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptides and the group in the agent of interest.

In a sixteenth aspect, the invention relates to a nanoparticle comprising multiple copies of the conjugate according to the sixth aspect of the invention, multiple copies of the polypeptide according to the eleventh aspect of the invention or which has been obtained by the method according to the twelfth or thirteenth aspect of the invention.

In a seventeenth aspect, the invention relates to a biparatopic nanoparticle that comprises multiple copies of a first and a second type of conjugates, wherein both said first and second types of conjugates are as defined in the sixth aspect of the invention or as the polypeptide defined in the eleventh aspect of the invention and wherein the first and

second types of conjugates differ in the polycationic peptide or a biparatopic nanoparticle that has been obtained by a method according to the fourteenth or fifteenth aspect of the invention.

- 5 In an eighteenth aspect, the invention relates to a biparatopic nanoparticle that comprises multiple copies of a conjugate according to the sixth aspect of the invention and multiple copies of a polypeptide according to the eleventh aspect of the invention, wherein the polycationic region of the conjugate is different from the first region of the polypeptide, or a biparatopic nanoparticle that has been obtained by a method according to the
10 fourteenth or fifteenth aspect of the invention.

In a nineteenth aspect, the invention relates to a conjugate according to the sixth aspect of the invention, a polypeptide according to the eleventh aspect of the invention or a nanoparticle according to the sixteenth, seventeenth or eighteenth aspect of the invention
15 for use in medicine.

In a twentieth aspect, the invention relates to a method for the imaging of a target cell which comprises specific binding sites for one or more components of the conjugate according to the sixth aspect of the invention, of the polypeptide according to the eleventh
20 aspect of the invention or of the nanoparticle according to the sixteenth, seventeenth or eighteenth aspect of the invention, the method comprising

- (i) contacting a sample containing said cell with a conjugate according to the sixth aspect of the invention, with a polypeptide according to the eleventh aspect of the invention or with a nanoparticle according to the sixteenth, seventeenth or eighteenth aspect of the invention under conditions
25 adequate for the binding of the conjugate, of the polypeptide or of the nanoparticle to the cell and wherein the agent of interest is an imaging agent and
- (ii) Imaging the cell by detecting the signal provided by the imaging agent.

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In a twenty-first aspect, the invention relates to a method for identifying a polypeptide that binds to a target peptide, said method comprising:

- i) contacting a target peptide with the polypeptide display library according to the second aspect of the invention conditions that allow a polypeptide to interact with the target peptide,
- ii) recovering those members of the library that have specifically interacted with the target peptide, and
- iii) identifying the sequence of the polypeptide that interacts with the target peptide.

In a twenty-second aspect, the invention relates to a use of a polypeptide according to the first aspect of the invention for presenting a peptide, wherein said peptide is found in one of the loop regions.

In a twenty-third aspect, the invention relates to a method for determining the presence of a target peptide in a sample comprising:

- i) contacting the proteins present in the sample with a polypeptide according to the first aspect of the invention, wherein the sequence of at least one of the loop regions in the polypeptide is a sequence that is capable of specifically binding to the target peptide,
- ii) determining if there is an interaction between the target peptide and the polypeptide,

wherein if there is an interaction between the polypeptide and the target peptide, then the target peptide is present in the sample.

DESCRIPTION OF THE FIGURES

Fig. 1 shows the structure of Human Nidogen-1 protein as depicted in Takagi J. et al. (Nature 424, 969-974, 2003). G1, G2 and G3 indicate the three main globular domains. EG represent EGF modules, TY represent thyroglobulin repeat and LY represents LDL receptor YWTD repeat.

Fig. 2 shows the characterization of T22-STM-H6 (A), T22-NIDOm2-H6 (B) and T22-GFP-H6 (C) nanoparticles. MALDI-TOF mass spectrometry spectrum is shown in (Top),

Western-blot immunodetection using anti-His monoclonal antibody is shown in the (inset) and Volume size distribution measured by DLS is shown (bottom) for each nanoparticle.

Fig. 3 shows the characterization of T22-NIDOMut2-H6 Nanoparticles characterization.
 5 A) Coomassie-blue staining of T22-NIDOMut2-H6 purification peaks 1 (Pico1) and peak 2 (Pico2) in Carbonate (-) and Carbonate + Salt (+) buffers upon SDS-PAGE electrophoresis. B) Western-blot immunodetection of T22-NIDOMut2-H6 protein by anti-His monoclonal antibody. C) Volume size distribution of T22-NIDOMut2-H6 nanoparticles determined by DLS. D) MALDI-TOF mass spectrometry spectrum of T22-
 10 NIDOMut2-H6 protein.

Fig. 4 shows T22-NIDOMut2-H6 labelling. A) MALDI-TOF mass spectrometry spectrum of labeled T22-NIDOMut2-H6-ATTO488 protein. Each peak over 30.3kDa correspond to an additional ATTO molecule incorporation. B) Volume size distribution of T22-
 15 NIDOMut2-H6-ATTO488 nanoparticles determined by DLS.

Fig. 5 shows the CXCR4-specific internalization of the T22-NIDOMut2-H6 protein in CXCR4+ cells. A) Labelled T22-NIDOMut2-H6-ATTO488 nanoparticles internalization and competition (+AMD) upon incubation for 24h over HeLa cells (CXCR4+) at different
 20 concentrations (1 nM and 10 nM). The % of cell uptake inhibition in presence of the CXCR4 receptor antagonist AMD3100 (+AMD) is indicated. B) Confocal laser microscopy images of HeLa cells incubated in presence of 25nM of T22-NIDOMut2-H6-ATTO488 for 24h. Cell nuclei were stained by Hoechst, cell membranes by CellMask and punctuated pattern inside cells correspond to protein nanoparticles.
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Fig. 6 shows T22-NIDOMut2-H6 nanoparticles conjugation with oligo-FdU. A) Schematic representation of the covalent binding of a thiol conjugated oligo 5'-(FdU)5-hexaethyleneglycolthiol-3' (oligo-FdU-SH) through protein lysine amines using a 6-Maleimidohexanoic acid N-hydroxysuccinimide ester (EMCS) bifunctional linker in a
 30 two-step reaction. B) MALDI-TOF mass spectrometry spectrum of T22-NIDOMut2-H6-FdU nanoconjugates. C) Volume size distribution of T22-NIDOMut2-H6-FdU nanoconjugates determined by DLS.

Fig. 7 shows cytotoxicity of oligo-FdU conjugated nanoparticles over CXCR4+ cells. Graph shows the viability (%) of HeLa cells (CXCR4+) upon incubation in presence of 25nM or 100nM of T22-STM-H6-FdU, T22-NIDOMut2-H6-FdU or two different stocks
5 of T22-GFP-H6-FdU nanoconjugates and 100nM of free oligo-FdU (FdU) for 48h measured by a MTT viability test assay.

Fig. 8 shows that the T22-NIDOMut2-H6-FdU nanoconjugate induces higher growth inhibition than T22-STM-H6-FdU or T22-GFP-H6-FdU in a CXCR4+ tumor model. The
10 Graphic displays the evolution of tumor volume along time for each nanoconjugate-treated group (n=4) at a dosage of 20 ug q3d x 5 doses, and its comparison with Buffer-treated (K, n=4) in the CXCR4+ M5 subcutaneous (SC) colorectal cancer (CRC) model.

Fig. 9 shows a higher apoptotic induction by the T22-NIDOMut2-H6-FdU nanoconjugate
15 in CXCR4+ M5 tumor tissue than T22-STM-H6-FdU or T22-GFP-H6-FdU. A) Graphic display of the number of apoptotic bodies observed in tumor sections in Buffer- or nanoconjugate-treated tumors at the end of the experiment. B) Representative microphotographs of H&E-stained sections identifying the apoptotic figures (Black arrows) observed in each compared group.

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Fig. 10 shows a lack of histological alterations in kidney or liver among the compared groups. Representative H&E-stained sections of kidney and liver tissues showing lack of altered architecture or histology (no inflammation or apoptotic figures observed) in both organs at the end of treatment in control buffer-treated or nanoconjugate-treated mice.

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Fig. 11 shows the correlation between intrinsic Fluorescence vs temperature for isolated hexahistidine-tagged versions of the human nidogen G2 domain, of the human nidogen G2 containing the H459A., R468N, F639S and R650A mutations and for stefin A. Arrow indicates the heating of the sample. Unfolding curves for Nidomut2H6 (B), NidoWTH6
30 (C) and STMH6 (D) with CSM values as a function of temperature. CSM values were calculated from experiment exemplified with Fig 1a and performed for each protein. Black and grey arrows indicate the T_m and T_{onset} values, respectively.

Fig. 12 shows the structure of Nidogen G2 domain. A) Schematic representation of multi-domain human Nidogen 1 protein and its interactions with natural ligands. β -barrel structure within G2 domain is depicted. B) Secondary structure of Nidogen G2 β -barrel domain. In grey different β -sheets (A-K) and in blue the α -helix. C) Cartoon representation of the Tertiary β -barrel structure of Nidogen G2 (1GL4 accession number of RCSB PDB database, version 1.2 from the 13-07-2011) on the left panel and Green fluorescent protein (1QYO accession number of RCSB PDB database chain A, version 1.2 from the 13-07-2011) on the right panel. D) Superposition of Nidogen G2 and GFP β -barrel structures.

Fig. 13 shows the design of NidoMut2 peptide. A) Similarity of human and mouse Nidogen G2 domain: Amino acid alignment between Human Nidogen 1 (P14543 from Uniprot Database, version dated July 7, 2009) and Mouse Nidogen 1 (P10493 from Uniprot Database version 27 July 2011) proteins analyzed by Clustal Omega (EMBL-EMI). Black lines indicate the start and finish point of the G2 β -barrel domain. “*” indicates an amino acid match, “:” indicates amino acids with strong similar properties and “.” indicates amino acids with weak similar properties. B) Amino acid sequence of Human Nidogen 1, HSNBT scaffold and T22-HSNBT-H6 protein. In Human Nidogen, G2 β -barrel domain is highlighted in black and candidate amino acids to be mutated indicated in bold black and underlined. In NIDOMut2 sequence, incorporated mutations are indicated in bold black. In T22-NIDOMut2-H6, N-terminal T22 ligand is indicated in underlined black, short linker is indicated in bold black, incorporated mutations are indicated in bold underlined and C-terminal poly-histidine tail is highlighted in bold black italics.

Fig. 14 shows the Western blots of the soluble fraction of cell lysates of the expression test of the proteins indicated in the figure.

Fig. 15 shows the MALDI-TOF mass spectrometry spectrum of the selected candidate proteins indicated in each panel of the figure after the expression test. Theoretical size of all proteins is approximately 30.3 kDa.

Fig. 16 shows the volume size distribution of T22-NIDOMut2-H6, T22-NIDOMut3-H6, T22-NIDOMut4_T215V-H6 and T22-NIDOMut5-H6 proteins and their nanoparticles, as determined by DLS.

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Fig. 17 A shows the precipitation profiles of T22-NIDOMut2-H6, T22-NIDOMut3-H6, T22-NIDOMut4_T215V-H6 and T22-NIDOMut5-H6 after incubation with increasing concentrations of ZnCl₂. **B.** Volume size distribution of T22-NIDOMut2-H6, T22-NIDOMut3-H6, T22-NIDOMut4_T215V-H6 and T22-NIDOMut5-H6 nanoparticles assembled at different ZnCl₂ concentrations, as determined by DLS.

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Fig. 18 A-C. CSM profiles of T22-NIDOMut2-H6, T22-NIDOMut3-H6 and T22-NIDOMut5-H6 in carbonate buffer and three FDA-approved buffers (A9, B6, D1). **D.** Graphical representation of key indicators (T_m, Tonset and ΔT) for each of the tested buffers.

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Fig. 19 A-B show the MALDI-TOF mass spectrometry spectrum of T22-NIDOMut3-H6-FdU and T22-NIDOMut2-H6-FdU. Each peak with additional 2 kDa over the protein weight (30.3 kDa) belongs to a conjugated protein with an extra oligoFdU attached. **C.** Citotoxicity assay after 48h incubation of the T22-NIDOMut3-H6-FdU nanoconjugate along with a non-conjugated negative control and the reference T22-NIDOMut2-H6-FdU positive control. **D.** Volume size distribution of T22-NIDOMut3-H6-FdU.

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Figure 20 shows structural and functional characterization of EPI-X4-based NPs. **A.** Scheme of the modular protein EPIX4-GFP-H6 (top) and EPIX4-(RK)-GFP-H6 (down) and amino acid sequence of the second one. **B.** Mass spectroscopy analysis of EPIX4-GFP-H6 (left) and EPIX4-(RK)-GFP-H6 (right). The molecular mass of proteins upon purification is shown by SDS-PAGE and Western blot (Anti-His). **C.** Hydrodynamic size and pdi (polydispersion index), determined by Dynamic Light Scattering (DLS). Values of peak size (mean) are indicated (in nm). **D.** Size exclusion chromatography (SEC) of EPIX4-GFP-H6 (black) and EPIX4-(RK)-GFP-H6 (grey) using a Superdex 200 increase 10/300GL column. **E.** Representative FESEM (direct deposition) of EPIX4-GFP-H6 (top)

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and EPIX4-(RK)-GFP-H6 (down) protein NPs. Size bars represent 50 nm. **F** Protein amounts internalized into CXCR4⁺ HeLa cells after 2 μ M of EPIX4-GFP-H6 and EPIX4-(RK)-GFP-H6 administration at 4 h (dark green). Uptake inhibition promoted by the natural CXCR4 ligand AMD3100 (light green). Intracellular fluorescence was corrected by their specific fluorescence to render values representative of protein amounts. Asterisk indicates significant difference between EPIX4-GFP-H6 and EPIX4-(RK)-GFP-H6 protein internalization and hash indicates significant difference between EPIX4-(RK)-GFP-H6 and the inhibition promoted by AMD3100 ($p \leq 0.001$). **G**. Confocal images of HeLa cells exposed to EPIX4-GFP-H6 (left) and EPIX4-(RK)-GFP-H6 (right) for 24 h. In blue: cell nuclei, in red: cell membrane, in green: internalized NPs. Size bars represent 10 μ m. All data are presented as mean $\pm$ SEM.

Fig. 21 shows biparatopic nanoparticles formation and characterization. **A**. Scheme of the hybrid NPs forming proteins EPIX4-(RK)-GFP-H6 (top) and T22-BFP -H6 (down). **B**. Controlled EPIX4-(RK)-GFP-H6 disassembled by 0.2 % SDS (black) and assembled removing SDS by dialysis (grey); determined by Dynamic Light Scattering (DLS) (top). Hydrodynamic size comparison of T22-BFP-H6 (grey), EPIX4-(RK)-GFP-H6 (black) and Biparatopic NP (dashed grey) (down). Values of peak size (mean) are indicated (in nm) and Pdi (polydispersion index). **C**. Representative FESEM (direct deposition) of Biparatopic NPs. Size bars represent 50 nm. **D**. FRET analysis of biparatopic NP formation. Samples of biparatopic NPs, T22-BFP-H6 and EPIX4-(RK)-GFP-H6 monomers mixture and T22-BFP-H6 and EPIX4-(RK)-GFP-H6 NPs mixture were excited with the 405 nm line and the emission was collected from 350–650nm. BFP was used as donor fluorochrome and GFP as acceptor. **E**. Time course kinetics of cell internalization of EPIX4-(RK)-GFP-H6, T22-GFP-H6 and Biparatopic NPs (1 μ M) in CXCR4⁺ HeLa cells (left) and SW1417 (right). Intracellular fluorescence was corrected by specific fluorescence to render values representative of protein amounts. Significant differences ($p < 0.05$) between Biparatopic NPs and both proteins forming them are depicted by *, significant differences between Biparatopic NPs and EPIX4-(RK)-GFP-H6 are depicted by #. **E**. Time course kinetics of cell internalization of EPIX4-(RK)-GFP-H6, T22-GFP-H6 and Biparatopic NPs (1 μ M) in CXCR4⁺ HeLa cells (left) and SW1417 (right). Intracellular fluorescence was corrected by specific fluorescence to render values

representative of protein amounts. Significant differences ($p < 0.05$) between Biparatopic NPs and both proteins forming them are depicted by *, significant differences between Biparatopic NPs and EPIX4-(RK)-GFP-H6 are depicted by #. **F.** Uptake inhibition in HeLa cells exposed to 1 μ M for 1 h, mediated by the CXCR4 antagonist AMD3100 (always at an excess molar ratio of 10:1). & indicates significant difference between NPs and the inhibition promoted by AMD3100 ($p \leq 0.001$).

Fig. 22 shows in vivo biodistribution and toxicity assessment in a subcutaneous mouse model of CXCR4+human colorectal cancer. **A.** Quantification of emitted fluorescence (measured as FLI ratio) at different times in tumours. **B.** Number of apoptotic cell bodies after nanoparticle administration. Significant differences ($p < 0.05$) between EPIX4-(RK)-GFP-H6 or Biparatopic NPs against the control are depicted by *, significant differences between Biparatopic NPs and EPIX4-(RK)-GFP-H6 are depicted by #. **C.** Mitotic bodies after nanoparticles administration. Significant differences between EPIX4-(RK)-GFP-H6 or Biparatopic NPs against are depicted (* $p < 0.05$). **D.** Lack of systemic toxicity in kidney, liver, kidney and spleen by histological analysis of tissue sections (H&E) 5 and 24 h after treatment. All pictures were taken at 400 x. All data are presented as mean $\pm$ SEM

20 DETAILED DESCRIPTION OF THE INVENTION

I - Polypeptide of the invention

The inventors have observed that the G2 domain from the nidogen protein, optionally modified so as to show a decreased affinity for its natural ligands, is sufficiently stable to act as a scaffold and to present peptides which have been inserted into one or more of the loop regions connecting the beta strands within said G2 domain. In addition, the inventors have also found that the G2 domain of the nidogen protein can also be used to deliver agents of interest, including therapeutic as well as diagnostic/imaging agents to cells of interest when conjugated to a ligand which shows affinity towards a receptor expressed by said cells. The inventors found that a fusion protein comprising the nidogen G2 domain and the CXCR4-specific ligand T22 and which has been coupled to an anticancer agent

was capable of delivering the anticancer agent to CXCR4-expressing cells resulting in an inhibition of the proliferation of the cells to an extent significantly superior to the inhibition achieved by comparable agents wherein the T22 ligand is fused to different proteins, such as GFP or Stefin A.

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Accordingly, in a first aspect, the invention relates to a polypeptide comprising:

- (i) eleven beta strand domains designated A, B, C, D, E, F, G, H, I, J and K and
- (ii) ten loop regions designated as AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK loops wherein each loop region connects each two consecutive beta strand domains;

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wherein at least one of the loop regions is a variant of the cognate loop region in SEQ ID NO: 62, wherein the cognate loop regions in SEQ ID NO:62 are as defined in SEQ ID NO:1 (loop region AB), SEQ ID NO:2 (loop region BC), SEQ ID NO:3 (loop region CD), SEQ ID NO:4 (loop region DE), SEQ ID NO:5 (loop region EF), SEQ ID NO:6 (loop region FG), amino acids 149 to 150 in SEQ ID NO:62 (loop region GH), SEQ ID NO:7 (loop region HI), SEQ ID NO:8 (loop region IJ) and SEQ ID NO:9 (loop region JK), and

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wherein at least one beta strand domain is a variant of the cognate beta strand in SEQ ID NO:62 and has at least 50% sequence identity with said cognate beta strand domain, wherein the cognate beta strand domains in SEQ ID NO:62 are as defined in SEQ ID NO:9 (beta strand domain A), SEQ ID NO:11 (beta strand domain B), SEQ ID NO:12 (beta strand domain C), SEQ ID NO:13 (beta strand domain D), SEQ ID NO:14 (beta strand domain E), SEQ ID NO:15 (beta strand domain F), SEQ ID NO:16 (beta strand domain G), SEQ ID NO:17 (beta strand domain H), SEQ ID NO:18 (beta strand domain I), SEQ ID NO:19 (beta strand domain J) and SEQ ID NO:20 (beta strand domain K).

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The polypeptide is referred hereinafter as the “polypeptide according to the first aspect of the invention” or the “polypeptide of the invention”.

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The term “polypeptide”, as used herein, generally refers to a linear chain of amino acid residues of any length, joined together with peptide bonds. The term “peptide” as used herein, refers to a linear chain of amino acids as a polypeptide, although shorter than that

of a polypeptide. It generally refers to amino acid chains of 2-50 amino acids. It will be understood that the terms “peptide bond”, “peptide”, “polypeptide” and “protein” are known to the person skilled in the art.

- 5 The polypeptide of the invention is a variant of the “nidogen G2 domain”.

The term “nidogen-1” as used herein, refers to the glycoprotein formerly known as entactin. Nidogen-1 is disclosed in Uniprot Database with accession number P14543-1 (version dated July 7, 2009) (SEQ ID NO:72).

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- The term “G2 domain of nidogen-1”, as used herein, refers to the domain G2 of the protein nidogen 1 as defined above. The nidogen-1 G2 domain is as shown in SEQ ID NO:62 which corresponds to amino acid numbers 430 and 667 of the amino acid sequence of the nidogen-1 protein, with identification number P14543-1 of the Uniprot Database (version dated July 7, 2009) (SEQ ID NO:72). In another embodiment, the domain G2 of nidogen 1 is as shown in SEQ ID NO:64, which lacks the first two amino acids of SEQ ID NO:62, and thus, corresponds to a region consisting on amino acid numbers 432 and 667 of the amino acid sequence of the nidogen-1 protein precursor with identification number P14543-1 of the Uniprot Database (version dated July 7, 2009) (SEQ ID NO:72). In the native nidogen-1 sequence, the G2 domain is flanked by short EGF-like domains. However, for the purposes of the present invention, the nidogen-1 G2 domain lacks EGF-like domains at the N-or at the C-terminus.
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- 20

- In one embodiment, the polypeptide of the invention contains an N-terminal methionine residue. In another embodiment, the polypeptide of the invention does not contain a methionine at the N-terminal position.
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- As used herein, an “amino acid residue” refers to any naturally occurring amino acid, any amino acid derivative or any amino acid mimic known in the art. In a particular embodiment, said amino acid residue is an amino acid, i.e. a naturally occurring amino acid. In certain embodiments, the residues of the protein or peptide are sequential, without any non-amino acid interrupting the sequence of amino acid residues. In other
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embodiments, the sequence may comprise one or more non-amino acid moieties. In a particular embodiments, the sequence of residues of the protein or peptide may be interrupted by one or more non-amino acid moieties.

- 5 The expression “beta strand”, a “beta strand domain” or “beta strand sequence”, as used herein, refers to an extended polypeptide strand, or sequence, which is connected to another one through hydrogen bonding, between the NH group from one strand and the CO from the other strand. Typically, beta strands are around 3-10 amino acids long, although they can be longer, for instance they can even be 13-15 amino acids long. As a
10 result of said strand connections between strands, beta strands form a secondary protein structure that resembles a sheet. Said secondary protein structure is referred herein as a “beta sheet”. Within a beta sheet, beta strands can be arranged in a parallel, anti-parallel or mixed (parallel and anti-parallel) manner. When arranged in a parallel manner, the beta strands are aligned in the same direction from one terminus (N or C) to the other. When
15 arranged in an anti-parallel manner, each beta strand is aligned in an opposite direction to that of the strand to which it is connected.

- The expression “beta barrel”, as used herein, refers to a protein secondary structure formed by a beta-sheet, wherein the first strand is bonded to the last strand by means of a
20 hydrogen bond, leading to a closed toroidal structure.

- The expression “ α -helix” or “alpha helix”, as used herein, refers to a protein secondary structure that consists on a right hand-helix in which the N-H group of an amino acid bonds a hydrogen to the backbone C=O group of the amino acid located three or four
25 residues earlier in a protein sequence.

- The expression “ α -helical segment” or “alpha helical segment” as used herein, refers to a motif in the secondary structure of proteins that essentially comprises one or several α -helices.

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As understood by a skilled person, beta strands as used herein refer to protein domains, and beta sheets as used herein, refer to protein secondary structures.

In a particular embodiment, the eleven beta strand domains of the polypeptide of the invention are comprised in, or constitute, a beta sheet secondary structure. In a preferred embodiment, the twelve beta strand domains of the polypeptide of the invention are
5 comprised in, or constitute, a beta barrel secondary structure.

The term “loop”, “loop region”, “loop sequence”, “omega loop”, “omega loop region” or “omega loop sequence”, as used herein, refers to a non-regular, non-repeating protein structural motif, consisting of a polypeptide chain of six or more amino acid residues with
10 any amino acid sequence. The residues that make up the beginning and the end of the loop are close together in space with no intervening regular secondary structural motifs in between. They generally connect two protein domains comprised in a secondary protein structure, such as beta strands, or directly secondary protein structures, such as alpha helices. Said loops often allow a protein domain or a protein secondary structure to
15 which they are connected by one end, to change its direction (N- to C- terminus, or C- to N- terminus) with respect to another protein domain or protein structure to which the loop is connected by its other end. They are most often located in the external surface of the protein and thus generally participate in interactions between the protein to which they belong and other molecules.

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In a particular embodiment, the polypeptide of the first aspect of the invention is a variant nitrogen G2 domain which contains an heterologous polypeptide within one or more of the loop regions. In one embodiment, the heterologous polypeptide is inserted within the loop region, i.e. the loop region conserves all the amino acids found in the cognate loop
25 domain in SEQ ID NO:62 or SEQ ID NO:63 but the heterologous polypeptide is inserted between two consecutive amino acids. In another embodiment, the heterologous polypeptide within one or more of the loop regions is found as an insertion within the loop region which replaces the partially or completely the sequence of the loop region.

30 The length of the heterologous polypeptide is not particularly limitative. Thus, the heterologous polypeptide may comprise at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at

least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 100 or more amino acids.

5 In another embodiment, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain which contains an heterologous polypeptide within one or more of the loop regions and which contains a mutation in one or more beta strands, wherein the mutation is located at position 9 in beta strand B as defined in SEQ ID NO:11 (corresponding to the amino acid at position 30 in SEQ ID NO:62 or at amino acid at
10 position 459 in the human nidogen 1 precursor as defined in the sequence provided in the Uniprot Database with accession number P14543-1 (version dated July 7, 2009) or SEQ ID NO:72), at position 1 in beta strand C as defined in SEQ ID NO:12 (corresponding to the amino acid at position 39 in SEQ ID NO:62 or at amino acid at position 468 in the human nidogen 1 precursor as defined in the sequence provided in the Uniprot Database
15 with accession number P14543-1 (version dated July 7, 2009) or SEQ ID NO:72), at position 10 in beta strand J as defined in SEQ ID NO:19 (corresponding to the amino acid position 210 in SEQ ID NO:62 or at amino acid at position 639 in the human nidogen 1 precursor as defined in the sequence provided in the Uniprot Database with accession number P14543-1 (version dated July 7, 2009) or SEQ ID NO:72) or at position 3 in
20 beta strand K as defined in SEQ ID NO:20 (corresponding to the amino acid position 221 in SEQ ID NO:62 or at amino acid at position 650 in the human nidogen 1 precursor as defined in the sequence provided in the Uniprot Database with accession number P14543-1 (version dated July 7, 2009) or SEQ ID NO:72).

25 In one embodiment, the mutation at position 9 in beta strand B as defined in SEQ ID NO:11 is a H459A mutation, the mutation at position 1 in beta strand C as defined in SEQ ID NO:12 is a R468N mutation, the mutation at position 10 in beta strand J as defined in SEQ ID NO:19 is a F639S mutation and/or the at position 3 in beta strand K as defined in SEQ ID NO:20 is a R650A.

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In one embodiment, the variant human nidogen G2 domain contains the H459A and the R468N mutations. In one embodiment, the variant human nidogen G2 domain contains

the H459A and the F639S mutations. In one embodiment, the variant human nidogen G2 domain contains the H459A and the R650A mutations. In one embodiment, the variant human nidogen G2 domain contains the R468N and the F639S mutations. In one embodiment, the variant human nidogen G2 domain contains the R468N and the R650A mutations. In one embodiment, the variant human nidogen G2 domain contains the H459A, the R468N, and the F639S mutations. In one embodiment, the variant human nidogen G2 domain contains the H459A, the R468N, and the R650A mutations. In one embodiment, the variant human nidogen G2 domain contains the R468N, the F639S and the R650A mutations. In one embodiment, the variant human nidogen G2 domain contains the H459A, the R468N, the F639S and the R650A mutations. In a preferred embodiment, the nidogen G2 domain variant has a sequence as defined in SEQ ID NO:64 or 65 (hereinafter referred to as NIDOMut2).

In another embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant as defined in any of the embodiments above and, in particular, the nidogen G2 domain variant having the H459A, the R468N, the F639S and the R650A mutations which, in addition, comprises a mutation at a position selected from the group consisting of 543 (corresponding to histidine at position 114 in SEQ ID NO:62) and position 545 (corresponding to histidine at position 116 in SEQ ID NO:62). In another embodiment, position H543 is mutated to Lys. In another embodiment, position H545 is mutated to Asn. In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain that comprises the H459A, the R468N, the F639S, the R650A and the H543K mutations. In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain that comprises the H459A, the R468N, the F639S, the R650A and the H545N mutations. In another embodiment, the nidogen G2 domain variant comprises a H543K mutation and a H545N mutation. In one embodiment, the nidogen G2 domain variant comprises or consists of SEQ ID NO: 87 (hereinafter referred to NIDOMut3), which is characterized in that it contains the H459A, the R468N, the F639S, the R650A, the H543K and the H545N mutations.

30

In another embodiment, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular, the

NIDOMut3 variant, which, in addition comprises a mutation selected from the group consisting of:

- a mutation at valine at position 449 (corresponding to position 20 in SEQ ID NO:62). Preferably, the valine at position 449 is mutated to Thr. In a preferred embodiment, the nidogen G2 domain variant has a sequence as defined in SEQ ID NO:88 (hereinafter referred to as NIDOMut3-V45T).
- a mutation at valine at position 525 (corresponding to position 96 in SEQ ID NO:62). Preferably, the valine at position 449 is mutated to Gln. In a preferred embodiment, the nidogen G2 domain variant has a sequence as defined in SEQ ID NO:89 (hereinafter referred to as NIDOMut3-V121Q).
- a mutation at the phenylalanine at position 561 (corresponding to position 142 in SEQ ID NO:62). Preferably, the phenylalanine at position 561 is mutated to Glutamic acid. In a preferred embodiment, the nidogen G2 domain variant has a sequence as defined in SEQ ID NO:90 (hereinafter referred to as NIDOMut3-F157E).
- a mutation at the valine at position 619 (corresponding to position 190 in SEQ ID NO:62). Preferably, the valine at position 619 is mutated to threonine. In a preferred embodiment, the nidogen G2 domain variant has a sequence as defined in of SEQ ID NO:91 (hereinafter referred to as NIDOMut3-V215T).

In another embodiment, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above, and in addition comprises the V449T, the V525Q, the F561E and the V619T mutations. In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain that comprises the H459A, the R468N, the F639S, the R650A, the H543K, the V449T, the V525Q, the F561E and the V619T mutations. In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain that comprises the H459A, the R468N, the F639S, the R650A, the V449T, the H545N, the V525Q, the F561E and the V619T mutations. In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain that comprises the H459A, the R468N, the F639S, the R650A, the H543K, the H545N, the V449T, the V525Q, the

F561E and the V619T mutations. In another embodiment, the variant nidogen G2 domain comprises or consists of the sequence as defined in SEQ ID NO:92 (hereinafter referred to as NIDOMut4).

- 5 In another embodiment, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular the NIDOMut4), which in addition comprises a mutation at the threonine at position 619 (corresponding to position 190 in SEQ ID NO:62). Preferably, the threonine at position 619 is mutated to valine. In another embodiment, the polypeptide of the first aspect of the
- 10 invention is a variant nidogen G2 domain as defined in any of the embodiments above in which the amino acid at position 619 (corresponding to position 190 in SEQ ID NO:62) is the same residue that appear in the human nidogen G2 domain as defined in the UniProt database under accession number P14534), i.e. a Valine. Accordingly, in one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain
- 15 variant having the H459A, the R468N, the F639S, the R650A, the H543K, the H545N, the V449T, the V525Q and the F561E mutations. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:93 (hereinafter referred to as NIDOMut4_T215V).
- 20 In another embodiment, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular, the NIDOMut4, which, in addition comprises a mutation at the cysteine at position 618 (corresponding to position 189 in SEQ ID NO:62). Preferably, the cysteine at position 618 is mutated to serine. Accordingly, in one embodiment, the polypeptide of the first
- 25 aspect of the invention is a nidogen G2 domain variant having the H459A, the R468N, the F639S, the R650A, the H543K, the H545N, the V449T, the V525Q, the V619T, the F561E and the C618S mutations. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:94 (hereinafter referred to as NIDOMut5).
- 30 In another embodiment, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular the

NIDOMut3 variant, which in addition comprises a mutation selected from the group consisting of:

- 5 - a mutation at valine at position 580 (corresponding to position 151 in SEQ ID NO:62). Preferably, the valine at position 580 is mutated to Thr. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:95 (hereinafter referred to as NIDOMut3-V176T).
- 10 - a mutation at isoleucine at position 604 (corresponding to position 175 in SEQ ID NO:62). Preferably, the isoleucine at position 604 is mutated to Thr. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:96 (hereinafter referred to as NIDOMut3-I200T).
- 15 - a mutation at the valine at position 638 (corresponding to position 209 in SEQ ID NO:62). Preferably, the valine at position 638 is mutated to tyrosine. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:97 (hereinafter referred to as NIDOMut3-V236Y).
- 20 - a mutation at the leucine at position 641 (corresponding to position 212 in SEQ ID NO:62). Preferably, the leucine at position 641 is mutated to threonine. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:98 (hereinafter referred to as NIDOMut3-L237T).
- 25 - a mutation at serine at position 469 (corresponding to position 40 in SEQ ID NO:62). Preferably, the serine at position 469 is mutated to Ile. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:99 (hereinafter referred to as NIDOMut3-S65I).
- 30 - a mutation at arginine at position 518 (corresponding to position 89 in SEQ ID NO:62). Preferably, the arginine at position 518 is mutated to Ile. In one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:100 (hereinafter referred to as NIDOMut3-R114I).

- a mutation at the cysteine at position 618 (corresponding to position 189 in SEQ ID NO:62). Preferably, the cysteine at position 618 is mutated to serine. Accordingly, in one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:101 (hereinafter referred to as NIDOMut3-C214S).

In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular the NIDOMut3 variant, which, in addition, contains mutations at the 469 (preferably a S469I mutation) and at the 518 position (preferably a R518I mutation). Accordingly, in one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the H459A, the R468N, the F639S, the R650A, the H543K, the H545N, the S469I and the R518I mutations and corresponds to the sequence of SEQ ID NO:102 (hereinafter referred to as NIDOMut3-S65I_R114I).

In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular the NIDOMut5 variant, which, in addition, contains mutations at the 469 (preferably a S469I mutation) and at the 518 position (preferably a R518I mutation). Accordingly, in one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the H459A, the R468N, the F639S, the R650A, the H543K, the H545N, the V449T, the V525Q, the V619T, the F561E, the S469I and the R518I mutations, as defined in SEQ ID NO:103 (hereinafter referred to as NIDOMut5-S65I_R114I).

In some embodiments, the polypeptide of the first aspect of the invention is a variant nidogen G2 domain as defined in any of the embodiments above and, in particular the NIDOMut5 variant, which, in addition, contains a mutation at serine at position 469 (corresponding to position 40 in SEQ ID NO:62). Preferably, the serine at position 469 is mutated to Ile. Accordingly, in one embodiment, the polypeptide of the first aspect of the invention is a nidogen G2 domain variant having the H459A, the R468N, the F639S, the R650A, the H543K, the H545N, the V449T, the V525Q, the V619T, the F561E and the S469I mutations. In one embodiment, the polypeptide of the first aspect of the invention

is a nidogen G2 domain variant having the sequence of SEQ ID NO:104 (hereinafter referred to as NIDOm5-S65I).

In some embodiments, the polypeptide of the first aspect of the invention is a variant
5 nidogen G2 domain as defined in any of the embodiments above and, in particular the
NIDOm5 variant, which, in addition, contains a mutation at arginine at position 518
(corresponding to position 89 in SEQ ID NO:62). Preferably, the arginine at position 518
is mutated to Ile Accordingly, in one embodiment, the polypeptide of the first aspect of
the invention is a nidogen G2 domain variant having the H459A, the R468N, the F639S,
10 the R650A, the H543K, the H545N, the V449T, the V525Q, the V619T, the F561E and
the R518I mutations. In one embodiment, the polypeptide of the first aspect of the
invention is a nidogen G2 domain variant having the sequence of SEQ ID NO:104
(hereinafter referred to as NIDOm5-R114I).

15 The nidogen G2 domain variants suitable for use in the present invention are summarized
in the Table below.

Nidogen G2 domain variant	Mutations with respect to the native human nidogen (numbering of positions as in the sequence with accession number P14543-1 in the Uniprot Database version dated July 7, 2009)
NIDOm2	H459A, R468N, F639S, R650A
NIDOm3	H459A, R468N, F639S, R650A, H543K, H545N
NIDOm3_V45T	H459A, R468N, F639S, R650A, H543K, H545N, V449T
NIDOm3_V121Q	H459A, R468N, F639S, R650A, H543K, H545N, V525Q
NIDOm3-F157E	H459A, R468N, F639S, R650A, H543K, H545N, F561E
NIDOm3-V215T	H459A, R468N, F639S, R650A, H543K, H545N, V619T
NIDOm4	H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, F561E, V619T
NIDOm4_T215V	H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, F561E
NIDOm5	H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, V619T, F561E, C618S
NIDOm3-V176T	H459A, R468N, F639S, R650A, H543K, H545N, V580T
NIDOm3-I200T	H459A, R468N, F639S, R650A, H543K, H545N, I604T
NIDOm3-V236Y	H459A, R468N, F639S, R650A, H543K, H545N, V640Y
NIDOm3-L237T	H459A, R468N, F639S, R650A, H543K, H545N, L641T
NIDOm3-S65I	H459A, R468N, F639S, R650A, H543K, H545N, S469I
NIDOm3-R114I	H459A, R468N, F639S, R650A, H543K, H545N, R518I
NIDOm3-C214S	H459A, R468N, F639S, R650A, H543K, H545N, C618S
NIDOm3-S65I_R114I	H459A, R468N, F639S, R650A, H543K, H545N, S469I, R518I
NIDOm5-S65I_R114I	H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, V619T, F561E, C618S, S469I, R518I
NIDOm5-S65I	H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, V619T, F561E, C618S, S469I
NIDOm5_R114I	H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, V619T, F561E, C618S, R518I

Table 1: Summary table of nidogen G2 domain variants suitable for use in the present invention. Numbering of positions in the G2 domain as in the sequence with accession number P14543-1 in the Uniprot Database version dated July 7, 2009.

- 5 The heterologous polypeptide may be inserted within the loop region, i.e. the loop region conserves all the amino acids found in the cognate loop domain in SEQ ID NO:62 or SEQ ID NO:63 but the heterologous polypeptide is inserted between two consecutive amino acids. The length of the heterologous polypeptide is not particularly limitative. Thus, the heterologous polypeptide may comprise at least two, at least 3, at least 4, at least 5, at
- 10 least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 100 or more amino acids.

In another embodiment, the heterologous polypeptide may be replacing part of the loop region, i.e. the loop region contains a deletion with respect to the sequence of the cognate loop domain in SEQ ID NO:62 or SEQ ID NO:63 and the deleted sequence is replaced by the heterologous polypeptide is inserted between two consecutive amino acids. It will
5 be understood that the length of the deletion need not be the same as the length of the heterologous peptide. Accordingly, the loop region may contain a deletion which is of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or of 100% of
10 the total length of the region. The length of the heterologous polypeptide is not particularly limitative. Thus, the heterologous polypeptide may comprise at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90,
15 at least 95, at least 100 or more amino acids.

Wherein the variant G2 nidogen domain contains more than one heterologous polypeptide, the heterologous polypeptides may be found within the same loop region or, preferably, in different loop regions. Moreover, wherein the variant G2 nidogen domain
20 contains more than one heterologous polypeptide, the heterologous polypeptides may be the same or different.

The heterologous polypeptide forming part of the variant G2 nidogen domain according to the invention specifically binds to a target peptide. More preferably, the heterologous
25 polypeptide specifically binds to a target peptide which does not show any specific binding for any other region of the variant G2 nidogen domain of the invention or of the first polypeptide of the invention.

The term “binding”, “bond”, “binds”, according to the invention refers to the interaction
30 of affinity binding molecules, or specific binding pairs, between them, as a result of non-covalent bonds, such as, but not limited to, hydrogen bonds, hydrophobic interactions, van der Waals bonds, ionic bonds or a combination of the above.

The expression “specifically binds”, “specifically binding”, “specifically recognizes”, or “specifically interacts”, when used in the present invention to refer to the binding of a polypeptide to a specific or target molecule, is understood as the capacity of said polypeptide to bind specifically to said molecule by means of complementarity between the three-dimensional structures of the two polypeptide and the target molecule, with a substantially high affinity such that the binding between said polypeptide and the target molecule preferably takes place before the binding of said polypeptide with other molecules present in its proximity, such as in a reaction mixture. The capacity of a polypeptide to specifically bind to a target molecule in a reaction mixture may be tested, for example, by assessing binding of said polypeptide under conventional conditions to the target molecule of interest as well as to a number of more or less (structurally and/or functionally) closely related molecules. Only if the polypeptide binds to the target molecule but does not or does not essentially bind to any other closely related molecules, said binding is considered specific for the target molecule. A binding between a polypeptide and a target molecule can be considered specific if the binding affinity between both has a dissociation constant (KD) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M. Methods to determine the binding between a polypeptide and a target molecule, and the KD of said binding, include methods well-known by an expert in the field. Non-limiting examples of such methods include gel-shift assays, such as electrophoretic mobility shift assay (EMSA), co-immunoprecipitation assays followed by: mass spectrometry, gas chromatography associated to mass spectrometry, liquid chromatography associated to mass spectrometry, or western blot analysis. An additional method is the oil-cushion method [see Hesselgesser et al, 1998, J.Immunol., 160:877-883].

In a particular embodiment, the binding of the polypeptide to a target molecule is considered specific, if the binding between said polypeptide and the target molecule has a dissociation constant (KD) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M. Similarly, the binding between a loop region and a

specific target molecule is considered specific, if the binding between said loop region and the target molecule has a dissociation constant (KD) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M.

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In the polypeptide of the invention, loop region AB connects beta strands A and B, loop region BC connects beta strands B and C, loop region CD connects beta strands C and D, loop region DE connects beta strands D and E, loop region EF connects beta strands E and F, loop region FG' connects beta strands F and G, loop region GH connects beta strands G and H, loop region

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HI connects beta strands H and I, loop region IJ connects beta strands I and J and loop region

JK connects beta strands J and K

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In a particular embodiment, beta strand A is connected to beta strand B by loop region AB in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand B is connected to beta strand C by loop region BC in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand C is connected to beta strand D by loop region CD in the polypeptide of the first

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aspect of the invention. In another particular embodiment, beta strand D is connected to beta strand E by loop region DE in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand E is connected to beta strand F by loop region EF in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand F is connected to beta strand G by loop region FG in the

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polypeptide of the first aspect of the invention. In another particular embodiment, beta strand G is connected to beta strand H by loop region GH in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand H is connected to beta strand I by loop region HI in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand I is connected to beta strand J by loop region

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IJ in the polypeptide of the first aspect of the invention. In another particular embodiment, beta strand J is connected to beta strand K by loop region JK in the polypeptide of the first aspect of the invention.

The expression “cognate loop region in SEQ ID NO:62” as used herein, refers to a loop region as it appears in SEQ ID NO:62, which is the wild-type nidogen G2 domain of human origin. As it will be understood by a skilled person, each loop region in the polypeptide of the first aspect of the invention has its cognate loop region in SEQ ID NO:62.

As it will be understood by a skilled person, two amino acid sequences are considered to encode the same protein domains or secondary protein structures, if they show a certain degree of sequence identity and they encode the same type of protein domain or of protein secondary structure, i.e. they both form a beta strand, a loop region, an alpha helix, an α -helical segment, a beta sheet, or beta barrel. In a particular embodiment, the amino acid sequence encoding two protein domains or secondary protein structures that are considered to be the same, show a degree of sequence identity of at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.5% at least 99.75%, at least 99.8%, at least 99.9%, at least 99.95%, at least 99.975%, at least 99.99%. The degree of identity between two amino acid sequences can be determined by conventional methods, for example, by means of standard sequence alignment algorithms known in the state of the art, such as, for example BLAST [Altschul S.F. *et al.*, J. Mol. Biol., 1990 Oct 5; 215(3):403-10]. Methods to determine whether an amino acid sequence within a protein forms, or maintains, a specific domain or protein secondary structure are well known by a skilled person, and include methods to determine the secondary structure of a protein such as the bioinformatics tool DSSPcont (Carter, Andersen, & Rost, 2003) and STRIDE (Heinig & Frishman, 2004), once the atomic coordinates of the protein has been obtained following methods well-known by a skilled person, such as X-ray crystallography or protein NMR.

The cognate loop region in SEQ ID NO:62 of loop regions AB comprises, essentially comprises or consists of SEQ ID NO:1. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region BC comprises, essentially comprises or consists of SEQ ID NO:2. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region CD comprises, essentially comprises or consists of SEQ

ID NO. 3. In another particular embodiment, the cognate loop region in SEQ ID NO. 62 of loop region DE comprises, essentially comprises or consists of SEQ ID NO:4. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region EF comprises, essentially comprises or consists of SEQ ID NO:5. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region FG comprises, essentially comprises or consists of SEQ ID NO:6. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region GH comprises, essentially comprises or consists of amino acids 149 to 150 in SEQ ID NO:62. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region HI comprises, essentially comprises or consists of SEQ ID NO:7. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region IJ comprises, essentially comprises or consists of SEQ ID NO:8. In another particular embodiment, the cognate loop region in SEQ ID NO:62 of loop region JK comprises, essentially comprises or consists of SEQ ID NO:9.

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In particular embodiment, a loop region of the polypeptide of the first aspect of the invention is a variant of its cognate loop region in SEQ ID NO:62.

The expression “a loop region variant”, as used herein, refers to a loop region of the polypeptide of the first aspect that comprises in its sequence a modification, insertion and/or deletion of one or more amino acids with respect to the sequence of its cognate loop region in SEQ ID NO:62. In a particular embodiment said loop region from the polypeptide of the first aspect is elected from the group of loop regions consisting of AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK.

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Thus, in a particular embodiment, the variant of at least one of the loop regions of the polypeptide of the first aspect of the invention results from the mutation by deletion, substitution or addition of at least one amino acid in the sequence of its cognate loop region in SEQ ID: 62.

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In a certain embodiment, the loop region variant of the polypeptide of the first aspect has a degree of sequence identity with the sequence of its cognate loop region in SEQ ID

NO:62 of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%. Methods to determine the degree of sequence identity between two sequences have been provided
5 above.

In a particular embodiment, loop region AB is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular embodiment, loop region BC is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular
10 embodiment, loop region CD is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular
embodiment, loop region DE is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular embodiment, loop region EF is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular embodiment, loop region
15 FG is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular embodiment, loop region GH is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular embodiment, loop region HI is a loop region variant of its cognate loop region in SEQ ID NO:62. In another particular embodiment, loop region IJ is a loop region variant of its cognate loop region in SEQ ID NO:62. In
20 another particular embodiment, loop region JK is a loop region variant of its cognate loop region in SEQ ID NO:62.

In a particular embodiment, the sequence of the AB loop region in the variant nitrogen G2 domain according to the invention is a variant of SEQ ID NO:1, whereas the sequence
25 of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8 or all of the BC, CD, DE, EF, FG', GH, HI, IJ and JK loop regions of the polypeptide of the first aspect of the invention are identical to the sequences of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region AB is a variant of SEQ ID NO:1, and the sequence of at least 1, at least 2, at least 3, at least 4, at
30 least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region AB is a variant of

SEQ ID NO:1, and the rest of the sequence of the polypeptide of the first aspect of the invention is identical to the remaining of the sequence in SEQ ID NO:62.

In a certain embodiment, the loop region AB variant of SEQ ID NO:1 has at least 30%,
5 at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:1.

In another particular embodiment, the sequence of loop region AB of the polypeptide of
10 the first aspect of the invention is SEQ ID NO:1.

In a particular embodiment, the sequence of loop region BC is a variant of SEQ ID NO:2.
In
another particular embodiment, the sequence of loop region BC is a variant of SEQ ID
15 NO:2, and the sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8 or all of the AB, CD, DE, EF, FG, GH, HI, IJ and JK loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of loop region BC is a variant of SEQ ID NO:2, and the sequence of at least 1, at least 2,
20 at least 3, at least 4, at least 5, at least 6, at least 7, at least 8 or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region BC is a variant of SEQ ID NO:2, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

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In a certain embodiment, the loop region BC variant of SEQ ID NO:2 has at least 30%,
at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:2.

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In another particular embodiment, the sequence of loop region BC of the polypeptide of the first aspect of the invention is SEQ ID NO:2.

- In a particular embodiment, the sequence of loop region CD is a variant of SEQ ID NO:3. In another particular embodiment, the sequence of loop region CD is a variant of SEQ ID NO:3, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the AB, BC, DE, EF, FG, GH, HI, IJ and JK, loop regions of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of loop region CD is a variant of SEQ ID NO:3, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention have the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region CD is a variant of SEQ ID NO:3, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.
- 15 In a certain embodiment, the loop region CD variant of SEQ ID NO:3 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:3.
- 20 In another particular embodiment, the sequence of loop region CD of the polypeptide of the first aspect of the invention is SEQ ID NO:3.

- In a particular embodiment, the sequence of the loop region CD is a variant of the cognate domain in SEQ ID NO:62 in which sequence is modified with respect to the cognate region in those regions of the loop which do not show alpha-helical structure. As shown by Hopf et al. (supra.), the CD loop region in the native G2 domain contains three regions with alpha helical structures, which are known as $\alpha 1$, $\alpha 2$ and $\alpha 3$. These regions are defined as SEQ ID NO:21, 22 and 23, respectively. These regions separate the CD loop region into 4 loop regions which correspond, respectively, to the region between the end of the beta strand C and $\alpha 1$ (hereinafter C α region) between $\alpha 1$ and $\alpha 2$, between $\alpha 2$ and $\alpha 3$ and between $\alpha 3$ and the beginning of beta strand D (hereinafter αD region). In one embodiment, the C α region comprises, essentially comprises or consists of SEQ ID
- 25
- 30

NO:24. In one embodiment, the α D region comprises, essentially comprises or consists of amino acids GG. In one embodiment, the sequence of the loop region is a variant of the cognate region SEQ ID NO:3 in which the sequences of SEQ ID NO:21, 22 and 23 are preserved with respect to the cognate region. In another embodiment, the sequence of
5 the loop region is a variant of the cognate region SEQ ID NO:3 contains one or more mutations in the C α region. In another embodiment, the sequence of the loop region is a variant of the cognate region SEQ ID NO:3 contains one or more mutations in the α D region. In another embodiment, the sequence of the loop region is a variant of the cognate region SEQ ID NO:3 contains one or more mutations in the C α region and in the α D
10 region.

In a certain embodiment, the C α region in the polypeptide of the invention has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least
15 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:24.

In another particular embodiment, the sequence of loop region C α of the polypeptide of the first aspect of the invention is SEQ ID NO:24.

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In a certain embodiment, the loop region α D variant has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with the sequence consisting of
25 amino acids GG.

In a particular embodiment, the sequence of loop region DE is a variant of SEQ ID NO:4. In another particular embodiment, the sequence of loop region DE is a variant of SEQ ID NO:4, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at
30 least 7, at least 8, at least 9, or all of the AB, BC, CD, EF, FG, GH, HI, IJ and JK loop regions of the polypeptide of first aspect of the invention contains the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment,

the sequence of loop region DE is a variant of SEQ ID NO:4, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the

5 sequence of loop region DE is a variant of SEQ ID NO:4, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the loop region DE variant of SEQ ID NO:4 has at least 30%,

10 at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:4.

In another particular embodiment, the sequence of loop region DE of the polypeptide of

15 the first aspect of the invention is SEQ ID NO:4.

In a particular embodiment, the sequence of loop region EF is a variant of SEQ ID NO:5. In another particular embodiment, the sequence of loop region EF is a variant of SEQ ID NO:5, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at

20 least 7, at least 8, at least 9, or all of the AB, BC, CD, DE, FG, GH, HI, IJ and JK loop regions of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of loop region EF is a variant of SEQ ID NO:5, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the

25 polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region EF is a variant of SEQ ID NO:5, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

30 In a certain embodiment, the loop region EF variant of SEQ ID NO:5 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least

65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:5.

In another particular embodiment, the sequence of loop region EF of the polypeptide of
5 the first aspect of the invention is SEQ ID NO:5.

In a particular embodiment, the sequence of loop region FG is a variant of SEQ ID NO:6.
In another particular embodiment, the sequence of loop region FG is a variant of SEQ ID
NO:6, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at
10 least 7, at least 8, at least 9, or all of the AB, BC, CD, DE, EF, GH, HI, IJ and JK is the
sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another
particular embodiment, sequence of loop region FG is a variant of SEQ ID NO:6, and
sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at
least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the
15 invention is the sequence of their cognate loop region in SEQ ID NO:62. In another
particular embodiment, the sequence of loop region FG is a variant of SEQ ID NO:6,
and the rest of the sequence of the polypeptide of the first aspect of the invention is as the
rest of the sequence in SEQ ID NO:62.

20 In a certain embodiment, the loop region FG variant of SEQ ID NO:6 has at least 30%,
at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least
65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at
least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:6.

25 In another particular embodiment, the sequence of loop region FG of the polypeptide of
the first aspect of the invention is SEQ ID NO:6.

In a particular embodiment, the sequence of loop region GH is a variant of the sequence
TS, corresponding to amino acids 149 to 150 in SEQ ID NO:62 (or amino acids 147 to
30 148 in SEQ ID NO:63). In another particular embodiment, the sequence of loop region
GH is a variant of the sequence corresponding to amino acids 149 to 150 in SEQ ID
NO:62, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at

least 7, at least 8, at least 9, or all of the AB, BC, CD, DE, EF, FG, HI, IJ and JK of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of loop region GH is a variant of the sequence corresponding to amino acids 149 to 150 in
5 SEQ ID NO:62, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region GH is a variant of the sequence corresponding to amino acids 149 to 150 in SEQ ID NO:62, and the rest of the
10 sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the loop region GH variant of the sequence corresponding to amino acids 149 to 150 in SEQ ID NO:62 has at least 30%, at least 35%, at least 40%, at
15 least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with the sequence corresponding to amino acids 149 to 150 in SEQ ID NO:62.

20 In another particular embodiment, the sequence of loop region GH of the polypeptide of the first aspect of the invention is the sequence corresponding to amino acids 149 to 150 in SEQ ID NO:62.

In a particular embodiment, the sequence of loop region HI is a variant of SEQ ID NO:7.
25 In another particular embodiment, the sequence of loop region HI is a variant of SEQ ID NO:7, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the AB, BC, CD, DE, EF, FG, GH, IJ and JK of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of
30 loop region HI is a variant of SEQ ID NO:7, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop

region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region HI is a variant of SEQ ID NO:7, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

- 5 In a certain embodiment, the loop region HI variant of SEQ ID NO:7 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:7.
- 10 In another particular embodiment, the sequence of loop region HI of the polypeptide of the first aspect of the invention is SEQ ID NO:7.

- In a particular embodiment, the sequence of loop region IJ is a variant of SEQ ID NO:8. In another particular embodiment, the sequence of loop region IJ is a variant of SEQ ID
- 15 NO:8, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the AB, BC, CD, DE, EF, FG, GH, HI, and JK loop regions of polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO: 62 indicated above. In another particular embodiment, the sequence of loop region IJ is a variant of SEQ ID NO:8, and sequence of at least 1, at
- 20 least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region IJ is a variant of SEQ ID NO:8, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ
- 25 ID NO:62.

- In a certain embodiment, the loop region IJ variant of SEQ ID NO:8 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at
- 30 least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:8.

In another particular embodiment, the sequence of loop region IJ of the polypeptide of the first aspect of the invention is SEQ ID NO:8.

In a particular embodiment, the sequence of loop region JK is a variant of SEQ ID NO:9.

- 5 In another particular embodiment, the sequence of loop region JK is a variant of SEQ ID NO:9, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the AB, BC, CD, DE, EF, FG, GH, HI, and IJ of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of
- 10 loop region JK is a variant of SEQ ID NO:9, and sequence of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the loop regions of the polypeptide of the first aspect of the invention is the sequence of their cognate loop region in SEQ ID NO:62. In another particular embodiment, the sequence of loop region JK is a variant of SEQ ID NO:9, and the rest of the sequence of the polypeptide of the
- 15 first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

- In a certain embodiment, the loop region JK variant of SEQ ID NO:9 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at
- 20 least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:10.

In another particular embodiment, the sequence of loop region JK of the polypeptide of the first aspect of the invention is SEQ ID NO:9.

- 25 In a particular embodiment, at least one of the sequences located upstream, immediately upstream, downstream or immediately downstream a variant loop region in the polypeptide of the first aspect of the invention comprises a modification, insertion and/or deletion of one or more amino acids with respect to the sequence (referred to as its reference sequence) in SEQ ID NO:62, placed in the same location with respect to the
- 30 cognate loop region of said loop region variant. However, said at least one sequence encode the same protein domain or secondary structures, as defined above, in the polypeptide of the first aspect of the invention as its reference sequence in the polypeptide

with SEQ ID NO:62. Methods to determine if two amino acid sequences form the same protein domain or secondary structure, such as a loop region, an α -helical segment or an α -helix, are those provided above to determine if two amino acid sequences form the same protein structure.

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In a particular embodiment, the at least one sequence comprising a modification, insertion and/or deletion of one or more amino acids with respect to its reference sequence, has at least at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least
10 96%, at least 97%, at least 98%, at least 99%, with its reference sequence in SEQ ID NO:62. Methods to determine the degree of sequence identity between two amino acid sequences have been provided above.

The expression “cognate beta strand domain in SEQ ID NO:62” as used herein, refers to
15 a beta strand domain in SEQ ID NO:62 located between amino acid sequences in the protein with SEQ ID NO:62 encoding the same loop regions or protein secondary structures as the sequences between which said beta strand domain variant is located in the polypeptide of the first aspect of the invention. As it will be understood by a skilled person, each beta strand domain in the first polypeptide of the invention has its cognate
20 beta strand domain in SEQ ID NO:62.

Each of the beta strands domains in the variant nidogen G2 domain may be identical to the cognate beta strand in SEQ ID NO:62 or may differ in one or more amino acids so that the overall sequence identity between the beta strand in the variant nidogen G2
25 domain and the cognate beta strand domain in SEQ ID NO:62 may be of at least 50%. In preferred embodiments, the sequence identity between the beta strand in the variant nidogen G2 domain and the cognate beta strand domain in SEQ ID NO:62 is of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%.

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In a particular embodiment, the beta strand domain A is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand

domain B is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain C is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain D is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain E is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain F is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain G is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain H is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain I is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain J is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, beta strand domain K is a beta strand domain variant of its cognate beta strand domain in SEQ ID NO:62.

In a particular embodiment, a beta strand domain of the polypeptide of the first aspect of the invention is a variant of its cognate beta strand in SEQ ID NO:62.

The expression “beta strand domain variant”, as used herein, refers to a beta strand domain from the polypeptide of the first aspect of the invention that comprises in its sequence a modification, insertion and/or deletion of one or more amino acids with respect to the sequence of its cognate beta strand domain in SEQ ID NO:62. In a particular embodiment, said beta strand domain from the polypeptide of the first aspect is selected from the group consisting of A, B, C, D, E, F, G, H, I, J or K,

In a certain embodiment, the beta strand domain variant of the polypeptide of the first aspect has a degree of sequence identity with the sequence of its cognate beta strand domain in SEQ ID NO:62 of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%. Methods to determine the degree of sequence identity have been provided above.

In a particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain A has SEQ ID NO:10. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain B has SEQ ID NO:11. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain C has SEQ ID NO:12. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain D has SEQ ID NO:13. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain E has SEQ ID NO:14. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain F has SEQ ID NO:15. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain G has SEQ ID NO:16. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain H has SEQ ID NO:17. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain I has SEQ ID NO:18. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain J has SEQ ID NO:19. In another particular embodiment, the cognate beta strand domain in SEQ ID NO:62 for beta strand domain K has SEQ ID NO:20.

Accordingly, in a particular embodiment, the sequence of the beta strand A is a variant of SEQ ID NO:10. In another particular embodiment, the sequence of beta strand domain A is a variant of SEQ ID NO:10, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the B-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand A is a variant of SEQ ID NO:10, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand A is a variant of SEQ ID NO:10, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain A variant of SEQ ID NO:10 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:11.

In another particular embodiment, the sequence of beta strand domain A of the polypeptide of the first aspect of the invention is SEQ ID NO:10.

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In a particular embodiment, the sequence of the beta strand B is a variant of SEQ ID NO:11. In another particular embodiment, the sequence of beta strand domain B is a variant of SEQ ID NO:11, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A, C-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand B is a variant of SEQ ID NO:11, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand B is a variant of SEQ ID NO:11, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

25 In a certain embodiment, the beta strand domain B variant of SEQ ID NO:11 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:12.

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In another particular embodiment, the sequence of beta strand domain B of the polypeptide of the first aspect of the invention is SEQ ID NO:11.

In a particular embodiment, the sequence of the beta strand C is a variant of SEQ ID NO:12. In another particular embodiment, the sequence of beta strand domain C is a variant of SEQ ID NO:12, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A, B, D-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand C is a variant of SEQ ID NO:12, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand C is a variant of SEQ ID NO:12, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

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In a certain embodiment, the beta strand domain C variant of SEQ ID NO:12 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:12.

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In another particular embodiment, the sequence of beta strand domain C of the polypeptide of the first aspect of the invention is SEQ ID NO:12.

25 In a particular embodiment, the sequence of the beta strand D is a variant of SEQ ID NO:13. In another particular embodiment, the sequence of beta strand domain D is a variant of SEQ ID NO:13, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-C, E-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand D is a variant of SEQ ID NO:13, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand

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domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand D is a variant of SEQ ID NO:13, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain D variant of SEQ ID NO:13 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:13.

In another particular embodiment, the sequence of beta strand domain D of the polypeptide of the first aspect of the invention is SEQ ID NO:13.

In a particular embodiment, the sequence of the beta strand E is a variant of SEQ ID NO:14. In another particular embodiment, the sequence of beta strand domain E is a variant of SEQ ID NO:14, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-D, F-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand E is a variant of SEQ ID NO:14, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand E is a variant of SEQ ID NO:14, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain E variant of SEQ ID NO:14 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least

95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:14.

In another particular embodiment, the sequence of beta strand domain E of the polypeptide of the first aspect of the invention is SEQ ID NO:14.

In a particular embodiment, the sequence of the beta strand F is a variant of SEQ ID NO:15. In another particular embodiment, the sequence of beta strand domain F is a variant of SEQ ID NO:15, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-E, G-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand F is a variant of SEQ ID NO:15, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand F is a variant of SEQ ID NO:15, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

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In a certain embodiment, the beta strand domain F variant of SEQ ID NO:15 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:15.

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In another particular embodiment, the sequence of beta strand domain F of the polypeptide of the first aspect of the invention is SEQ ID NO:15.

In a particular embodiment, the sequence of the beta strand G is a variant of SEQ ID NO:16. In another particular embodiment, the sequence of beta strand domain G is a variant of SEQ ID NO:16, and at least 1, at least 2, at least 3, at least 4, at least 5, at least

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6, at least 7, at least 8, at least 9, or all of the A-F and H-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand G is a variant of SEQ ID NO:16, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand G is a variant of SEQ ID NO:16, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain G variant of SEQ ID NO:16 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:16.

In another particular embodiment, the sequence of beta strand domain G of the polypeptide of the first aspect of the invention is SEQ ID NO:16.

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In a particular embodiment, the sequence of the beta strand H is a variant of SEQ ID NO:17. In another particular embodiment, the sequence of beta strand domain H is a variant of SEQ ID NO:18, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-G, I-K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand H is a variant of SEQ ID NO:17, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand H is a variant of SEQ ID NO:17, and the rest of the sequence of

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the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain H variant of SEQ ID NO:17 has at least
5 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:17.

10 In another particular embodiment, the sequence of beta strand domain H of the polypeptide of the first aspect of the invention is SEQ ID NO:17

In a particular embodiment, the sequence of the beta strand I is a variant of SEQ ID NO:18. In another particular embodiment, the sequence of beta strand domain I is a
15 variant of SEQ ID NO:18, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-H, J, K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand I is a variant of SEQ ID NO:18, and at least 1, at least 2, at least
20 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand I is a variant of SEQ ID NO:18, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ
25 ID NO:62.

In a certain embodiment, the beta strand domain I variant of SEQ ID NO:18 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least
30 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:19.

In another particular embodiment, the sequence of beta strand domain I of the polypeptide of the first aspect of the invention is SEQ ID NO:18.

In a particular embodiment, the sequence of the beta strand J is a variant of SEQ ID NO:19. In another particular embodiment, the sequence of beta strand domain J is a variant of SEQ ID NO:19, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-I, K beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand J is a variant of SEQ ID NO:19, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand J is a variant of SEQ ID NO:19, and the rest of the sequence of the polypeptide of the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain J variant of SEQ ID NO:19 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:19.

In another particular embodiment, the sequence of beta strand domain J of the polypeptide of the first aspect of the invention is SEQ ID NO:19.

In a particular embodiment, the sequence of the beta strand K is a variant of SEQ ID NO:20. In another particular embodiment, the sequence of beta strand domain K is a variant of SEQ ID NO:20, and at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or all of the A-J beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62 indicated above. In another particular embodiment, the sequence of beta strand K is a variant of SEQ ID NO:20, and at least 1, at least 2, at least 3, at least 4,

at least 5, at least 6, at least 7, at least 8, at least 9, or all of the beta strand domains of the polypeptide of the first aspect of the invention have the sequence of their cognate beta strand domain in SEQ ID NO:62. In another particular embodiment, the sequence of beta strand K is a variant of SEQ ID NO:20, and the rest of the sequence of the polypeptide of
5 the first aspect of the invention is as the rest of the sequence in SEQ ID NO:62.

In a certain embodiment, the beta strand domain K variant of SEQ ID NO:20 has at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least
10 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:20.

In another particular embodiment, the sequence of beta strand domain K of the polypeptide of the first aspect of the invention is SEQ ID NO:20.

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In one embodiment, the polypeptide according to first aspect of the present invention contains a variant of at least one of the loop regions with respect with the cognate loop region in SEQ ID NO:62 which results from the mutation by deletion, substitution or addition of at least one amino acid with respect to the sequence of the cognate loop region.

20 In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region AB with respect to the cognate AB loop region SEQ ID NO:1. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region BC with respect to the cognate BC loop region SEQ ID NO:2. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region CD with respect to the cognate CD loop
25 region SEQ ID NO:3. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region DE with respect to the cognate DE loop region SEQ ID NO:4. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region EF with respect to the cognate EF loop region SEQ ID NO:5. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region FG
30 with respect to the cognate FG loop region SEQ ID NO:6. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region GH with respect to the cognate GH loop region corresponding to amino acids 149 to 150 in SEQ ID NO:62. In

one embodiment, the variant nidogen G2 domain contains a mutation in the loop region HI with respect to the cognate HI loop region SEQ ID NO:7. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region IJ with respect to the cognate IJ loop region SEQ ID NO:8. In one embodiment, the variant nidogen G2 domain contains a mutation in the loop region JK with respect to the cognate JK loop region SEQ ID NO:9.

The α -helical segment C α D of the variant nidogen G2 domain may be identical to the cognate α -helical segment in SEQ ID NO:62 or may differ in one or more amino acids so that the overall sequence identity between the α -helical segment C α D in the variant nidogen G2 domain and the cognate α -helical segment in SEQ ID NO:62 with SEQ ID NO:26 may be of at least 50%.

In preferred embodiment, the sequence identity between the α -helical segment C α D in the variant nidogen G2 domain and the cognate α -helical segment in SEQ ID NO:62 with SEQ ID NO:24 is of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%.

Methods to determine the degree of sequence identity have been provided above. In a particular embodiment, said α -helical segment C α D that differs in one or more amino acids as indicated just above is referred to as an α -helical segment variant of the cognate α -helical segment in SEQ ID NO:62.

In a particular embodiment, the polypeptide of the first aspect has a beta barrel structure. In a preferred embodiment, it has the beta barrel structure of the beta barrel domain of the G2 domain of nidogen-1. In a particular embodiment, it has the beta barrel structure of the sequence with SEQ ID NO:62.

The beta barrel domain of G2 domain of nidogen-1, and the beta barrel structure of the sequence with SEQ ID NO:62, consists of an 11-stranded beta-barrel. The beta strands of said beta barrel are herein referred to as I, II, III, IV, V, VI, VII, VIII, IX, X, XI. They

correspond to the cognate beta strands in SEQ ID NO:62 of the beta strands A-K of the polypeptide of the first aspect. The interior of the beta barrel is traversed by the hydrophobic, predominantly alpha helical segment connecting strands III and IV. The N-terminal half of the barrel comprises two beta-meanders (strands I-III and IV-VI) linked
5 by the buried alpha-helical segment. The polypeptide chain then crosses the bottom of the barrel and forms a five-stranded Greek key motif in the C-terminal half of the domain.

The expression “beta-meander”, as used herein, refers to 2 or more consecutive antiparallel β -strands linked together by hairpin loops. The term “hairpin loop” as used
10 herein, refers to two antiparallel strands linked by a short loop of two to five residues, of which one is frequently a glycine or a proline, both of which can assume the dihedral-angle conformations required for a tight turn or a β -bulge loop.

The expression “Greek key”, as used herein, refers to a secondary protein structure
15 consisting of four adjacent antiparallel strands and their linking loops. In this structure, three antiparallel strands are connected by hairpins, while the fourth is adjacent to the first and linked to the third by a longer loop.

Thus, in a particular embodiment, beta strands A-C as well as beta strands D-F of the
20 variant of domain G2 form a beta meander. In another particular embodiment, said beta meanders are connected by the α -helical segment C α D of the variant of domain G2. In another particular embodiment, beta strands G-K of the variant of domain G2 form a five-stranded Greek key motif. In another particular embodiment, the beta strands of the variant of domain G2 are arranged in an antiparallel fashion, except beta strands A and F.

25

Methods to determine the secondary structure of a polypeptide, or whether two amino acid sequences encode the same domain or secondary protein structure have been provided above.

30 *II - Polypeptide display library*

In a second aspect, the invention relates to a polypeptide display library comprising a plurality of polypeptides according to the first aspect of the invention, wherein the plurality of polypeptides is formed by polypeptides that differ in the sequence of one or more loop regions.

5

The expression “polypeptide display library” as used herein, refers to a library, or pool, of polypeptides, comprising a plurality of polypeptides with different amino acid sequences. Each polypeptide of the library is as defined in the first aspect of the invention and differs from at least another polypeptide of the library in the sequence of one or more
10 loop regions.

The expression “polypeptides that differ in the sequence of one or more loop regions”, as used herein, refers to the fact that each polypeptide of the library shows at least one difference in its amino acid sequence with respect to the amino acid sequence of at least
15 another polypeptide of the library, wherein said at least one difference is comprised in the amino acid sequence of a loop region of the polypeptides. Thus, in a particular embodiment, the polypeptides of the library are polypeptides of the first aspect comprising at least one loop region variant, as defined in the first aspect, which is different to the corresponding loop region in another polypeptide of the library. In a particular
20 embodiment, said loop region variant is selected from the group consisting of A, B, C, D, E, F, G, H, I, J or K and is as described in the definition and embodiments of “loop region variant” of the first aspect of the invention.

The expression “show at least one difference in its amino acid sequence with respect to
25 the amino acid sequence of at least another polypeptide of the library, wherein said sequence is comprised in the amino acid sequence of a loop region of the polypeptides”, as used herein, refers to the fact that a loop region variant, as defined above, of a first polypeptide of the library comprises at least one insertion, deletion, or modification of at least one amino acid in its sequence with respect to the amino acid sequence of the
30 corresponding loop region in a second polypeptide of the library. As understood by a skilled person, when the loop region variant in the first polypeptide is loop region AB, the corresponding loop region in the second polypeptide is also loop region AB in the

second polypeptide. When the loop region variant in the first polypeptide is loop region BC, the corresponding loop region in the second polypeptide is also loop region BC in the second polypeptide. When the loop region variant in the first polypeptide is loop region CD, the corresponding loop region in the second polypeptide is also loop region CD in the second polypeptide. When the loop region variant in the first polypeptide is loop region DE, the corresponding loop region in the second polypeptide is also loop region DE in the second polypeptide. When the loop region variant in the first polypeptide is loop region EF, the corresponding loop region in the second polypeptide is also loop region EF in the second polypeptide. When the loop region variant in the first polypeptide is loop region FG, the corresponding loop region in the second polypeptide is also loop region FG in the second polypeptide. When the loop region variant in the first polypeptide is loop region GH, the corresponding loop region in the second polypeptide is also loop region GH in the second polypeptide. When the loop region variant in the first polypeptide is loop region HI, the corresponding loop region in the second polypeptide is also loop region HI in the second polypeptide. When the loop region variant in the first polypeptide is loop region IJ, the corresponding loop region in the second polypeptide is also loop region IJ in the second polypeptide. When the loop region variant in the first polypeptide is loop region JK, the corresponding loop region in the second polypeptide is also loop region JK in the second polypeptide

20

In a particular embodiment, said loop region variant of the first polypeptide of the library shows a degree of sequence identity with the corresponding loop region of the said second polypeptide of the library of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%. In another particular embodiment, the whole sequence of said first polypeptide shows a degree of sequence identity with the whole sequence of the second polypeptide of at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.75%, at least 99.9%, at least 99.95%, at least 99.975%, at least 99.98%, at least 99.99%, at least 99.999%. Methods to determine the percentage of sequence identity have been provided in the first aspect of the invention.

30

In a particular embodiment, the first polypeptide of the library shows a difference in the amino acid sequence of at least 2 loop regions, at least 3 loop regions, at least 4 loop regions, at least 5 loop regions, at least 6 loop region, at least 7 loop regions, at least 8
5 loop regions, at least 9 loop regions, at least 10 loop regions, at least 11 loop regions with respect to the corresponding loop region in at least another polypeptide of the library, referred to as the second polypeptide of the library. The term corresponding loop region in the second polypeptide is as indicated above for each loop region of the first polypeptide. The difference in the amino acid sequence is as defined above as well.
10 Additionally, each of said amino acid sequences show the degree of sequence identity with that of the corresponding loop region in the second polypeptide indicated above as well.

In a particular embodiment, the polypeptides of the library are capable of specifically
15 binding other molecules, preferably peptides or proteins, present in their proximity, for instance within a sample, by means of at least one of their loop regions. In a preferred embodiment, the polypeptide of the library showing a difference in the amino acid sequence in one or more of their corresponding loop regions, specifically bind different molecules, preferably peptides or proteins, present in their proximity, for instance within
20 a sample. As understood by a skilled person, said difference in their binding capacity can simply consist in that one polypeptide is capable to specifically bind one molecule, preferably a peptide or protein of a sample, and another polypeptide of the library with a difference in one or more loop regions is not. Alternatively, it can consist in that one polypeptide is capable of specifically binding one or more molecules, preferably peptides
25 or proteins, while another polypeptide of the library with a difference in one or more loop regions is not capable of specifically binding said one or more molecules, but is capable of binding other one or more molecules, preferably peptides present in a sample. In a particular embodiment, the first polypeptide of the library is capable of specifically binding a target molecule of interest, preferably a peptide or protein of interest, by means
30 of at least one loop region variant showing a difference in its sequence with respect to the corresponding loop region in the second polypeptide of the library, while the second polypeptide of the library is not capable to specifically bind to said target molecule.

In another particular embodiment, a loop region variant of a first polypeptide of the library specifically binds a target peptide, whereas its cognate loop region in SEQ ID NO:62 is not capable of specifically binding to said target peptide.

5

In another particular embodiment, the polypeptides of the polypeptide library having the same loop region variant in one of the loop regions selected from AB, BC, CD, DE, EF, FG, GH, HI, IJ, JK, specifically bind a specific target peptide through said loop region variant, whereas the polypeptides of the library not having said specific loop region variant do not.

10

The terms “bind”, “binding”, “specifically binds”, “specifically binding”, “specifically interacts”, have been defined in the first aspect of the invention. Methods to determine the binding between a polypeptide and a target molecule, as well as the K of such binding have also been provided in said definition.

15

In a particular embodiment, the binding of a polypeptide of the library to a target molecule is considered specific, if the binding between said polypeptide and the target molecule has a dissociation constant (K_d) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M. Similarly, the binding between a loop region, preferably a loop region variant, and a specific target molecule is considered specific, if the binding between said loop region and the target molecule has a dissociation constant (KD) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M.

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25

In a particular embodiment, each polypeptide of the library of the second aspect of the invention, as a phenotype, is linked directly or indirectly to a nucleic acid as a genotype corresponding to said phenotype.

30

The term “genotype”, when used in the present invention, refers to the nucleic acid molecules encoding, or comprising a sequence encoding, one or several peptides, polypeptides or proteins. Said group of peptides, polypeptides or proteins conform the phenotype corresponding to said genotype. As understood by a skilled person, the
5 genotype can be formed by a single nucleic acid molecule, encoding a single peptide, polypeptide or protein. In this case, the phenotype is conformed by said peptide, polypeptide or protein. Said nucleic acid can be any of the nucleic acids specified in the definition of nucleic acid below.

10 In a particular embodiment, the genotype is formed by a single nucleic acid molecule encoding a single polypeptide of the polypeptide display library. In another particular embodiment, the genotype is formed by several nucleic acid molecules encoding the same polypeptide of the polypeptide display library. In another particular embodiment, said nucleic acid molecules have the same nucleic acid sequence.

15

The definition of the terms peptide, polypeptide and protein has been provided in the first aspect of the invention.

The term “nucleic acid”, “nucleotide sequence”, or “polynucleotide” is used
20 interchangeably in the present invention to refer to the polymeric form of the ribonucleoside phosphate ester (adenosine, guanosine, uridine or cytidine; “RNA molecules”) or deoxy ribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine or deoxycytidine; “DNA molecules”) or any phosphoester analog thereof such as phosphorothioates and thioesters, in a single stranded or double stranded form.
25 Thus, the term includes single stranded DNA or RNA molecules. It also includes double stranded molecules formed by DNA-DNA, DNA-RNA and RNA-RNA strands. The term “nucleic acid sequence” and, in particular, the DNA or RNA molecule, refers only to the primary or secondary structure of the molecule and does not limit any particular type of tertiary structure. Thus, this term encompasses double stranded DNA, as comprised in
30 linear or circular DNA molecules, supercoiled DNA plasmids and chromosomes. IN a particular embodiment, the nucleic acid is a DNA molecule. In another particular embodiment, it is an RNA molecule.

The term “phenotype”, when used in the present invention, refers to a peptide, polypeptide, or protein, or group of peptide, polypeptides or proteins. The nucleic acid/s encoding, or comprising the sequence encoding it/them is the genotype corresponding to
5 said phenotype. As understood by a skilled person, in the context of the present invention, a phenotype can consist on a single peptide, polypeptide or protein. The genotype associated to said phenotype is the nucleic acid molecule, or a group of nucleic acid molecules, encoding it.

10 In a particular embodiment, the phenotype is a polypeptide of the polypeptide library as defined in the first aspect of the invention and above in this aspect of the invention. In a particular embodiment, it is the first polypeptide of the library as defined above.

The expression “a phenotype linked directly or indirectly to a nucleic acid as a genotype
15 corresponding to said phenotype” when used in the present invention, is understood as a a polypeptide of the polypeptide display library (i.e. the phenotype as defined above), that is linked to the nucleic acid encoding it (i.e. the genotype as defined above). Said linkage leads to a complex formed by the polypeptide of the library and nucleic acid/s encoding it. In a particular embodiment, the polypeptide is exposed in the outer surface of said
20 complex. Therefore, in a particular embodiment, the polypeptide display library is formed by complexes comprising a polypeptide of the first aspect of the invention, directly or indirectly linked to a nucleic acid encoding it. Said polypeptide is considered a phenotype, and said nucleic acid is considered the genotype corresponding to the phenotype.

25 In a particular embodiment, the polypeptides of the library are not inked to any nucleic acid encoding them.

Each polypeptide of the library, whether being part of a complex as described above or not, is referred to as a “member of the library”. Therefore, said term, as used herein, refers
30 to any polypeptide of the library, wherein said polypeptide can be directly or indirectly linked to a nucleic acid, wherein the nucleic acid encodes or comprises a sequence encoding said polypeptide, or be simply a polypeptide of the library that is not linked by

any means to a nucleic acid comprising a sequence encoding it. Thus, in a particular embodiment, the member of the library is the polypeptide of the library as defined above. In another particular embodiment, it is a complex comprising a polypeptide of the first aspect of the invention, directly or indirectly linked to a nucleic acid encoding it.

5

A direct link consists on a direct interaction between the polypeptide of the library and the nucleic acid encoding it, resulting on a polypeptide-nucleic acid complex wherein the polypeptide binds or is covalently attached to the nucleic acid/s encoding it. , wherein the polypeptide is comprised in the outer surface of said polypeptide – nucleic acid complex.

10 As understood by a skilled person, said complex can also comprise additional proteins and/or nucleic acids.

In a particular embodiment, the binding of the polypeptide of the complex to the nucleic acid is direct. In another particular embodiment, it is indirect, so that the polypeptide
15 binds or is covalently to the nucleic acid encoding it by means of another peptide, protein, protein complex, or molecule, binding to said nucleic acid.

The terms “covalently attached”, “covalent attachment”, or “covalently coupled”, as used herein, refers to the interaction between two molecules, either directly covalently joined
20 through a chemical covalent bond to one another, or indirectly covalently joined to one another through an intervening moiety or moieties, such as a linker, a bridge, or a spacer.

In another particular embodiment, the covalent attachment between the polypeptide of the library and the nucleic acid encoding it is direct, so that the polypeptide is covalently
25 joined to the nucleic acid/s encoding it. In another particular embodiment, it is indirect, so that the polypeptide is joined to the nucleic acid/s encoding it through an intervening moiety or moieties, such as a linker, a bridge, or a spacer. In preferred embodiment, it is joined through a linker.

30 The term “linker moiety”, or “linker”, as used herein, refers to a molecule connecting two molecules, or compounds. It is also intended that the linking moiety is not limited in its chemical nature and/or structure; therefore, the linking moiety may be a polysaccharide,

a polypeptide, a fatty acid, a phospholipid, or a chemical derivative thereof, among others. It is further intended that the at least one of the molecules covalently attached to another one through a linker, or even both of said molecules, is/are bound to said linker through any chemical bond, such as peptide bond, isopeptide bond, amide bond, imine bond, and
5 etcetera.

In a particular embodiment, the polypeptide display library is formed by complexes comprising a polypeptide of the first aspect of the invention directly linked to a nucleic acid encoding said polypeptide, wherein the polypeptide-nucleic acid complex is selected
10 from the group consisting of:

- a complex consisting on the polypeptide of the library binding to the nucleic acid encoding it
- a complex consisting on the polypeptide of the library binding to the nucleic acid encoding it and on additional proteins, peptides and/or nucleic acids,
- 15 - a polypeptide-nucleic acid conjugate,
- a ribosome, or a portion of a ribosome,

The term “conjugate”, as used herein, refers to any compound resulting from the covalent attachment of two or more individual compounds, or molecules, being said covalent
20 attachment as define above. The conjugate, per definition, is never found in nature as such.

The individual compounds covalently attached in the conjugate of the second aspect of the invention are a polypeptide of the library and the nucleic acid encoding it. Thus, in a
25 particular embodiment, the conjugate of the second aspect of the invention comprises the polypeptide of the library directly attached to the nucleic acid encoding it through a chemical covalent bond. In another particular embodiment, the conjugate of the second aspect of the invention comprises the polypeptide of the library attached to the nucleic acid encoding it through an intervening moiety or moieties, such as a linker, a bridge, a
30 spacer, a moiety or moieties. In a particular embodiment they are attached through a linker.

The term “ribosome”, as used herein refers to a highly complex cellular machine, essential for protein synthesis. Ribosomes link amino acids together in the order specified by messenger RNA (mRNA) molecules. It is made up of specialized RNA known as ribosomal RNA (rRNA) as well as dozens of distinct proteins (the exact number varies
5 between species). The ribosomal proteins and rRNAs are arranged into two distinct ribosomal pieces of different size, known generally as the large and small subunit of the ribosome.

The expression “ribosome portion”, as used herein, refers to an isolated part of a
10 ribosome, which can consist for instance on the isolated large or small subunit of a ribosome. It also refers to a ribosome that only comprises a part of its ribosomal proteins, or a part of its rRNAs.

In another particular embodiment, the polypeptide-nucleic acid complex of the second
15 aspect of the invention, comprises in its outer surface only one polypeptide of the library. In another particular embodiment, it comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 250, at least 500, at least 1×10^3
20 copies of a polypeptide of the library. In a particular embodiment, said copies have the same amino acid sequence. In another particular embodiment, said complex does not comprise any other polypeptide of the library.

In another particular embodiment, the polypeptide-nucleic acid complex of the second
25 aspect of the invention comprises only one nucleic acid encoding the amino acid sequence of the polypeptide of the library comprised in the complex. Said polypeptide and said polypeptide sequence is that specified in the embodiment above.

In a particular embodiment, the complex of the second aspect of the invention comprises
30 at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 copies of a

nucleic acid encoding the amino acid sequence of the polypeptide of the library comprised in the complex. Said polypeptide and said polypeptide sequence is that specified in the embodiment above. In a particular embodiment, said nucleic acids have the same nucleotide sequence.

5

An indirect link consists on a genetic fusion between the polypeptide of the library and the nucleic acid by a microorganism that comprises both the polypeptide and the nucleic acid. The polypeptide of the library is comprised in the outer surface of the microorganism. The nucleic acid is preferably comprised in the interior part of the

10 microorganism.

In a particular embodiment, said microorganism comprises in its outer surface, only one polypeptide of the library. In a particular embodiment, it comprises in its outer surface at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9,

15

at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 250, at least 500, at least $1 \cdot 10^3$ copies of a polypeptide of the library. In a particular embodiment, said copies have the same amino acid sequence. In another particular embodiment, said microorganism does not comprise any other polypeptide of the library.

20

In another particular embodiment, the microorganism comprises only one nucleic acid encoding the amino acid sequence of the polypeptide of the library comprised in the microorganism. Said polypeptide and said polypeptide sequence is that specified in the embodiment above.

25

In another particular embodiment, the microorganism comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 copies of a nucleic acid encoding

30

the amino acid sequence of the polypeptide of the library comprised in the microorganism. Said polypeptide and said polypeptide sequence is that specified in the

embodiment above. In a particular embodiment, said nucleic acids have the same nucleotide sequence.

In a particular embodiment, the microorganism is capable to replicate. In a particular
5 embodiment, the replicated microorganism is an exact copy of the microorganism from
which it derives. In another particular embodiment, replication of said microorganism
leads to a microorganism with the same polypeptide of the library and the same nucleic
acid encoding it comprised in the microorganism from which it derives. Thus, as it will
be understood by a skilled person, the replication of the microorganism allows the
10 amplification of the polypeptide of the library, as well as of the nucleic acid encoding it.

In a particular embodiment, the microorganism is selected from the group consisting of a
phage, a bacteriophage, a virus, a bacterium, and a yeast. In a preferred embodiment, it is
a phage. In another preferred embodiment, it is a bacteriophage.

15

In a particular embodiment, the bacteriophage is selected from the group consisting of an
Enterobacteria phage M13, T4 bacteriophage, T7 bacteriophage, or an Escherichia
Lambda virus.

20 In a certain embodiment, all the terms and embodiments described in the first aspect of
the invention are equally applicable to this aspect of the invention.

III - Polynucleotides, vectors and host cells

25 In a third aspect, the invention relates to a polynucleotide encoding the polypeptide
according to the first aspect of the invention, or encoding a polypeptide of the polypeptide
display library according to the second aspect of the invention.

The term polynucleotide has been defined in the second aspect of the invention.

30

In a certain embodiment, all the terms and embodiments described in any of the previous
aspects of the invention are equally applicable to the third aspect of the invention.

In a fourth aspect, the invention relates to a vector comprising the polynucleotide according to the third aspect of the invention.

5 The term “vector”, as used herein, refers to a vehicle through which a polynucleotide or a DNA molecule can be manipulated or introduced into a cell. The vector can be a linear or circular polynucleotide or it can be a larger polynucleotide or any other type of construction such as the DNA or RNA of a viral genome, a virion or any other biological construct that allows the manipulation of DNA or its introduction in a cell. It is understood
10 that the terms "recombinant vector", "recombinant system" can be used interchangeably with the term vector. A person skilled in the art will understand that there is no limitation as regards the type of vector which can be used because said vector can be a cloning vector suitable for propagation and for obtaining the polynucleotides or suitable gene constructs or expression vectors in different heterologous organisms suitable for purifying
15 the polynucleotides of the invention. Thus, suitable vectors according to the present invention include expression vectors in prokaryotes such as pET (such as pET14b), pUC18, pUC19, Bluescript and their derivatives, mp18, mp19, pBR322, pMB9, CoIE1, pCR1, RP4, phages and shuttle vectors such as pSA3 and pAT28, expression vectors in yeasts such as vectors of the type of 2 micron plasmids, integration plasmids, YEP
20 vectors, centromeric plasmids and the like, expression vectors in insect cells such as the pAC series and pVL series vectors, expression vectors in plants such as vectors of expression in plants such as pIBI, pEarleyGate, pAVA, pCAMBIA, pGSA, pGWB, pMDC, pMY, pORE series vectors and the like and expression vectors in superior eukaryotic cells based on viral vectors (adenoviruses, viruses associated to adenoviruses
25 as well as retroviruses and lentiviruses) as well as non-viral vectors such as pSilencer 4.1-CMV (Ambion), pcDNA3, pcDNA3.1/hyg pHCMV/Zeo, pCR3.1, pEF1/His, pIND/GS, pRc/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAX1, pZeoSV2, pCI, pSVL and pKSV-10, pBPV-1, pML2d and pTDT1.

The vector of the invention can be used to transform, transfect, or infect cells which can
30 be transformed, transfected or infected by said vector. Said cells can be prokaryotic or eukaryotic. By way of example, the vector wherein said DNA sequence is introduced can be a plasmid or a vector which, when it is introduced in a host cell, is integrated in the

genome of said cell and replicates together with the chromosome (or chromosomes) in which it has been integrated. Said vector can be obtained by conventional methods known by the persons skilled in the art (Sambrook *et al.*, 2001, “Molecular cloning, to Laboratory Manual”, 2nd ed., Cold Spring Harbor Laboratory Press, N.Y. Vol 1-3 a).

5

Therefore, in a fifth aspect, the invention relates to a host cell comprising the polynucleotide according to the third aspect of the invention, or the vector according to the fourth aspect of the invention.

- 10 The transformed, transfected or infected cells can be obtained by conventional methods known by persons skilled in the art (Sambrook *et al.*, 2001, mentioned above). In a particular embodiment, said host cell is a animal cell transfected or infected with a suitable vector.
- 15 Host cells suitable for the comprising the polynucleotide of the third aspect of the invention, or the vector of the fourth aspect of the invention include, without being limited to, mammal, plant, insect, fungal and bacterial cells. Bacterial cells include, without being limited to, Gram-positive bacterial cells such as species of the *Bacillus*, *Streptomyces*, *Listeria* and *Staphylococcus* genus and Gram-negative bacterial cells such as cells of the
- 20 *Escherichia*, *Salmonella* and *Pseudomonas* genera. Fungal cells preferably include cells of yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris* and *Hansenula polymorpha*. Insect cells include, without being limited to, *Drosophila* and Sf9 cells. Plant cells include, among others, cells of crop plants such as cereals, medicinal, ornamental or bulbous plants. Suitable mammal cells in the present invention include epithelial cell lines
- 25 (human, ovine, porcine, etc.), osteosarcoma cell lines (human, etc.), neuroblastoma cell lines (human, etc.), epithelial carcinomas (human, etc.), glial cells (murine, etc.), hepatic cell lines (from monkey, etc.), CHO (Chinese Hamster Ovary) cells, COS cells, BHK cells, HeLa cells, 911, AT1080, A549, 293 or PER.C6, NTERA-2 human ECC cells, D3 cells of the mESC line, human embryonic stem cells such as HS293, BGV01, SHEF1,
- 30 SHEF2, HS181, NIH3T3 cells, 293T, REH and MCF-7 and hMSC cells.

In a certain embodiment, all the terms and embodiments described in the first and second aspects of the invention are equally applicable to the third aspect of the invention. In another embodiment, all the terms and embodiments described in the first, second and third aspects of the invention are equally applicable to the fourth aspect of the invention.

- 5 In another particular embodiment, all the terms and embodiments of the first, second, third and fourth aspects of the invention are equally applicable to the fifth aspect of the invention.

IV - Conjugates of the invention

10

In a further aspect, the invention relates to a conjugate comprising

- (i) a polypeptide comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- (ii) an agent of interest.

15

The polypeptide forming part of the conjugate and which comprises the G2 domain of nidogen-1 or a functionally equivalent variant thereof (as specified in point (i) above) is also referred to as the “polypeptide of the conjugate of the sixth aspect of the invention”, “the polypeptide of the conjugate of the invention”, or “the polypeptide of the conjugate”.

20

The G2 domain of nidogen-1 or a functionally equivalent variant thereof comprised in the polypeptide of the conjugate of the sixth aspect of the invention is also referred to as the “first polypeptide of the conjugate”, “first polypeptide region of the conjugate”, “first polypeptide region”, or “first polypeptide region comprised in the polypeptide of the

25 conjugate”.

The term “conjugate” has been defined in the second aspect of the invention. The two components covalently attached in the conjugate of the sixth aspect of the invention are the polypeptide of the conjugate and an agent of interest. From now on, the conjugate of

30 the sixth aspect of the invention is also referred to as “the conjugate of the invention”.

The term “polypeptide” has been defined in the first aspect of the invention. The definition and embodiments indicated in the first aspect of the invention for the term “amino acid residue” also apply to the present aspect of the invention.

- 5 The expression “agent of interest”, as used herein, refers to any compound, without chemical structure limitations, provided it can be covalently attached to the polypeptide of the conjugate. In a particular embodiment, said agent is a therapeutic agent. In another particular embodiment, it is an imaging agent. The term “therapeutic agent” and “imaging agent” are defined below in sections IV-E.1 and IV-E.2.

10

IV.A- The first polypeptide of the conjugate

The conjugate of the invention contains a polypeptide comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof.

15

The term “nidogen-1” as used herein has been defined above in the context of the variant nidogen G2 domain and applies equally to the conjugate of the invention.

20

The term “G2 domain of nidogen-1”, as used herein, refers to the domain G2 of the protein nidogen 1 as defined above. In the native nidogen-1 sequence, the G2 domain is flanked by short EGF-like domains. However, for the purposes of the present invention, the nidogen-1 G2 domain is formed by amino acid numbers 430 and 667 of the amino acid sequence of the nidogen-1 protein, with identification number P14543-1 of the Uniprot Database (version dated July 7, 2009) (SEQ ID NO:62) and lacks EGF-like domains at the N-or at the C-terminus. In another embodiment, the domain G2 of nidogen 1 lacks the first two amino acids of SEQ ID NO:62 (SEQ ID NO:63), and thus, corresponds to a region consisting on amino acid numbers 432 and 667 of the amino acid sequence of the nidogen-1 protein, with identification number P14543-1 of the Uniprot Database (version dated July 7, 2009) (SEQ ID NO:72).

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The expression “functionally equivalent variant”, as used herein, refers to all those peptides showing a certain degree of sequence identity with the sequence of nidogen-1

G2 domain, preferably with SEQ ID NO:63, more preferably with SEQ ID NO:62, and that substantially maintain the function of the G2 domain of nidogen-1. The function of domain G2 to be maintained in the conjugate of the sixth aspect of the invention is considered to be the tertiary structure of the domain, preferably when it is not part of the conjugate. Accordingly, said functionally equivalent variant of domain G2 substantially maintains the tertiary structure of domain G2 of nidogen-1, preferably when it is not part of the conjugate. As understood by a skilled person, the tertiary structure of the G2 domain to be maintained is preferably the tertiary structure of the beta barrel domain of the G2 domain of nidogen-1. Said structure has been defined in the first aspect of the invention.

The expression “substantially maintained”, as used herein, is understood as that the structure of domain G2 of nidogen-1, preferably the structure of the beta barrel domain of G2 of nidogen-1 is maintained at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9%, preferably 95%, more preferably 99%, even more preferably 100%.

When expressed as a percentage, the maintenance of the tertiary structure of a protein domain is understood as the percentage of amino acids from the domain that maintain their relative position with respect to the rest of amino acids of the domain within the tertiary structure of the domain. Methods to determine the tertiary structure of a protein allowing determining the atomic coordinates of a protein are well-known by an expert on the field and include circular dichroism, X-ray crystallography or protein NMR.

In a particular embodiment, the functionally equivalent variant of domain G2 substantially maintains the percentage of the structure of domain G2 of nidogen-1 indicated above, once it is incorporated in the conjugate of the sixth aspect of the invention. In a preferred embodiment, it is substantially maintained when it is not part of the conjugate. In a preferred embodiment, the structure of domain G2 substantially maintained is the structure of the beta barrel domain of domain G2 of nidogen-1 described

in the first aspect of the invention. In a particular embodiment, it is the structure of the beta barrel domain of nidogen-1 domain G2 when it is the first polypeptide of the conjugate.

- 5 In a particular embodiment, the degree of sequence identity between the G2 domain of nidogen 1 with SEQ ID NO:62 and the functionally equivalent variant is of at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4% at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%,
10 or at least 99.9%. In another particular embodiment, the degree of sequence identity between the G2 domain of nidogen 1 with SEQ ID NO:63 and the functionally equivalent variant is of at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4% at least 99.5%, at least 99.6%,
15 at least 99.7%, at least 99.8%, or at least 99.9%. Methods to determine the degree of sequence identity between two amino acid sequences have been provided in the first aspect of the invention.

- Although once incorporated into the conjugate, the first polypeptide region of the
20 conjugate need not maintain the cellular or physiological function of domain G2 of nidogen. Thus, in a particular embodiment the first polypeptide region is the G2 domain of nidogen-1 or a functionally equivalent variant thereof, with a reduced physiological function once it is incorporated into the fusion protein of the invention. In another embodiment, the first polypeptide region is the G2 domain of nidogen-1 or a functionally
25 equivalent variant thereof, which does not have any physiological function of the G2 nidogen-1 domain, or of the beta barrel domain of the G2 nidogen-1 domain outside the conjugate. In a preferred embodiment, the first polypeptide region is a functionally equivalent variant of the G2 domain of nidogen with an already reduced physiological function, as compared to the wild-type G2 domain of nidogen-1, or to the wild-type beta
30 barrel domain of the G2 domain of nidogen-1, already before being incorporated into the conjugate of the invention. More preferably, the first polypeptide region is a protein which does not have any of the physiological function of the G2 domain of nidogen-1, or of the

wild type beta barrel domain of the G2 domain of nidogen-1, already before being incorporated into the conjugate of the invention, due to the presence of inactivating mutations.

- 5 The expression “physiological function” or “cellular function”, as used herein, refers to the function of a peptide within a cell or an organism. Thus, when referred to physiological function of domain G2 of nidogen-1, or of the beta barrel domain of the G2 domain of nidogen-1 it is understood as the function of said domain when part of nidogen-1 protein. Thus, it refers to the role of the domain in the biochemical pathways, or
- 10 molecular mechanisms, in which the protein nidogen-1 participates in a cell. Therefore, it is directly related with the capacity of the domain to interact with specific peptides or proteins of the cell, outside the cell, or in the outer surface of another cell. Thus, in a particular embodiment, it refers to the capacity of G2 domain, or of the beta barrel domain of the G2 domain, to interact with its usual protein binding partners. Non limiting
- 15 examples of such binding partners include collagen IV and perlecan. Thus, in a particular embodiment, the first polypeptide of the conjugate is a functionally equivalent variant of domain G2 of nidogen-1 comprising mutations that inhibit the interaction of the G2 domain of nidogen-1, or of the beta barrel domain of the G2 domain of nidogen-1, with the usual binding partners of the G2 domain of nidogen-1, or of the beta barrel domain of
- 20 the G2 domain of nidogen-1, preferably with collagen IV and/or Perlecan.

In another embodiment of the invention, the first polypeptide of the conjugate is a functionally equivalent variant of the G2 domain of nidogen-1 that is inert. In a particular embodiment, it is inert once incorporated in the conjugate. In another particular, it is inert

25 already before being incorporated in the conjugate.

As used herein, the term “inert” when referred to a polypeptide, protein, fragment or domain of proteins, it is understood as a polypeptide, protein, fragment or domain of proteins without physiological or biological activity, or without the ability to specifically

30 interact with other macromolecules for a biological function, and fragments or domains of proteins devoid of known therapeutic activity (e.g. antitumor activity). The inert polypeptide part of the conjugate is non-reactive and functions as a physical structure for

the binding of the agents of interest. It is intended that the inert polypeptides do not comprise any motifs that have intrinsic enzymatic, physiological, or biological activity on their own, nor do they present immune reactivity, meaning that they stimulate neither the adaptive, nor the innate immune responses.

5

In general, it is intended that any intrinsic activity of the first polypeptide of the conjugate is irrelevant for the purposes of the invention and does neither contribute, nor hinder the biological activity of the agent of interest.

10 In a particular embodiment, the first polypeptide of the conjugate is a functionally equivalent variant of the G2 domain of nidogen-1 as any of the polypeptides described in the first aspect of the invention. Thus, in a particular embodiment, the polypeptide of the conjugate is a functionally equivalent variant of the G2 domain of nidogen-1 as any of the polypeptides described in the first aspect of the invention.

15

In another particular embodiment, the first polypeptide region has a sequence corresponding to amino acids 430 to 667 with respect to the sequence of human nidogen-1 defined in the Uniprot Database with accession number P14543-1 (version dated July 7, 2009), i.e. it has SEQ ID NO:62. In another particular embodiment, the first
20 polypeptide region has a sequence corresponding to amino acids 432 to 667 with respect to the sequence of human nidogen-1 defined in the Uniprot Database with accession number P14543-1 (version dated July 7, 2009), i.e. it has SEQ ID NO:63.

In another particular embodiment, the first polypeptide of the conjugate is a functionally
25 equivalent variant of the domain G2 of nidogen-1 comprising a mutation in one or more amino acid residues at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 151, 604, 638, 641, 469 and 518 with respect to the numbering of the sequence of human nidogen-1 defined under the UniProt database with accession number P14543-1 (version dated July 7, 2009). Thus, in another particular embodiment, the polypeptide of the
30 conjugate of the sixth aspect of the invention is a functionally equivalent variant of the domain G2 of nidogen-1 comprising a mutation in one or more amino acid residues at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 151, 604, 638, 641, 469

and 518 with respect to the numbering of the sequence of human nidogen-1 defined under the UniProt database with accession number P14543-1 (version dated July 7, 2009).

The term “mutation”, as used herein refers to any modification, or deletion of an amino acid in an amino acid sequence, or the insertion of at least one amino acid before or after an amino acid in an amino acid sequence. As understood by a skilled person, the location of a modification or deletion of an amino acid is referred by the location of the modified or deleted amino acid in the amino acid sequence before said mutation. In a particular embodiment, when the mutation is an insertion, the location of said mutation is defined by reference to the amino acid at the N-terminus of the inserted amino acid/s. In another particular embodiment, when the mutation is an insertion, the location of said mutation is defined by reference to the amino acid at the C-terminus of the inserted amino acid/s. Thus, as understood by a skilled person, a mutation in one or more amino acid residues at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 151, 604, 638, 641, 469 and 518 of the protein sequence indicated above, refers to a modification or deletion of the amino acids at said positions in the amino acid sequence of human nidogen-1 defined under the UniProt database with accession number P14543-1 (version dated July 7, 2009). In a particular embodiment, it refers to an insertion of at least one amino acid at the C-terminus of one or more of said amino acids. In another particular embodiment, it refers to an insertion of at least one amino acid at the N-terminus of one or more of said amino acids.

In a preferred embodiment, the mutation is an amino acid modification. In a particular embodiment, the mutation at position 459 indicated above is a H459A mutation. In another preferred embodiment, the mutation at position 468 indicated above is a R468N mutation. In another preferred embodiment, the mutation at position 639 indicated above is a F639S mutation. In another particular embodiment, the mutation at position 650 indicated above is a R650A mutation.

Therefore, in a particular embodiment, the one or more mutations at positions 459, 468, 639 or 650 indicated above in the first polypeptide of the conjugate are a H459A mutation, a R468N mutation, a F639S mutation or a R650A mutation. In another particular

embodiment, the one or more mutations at positions 459, 468, 639 or 650 indicated above in the polypeptide of the conjugate are a H459A mutation, a R468N mutation, a F639S mutation or a R650A mutation.

- 5 Suitable nidogen G2 domain variants that can be included in the first polypeptide region of the conjugate include, without limitation, any of the nidogen G2 domain variants defined above in the context of the first aspect of the invention including the variant carrying the NIDOMut2, NIDOMut3, the NIDOMut3-V45T, the NIDOMut3_V121Q, the NIDOMut3-F157E, the NIDOMut3-V215T, the NIDOMut4, the NIDOMut4_T215V, the
- 10 NIDOMut5, NIDOMut3-V176T, the NIDOMut3-I200T, the NIDOMut3-V236Y, the NIDOMut3-L237T, the NIDOMut3-S65I, the NIDOMut3-R114I, the NIDOMut3-C214S, the NIDOMut3-S65I_R114I, the NIDOMut5-S65I_R114I, the NIDOMut3-S65I_R114I and the NIDOMut5-S65I_R114I as defined, respectively, as SEQ ID NO: 64, 65 and 87 to 104.

15

IV-B. The second polypeptide region of the conjugate

The polypeptide of the conjugate of the sixth aspect of the invention optionally comprises a second polypeptide region which is capable of specifically binding to a target of interest.

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The second polypeptide region which is capable of specifically binding to a target of interest is also referred to as the “second polypeptide of the conjugate”, “second polypeptide region of the conjugate”, “the second polypeptide region”, or “second polypeptide region comprised in the polypeptide of the conjugate”.

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- The expression “specifically binding” has been defined in the second aspect of the invention. In a particular embodiment, the second polypeptide of the conjugate is considered to specifically bind to a target of interest if it binds to said target with a dissociation constant (K_D) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than
- 30 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M. Methods to determine if a polypeptide is capable of binding

to a target molecule, as well as to determine the dissociation constant of said binding are provided in the definition of “specifically binding” in the second aspect of the invention.

In a particular embodiment, the second polypeptide region within the conjugate of the invention is a ligand for a cell receptor. The term "cell receptor", or “receptor on the surface of a cell” denotes a cell-associated protein that binds to "ligand”. Non-limiting examples of agents of interest which are specific ligands for cell receptors and the specific receptors or cell types to which they bind are those shown in table 2 below:

Ligand	Cell receptor/cell type or microorganism
Folic acid or folic acid targeting ligand, including methotrexate or aminopterin.	Folate receptor in leucocyte
Hyalurinic acid (HA)	CD44
Xanthine scaffold, including lingalipatin	fibroblast activation protein (FAP) on wound fibroblast cells
IgG Fc	Protein A in <i>S.aureus</i>
Ligands of Her 2 are well-known in the art and can be any of those described in Wikman M et al., Protein Eng Des Sel 17: 455-62 (2004); Orlova A et al. Cancer Res 66: 4339-8 (2006); Ahlgren S et al., Bioconjug Chem 19: 235-43 (2008); Feldwisch J et al., JMol Biol 398: 232-47 (2010); US patents with patent number: 5,578,482; 5,856,110; 5,869,445; 5,985,553; 6,333, 169; 6,987,088; 7,019,017; 7,282,365; 7,306,801; 7,435,797; 7,446, 185; 7,449,480; 7,560,111; 7,674,460; 7,815,906; 7,879,325; 7,884, 194; 7,993,650; 8,241,630; 8,349,585; 8,389,227; 8,501,909; 8,512,967; 8,652,474; and U.S. patent application US20110059090A1)	HER2
CRPPR peptide	Cardiac endothelial cell
Angiotensin	angiotensin II type 1 (AT1) receptor

Ligand for interleukin-6 (IL-6), such as: - the S7 peptide described in Su JL et al. (Cancer Res. 2019 Jul 15;79(14):3791) having SEQ ID NO:74 (LSLITRL), - The IL6 ligand described in Weiergräber O et al. (FEBS Lett. 1996 Jan 29;379(2):122-6 and with SEQ ID NO:75 (WQDPHSWNSSFYRLRFELRYRAERSKTFTTW), interleukin-6 receptor (IL-6R); PDGF; angiopoietin; angiopoietin 2; Tie2; S1P; integrins α v β 3, α v β 5, and α 5 β 1; betacellulin; apelin/APJ; erythropoietin; complement factor D; TNFα; HtrA1; a VEGF receptor; ST-2 receptor; and proteins genetically linked to age-related macular degeneration (AMD) risk such as complement pathway components C2, factor B, factor H, CFHR3, C3b, C5, C5a, C3a, HtrA1, ARMS2, TIMP3, HLA, interleukin-8 (IL-8), CX3CR1, TLR3, TLR4, CETP, LIPC, COL10A1, and TNFRSF10A	Cells from the eye, such as cells from the retinal pigment epithelium.
Opsonins, including vitronectin, fibronectin, complement components such as Clq (including any of its component polypeptide chains A, B and C), complement fragments such as C3d, C3b and C4b, mannose-binding protein, conglutinin, surfactant proteins A and D, C-reactive protein (CRP), alpha2-macroglobulin, and immunoglobulins, for example, the Fc portion of an immunoglobulin	Microbe (virus, bacterial cell, fungal cell, protozoa)
Mannose-binding lectin (MBL)	Microbe (virus, bacterial cell, fungal cell, protozoa)

Table 2. Ligands suitable for use in the present invention and receptors, cells or microorganisms, to which said ligands bind. On the left column, a specific ligand binding to a certain receptor is provided. The receptor to which each ligand provided in a certain row in the left column binds is indicated on the same row on the right column. Alternatively, the cell comprising said receptor, or the microorganism comprising said receptor, are indicated.

In a particular embodiment, the second polypeptide of the conjugate is selected from the group of ligands provided in table 2.

10

In a particular embodiment, the target of interest is a receptor in the surface of a cell and the second polypeptide of the conjugate is capable of promoting internalization of the conjugate in said cell. The expression “promoting internalization of the conjugate in said cell”, when referred to the second polypeptide of the conjugate, refers to a polypeptide binding to a receptor in a cell surface that undergoes endocytosis in response to the binding of said polypeptide. This binding specificity allows the delivery of the second polypeptide of the conjugate, as well as the rest of the conjugate comprising it, to the cell,

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tissue or organ which expresses said receptor. In this way, a conjugate comprising said polypeptide region will be directed specifically to said cells when administered to an animal or contacted *in vitro* with a population of cells of different types.

- 5 As used herein, "internalization" refers to a process by which a molecule or a construct comprising a molecule binds to a target element on the outer surface of the cell membrane and so that the resulting complex is internalized by the cell. Internalization may be followed up by dissociation of the resulting complex within the cytoplasm. The target element, along with the molecule or the construct, may then localize to a specific cellular
10 compartment. Preferably, the second polypeptide of the conjugate of the invention, besides promoting internalization, will facilitate endosomal escape of the conjugate.

- The expression "facilitate endosomal escape", as used herein, refers to the ability of the second polypeptide of the conjugate to induce the release of the conjugate from the
15 endosomal compartment after internalization by receptor-mediated endocytosis.

- The ability of the conjugate of the invention to be internalized by cells expressing the receptor to which the second polypeptide of the conjugate binds may be conveniently determined by fluorescence methods in the case that the polypeptide of the conjugate
20 comprises a fluorescent protein, such as GFP. Such fusion proteins can be obtained by preparing a recombinant nucleic acid wherein the nucleic acids encoding the polypeptide of the conjugate and the fluorescent protein are fused in frame and expressed in an adequate host cell or organism. The fusion protein is then contacted with a culture of cells expressing the aforementioned receptor or *in vivo* with a tissue which expresses said
25 receptor for an appropriate amount of time, after which fluorescence microscopy may be used to determine whether the construct penetrated the cell. Presence of fluorescence in the cytoplasm may be further investigated by comparing the fluorescence microscopy image resulting from the fluorescent protein to that obtained with a known cytoplasmic stain.

30

A wide array of uptake receptors and carriers, with an even wider number of receptor-specific ligands, are known in the art.

Non-limiting examples of receptors which may be targeted by the second polypeptide have been provided above.

- 5 In a particular embodiment, the second polypeptide of the conjugate is a polycationic peptide.

The term “polycationic peptide”. or “polycationic region” as used herein, corresponds to a polypeptide sequence containing multiple positively charged amino acids. The
10 polycationic peptide may be formed exclusively by positively charged amino acids or may contain other amino acids provided that the overall net charge of the region at pH 7 is positive.

It is well known in the art that amino acids and their corresponding amino acid residues
15 possess different properties depending on their side chains and they may be grouped depending on those properties. Thus, at physiological pH, five amino acids show an electrical charge: arginine, histidine, and lysine are positively charged while aspartic acid and glutamic acid are negatively charged. The person skilled in the art will acknowledge then that the polycationic peptide of the invention corresponds to a polypeptide with a net
20 electrical charge of more than one positive charge in physiological pH conditions. Accordingly, the polycationic peptide of the invention is not limited by the presence of one or more negatively charge amino acid residues as long as there are always enough positively charged amino acid residues to result in a net positive electrical charge of two or more.

25

Thus, in one embodiment of the invention, the polycationic peptide of the invention is selected from the group consisting of

- (i) a sequence which is capable of specifically interacting with a receptor on a cell surface and promoting internalization of the conjugate on said cell,
- 30 (ii) an arginine-rich sequence,
- (iii) the GW-H1 peptide,
- (iv) a CD44 ligand,

- (v) a peptide capable of crossing the blood-brain barrier,
- (vi) a cell penetrating peptide and
- (vii) a nucleolin-binding peptide.

- 5 (i) *Sequence which is capable of specifically binding to a receptor on a cell surface and promoting internalization of the conjugate on said cell*

The terms “sequence which is capable of specifically binding to a receptor on a cell surface and promoting internalization of the conjugate on said cell”, as used herein, refers
 10 to any sequence encoding a polypeptide capable of specifically binding to a target of interest, as defined above, wherein said target is a receptor on the surface of a cell, as defined above, and wherein the polypeptide encoded by said sequence promotes internalization of the conjugate on said cell, as defined above.

- 15 The embodiments provided above for the second polypeptide of the conjugate also apply to said polycationic peptide.

Non-limiting examples of receptors which may be targeted by the polycationic peptide of the invention, preferably the sequence which is capable of specifically binding to a
 20 receptor on a cell surface referred above, include any of the cell receptors provided above. In a particular embodiment, said receptors are selected from the group consisting of a CXCR4 receptor, an angiotensin receptor, a bombesin receptor, a bradykinin receptor, a calcitonin receptor, a chemokine receptor, a cholecystokinin receptor, a corticotropin-releasing factor receptor, an endothelin receptor, an ephrin receptor, a formylpeptide
 25 receptor, a Frizzled receptor, a galanin receptor, a the growth hormone secretagogue receptor (Ghrelin) receptor, a Kisspeptin receptor, a melanocortin receptor, Neuropeptide FF/neuropeptide AF receptor, a neuropeptide S receptor, a neuropeptide W/neuropeptide B receptor, a neuropeptide Y receptor, a neurotensin receptor, an orexin receptors, a peptide P518 receptor, a somatostatin receptor, a tachykinin receptor, a Toll-like receptor,
 30 a vasopressin and oxytocin receptor and a VEGF receptor.

In a preferred embodiment of the invention, the polycationic peptide comprising a sequence which is capable of specifically binding to a receptor on a cell surface and promoting internalization of the conjugate on said cell is a CXCR4 ligand.

5 The term “CXCR4”, as used herein, refers to a G protein-coupled, seven-transmembrane chemokine receptor. Like other chemokine receptors, CXCR4 plays an important role in immune and inflammatory responses by mediating the directional migration and activation of leukocytes CXCR4 is expressed or overexpressed in a variety of cancer cell lines and tissues including breast, prostate, lung, ovarian, colon, colorectal, pancreatic,
10 kidney, and brain, as well as non-Hodgkin's lymphoma and chronic lymphocytic leukemia. The only known ligand to CXCR4 is stromal cell-derived factor-1 (SDF-1, or CXCL12). The interaction between CXCR4 and SDF-1 plays an important role in multiple phases of tumorigenesis, including tumor growth, invasion, angiogenesis, and metastasis.

15

The expression “specifically binding” has been defined in the second aspect of the invention. As understood by a skilled person, the expression “specifically binding to CXCR4”, as used herein refers to the ability of the conjugates of the invention to bind more frequently, more rapidly, with greater duration and/or with greater affinity to
20 CXCR4 or cell expressing same than it does with alternative receptors or cells without substantially binding to other molecules.

Binding affinity is measured, for instance, by any of the methods provided in the definition of “specifically binding” in the second aspect of the invention, preferably, as
25 described by Tamamura et al. by the oil-cushion method [see Hesselgesset et al, 1998, J.Immunol., 160:877-883]. Said method comprises contacting the peptide with CXCR4-transfected cell line (e.g. CHO cells) and a labeled CXCR4 ligand (e.g. ¹²⁵I-SDF-1 α) and measuring the inhibition percentage of the targeting peptide against the binding of the labeled CXCR4 ligand.

30

Specific binding can be exhibited, e.g., by a low affinity targeting agent having a K_d of at least about 10⁻⁴ M. e.g., if CXCR4 has more than one binding site for a ligand, a ligand

having low affinity can be useful for targeting. Specific binding also can be exhibited by a high affinity ligands, e.g. a ligand having a K_d of at least about of 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, at least about 10^{-10} M, or can have a K_d of at least about 10^{-11} M or 10^{-12} M or greater. Both low and high affinity-targeting ligands are useful for
5 incorporation in the conjugates of the present invention

The ability of the conjugate of the invention to be internalized by cells expressing CXCR4 may be determined by fluorescence methods where the conjugate comprises a fluorescent protein, such as GFP, as indicated above for any second polypeptide of the conjugate
10 binding to any cell receptor. More specifically, conjugates to be internalized by cells expressing CXCR4 can be obtained by preparing a recombinant nucleic acid wherein the nucleic acids encoding the polycationic peptide and the fluorescent protein are fused in frame and expressed in an adequate host cell or organism. The fusion protein is then contacted with a culture of cells expressing CXCR4 or in vivo with a tissue which
15 expresses CXCR4 for an appropriate amount of time, after which fluorescence microscopy may be used to determine whether the construct penetrated the cell. Presence of fluorescence in the cytoplasm may be further investigated by comparing the fluorescence microscopy image resulting from the fluorescent protein to that obtained with a known cytoplasmic stain.

20

In an even more preferred embodiment of the invention, the CXCR4 ligand is selected from the group comprising the RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 peptide (SEQ ID NO:27), the vCCL2 peptide (SEQ ID NO:28), the EPI-X4 sequence (SEQ ID NO:29) or a functionally equivalent
25 variant thereof, such as the peptide of SEQ ID NO:132.

The sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25) is the amino acid sequence of the T22 peptide. Said peptide corresponds to a peptide derived from the protein polyphemus II (extracted from hemocyte debris from *Limulus polyphemus*).
30 The vCCL2 corresponds to the viral macrophage inflammatory protein-II, an homologue of human chemokine CCL2 encoded by human herpesvirus 8. The V1 peptide corresponds to residues 1-21 of the N-terminus of vCCL2. CXCL12, C-X-C motif

chemokine 12, also known as stromal cell-derived factor 1 (SDF1), is a member of the chemokine family that acts as a pro-inflammatory mediator. All four peptides are known to have interactions with the CXCR4 receptor, as shown in Liang, X. 2008. Chem. Biol. Drug. Des. 72:91-110.

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EPI-X4 corresponds to residues 408–423 of human serum albumin (HSA). It has also been described to bind to the CXCR4 receptor (Zirafi *et al.*, 2015, Cell reports, 11:1-11). In one embodiment, an optimized EPI-X4 tandem version with higher receptor affinity and serum stability (SEQ ID NO:132) is used.

10

In one embodiment, the polycationic peptide is the one selected from the group consisting of:

- the T140 peptide having the sequence $RRX_1CYRKX_2PYRX_3CR$ (SEQ ID NO:41) wherein X_1 is L-3-(2-naphtyl)alanine, X_2 is D-Lys and X_3 is L-Citrulline.
- 15 - the TN14003 peptide having the sequence $RRX_1CYX_2KX_3PYRX_4CR$ (SEQ ID NO:42) wherein X_1 is L-3-(2-naphtyl)alanine, X_2 is L-Citrulline, X_3 is dLys and X_4 is L-Citrulline,
- the TC14012 peptide having the sequence $RRX_1CYEKX_2PYRX_3CR$ (SEQ ID NO:43) wherein X_1 is L-3-(2-naphtyl)alanine, X_2 is D-Citrulline and X_3 is L-
- 20 - Citrulline,
- the TE14011 peptide having the sequence $RRX_1CYX_2KX_3PYRX_4CR$ (SEQ ID NO:44) wherein X_1 is L-3-(2-naphtyl)alanine, X_2 is L-Citrulline, X_3 is D-Glu and X_4 is L-Citrulline and
- the TZ14011 peptide having the sequence $RRX_1CYX_2KX_3PYRX_4CR$ (SEQ ID
- 25 NO:45) wherein X_1 is L-3-(2-naphtyl)alanine, X_2 is L-Citrulline, X_3 is D-Lys and X_4 is L-Citrulline or the variant thereof wherein the N-terminal Arginine residue is acetylated (known Ac-TZ14011),

The terms “functional variant” and “functionally equivalent variant” are interchangeable and are herein understood as all those peptides derived from the T22, the V1, the CXCL12, the vCCL2, and/or the EPI-X4 peptides by means of modification, insertion

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and/or deletion of one or more amino acids, provided that the function of binding to CXCR4 and internalizing the conjugate is substantially maintained.

In one embodiment, functionally equivalent variants of the cationic polypeptides are those
5 showing a degree of identity with respect to the human T22, V1, CXCL12 vCCL2, and/or the EPI-X4 peptides, according to their respective SEQ ID NOs, greater than at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%. Methods to determine the degree of identity between two amino acid sequences have been provided in the first
10 aspect of the invention. The cationic polypeptides of the invention may include post-translational modifications, such as glycosylation, acetylation, isoprenylation, myristoylation, proteolytic processing, etc.

Alternatively, suitable functional variants of the cationic polypeptide are those wherein
15 one or more positions contain an amino acid which is a conservative substitution of the amino acid present in the T22, V1, CXCL12, vCCL2 and/or EPI-X4 peptides mentioned above. "Conservative amino acid substitutions" result from replacing one amino acid with another having similar structural and/or chemical properties. For example, the following six groups each contain amino acids that are conservative substitutions for one another:
20 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). Selection of such conservative amino acid substitutions is within the skill of one of ordinary skill in the art and is described, for example by Dordo et al. et al., [J. Mol. Biol.,
25 1999, 217:721-739] and Taylor et al., [J. Theor. Biol., 1986, 119:205-218].

A suitable assay for determining whether a given peptide can be seen as a functionally equivalent variant thereof is, for instance, the following assay: a putative T22, V1, CXCL12, vCCL2, or EPI-X4 peptide variant is fused in frame with a marker polypeptide
30 (e.g. a fluorescent protein). Such fusion proteins can be obtained by preparing a recombinant nucleic acid wherein the nucleic acids encoding the peptide and the fluorescent protein are fused in frame and expressed in an adequate host cell or organism.

The fusion protein is then contacted with a culture of cells CXCR4 (e.g. HeLa cells) for an appropriate amount of time after which fluorescence microscopy may be used to determine whether the construct penetrated the cell. If the peptide is a functionally equivalent variant of the corresponding peptide, the marker protein will be internalized and presence of fluorescence in the cytoplasm of the cell will be visible. Furthermore, the performance of the functionally equivalent variant can be assayed by comparing the fluorescence microscopy image resulting from the fluorescent protein to that obtained with a known cytoplasmic stain (e.g. DAPI).

10 (ii) *Arginine-rich sequence*

As aforementioned, the arginine amino acid and its residue present positive charge at physiological pH. It will be understood that an “arginine-rich sequence” refers to a polypeptide sequence containing multiple arginine residues. Thus, the polypeptide sequence may comprise 33%, preferably 40%, preferably 45%, preferably 50%, preferably 55%, preferably 60%, preferably 65%, preferably 70%, preferably 75%, preferably 80%, preferably 85%, more preferably 90%, more preferably 95%, even more preferably 99%, yet even more preferably 100% of the amino acid residues of its complete sequence as arginine residues. It will be understood that whenever the sequence of the arginine-rich sequence comprises less than the 100% of the sequence as arginine residues, these residues do not need to be all adjacent or contiguous with respect to each other.

The person skilled in the art will recognize that a polypeptide with one or more arginine residues will be a polycationic peptide as long as the total positive electrical charge of the polypeptide at physiological pH is 2 or more, resulting not only from the positive electrical charges of the arginine residues but also from any other positively charged amino acids.

In an embodiment of the invention, the polycationic peptide of the invention is an arginine-rich sequence.

In a preferred embodiment of the invention, the arginine-rich sequence of the polycationic peptide of the invention is selected from the group consisting of SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32 and SEQ ID NO:33.

5 (iii) *The GW-H1 peptide*

The GW-H1 peptide was previously described by Chen and colleagues [Chen, Y-L.S. *et al.* 2012. *Peptides*, 36:257-265]. The GW-H1 peptide was first selected as an antimicrobial peptide but it is also characterized by its capability to bind to cell membranes, internalize itself to the cytoplasm, and migrate to the nuclei in eukaryotic
10 cells. Once inside the cell, GW-H1 is capable to induce apoptosis. It has been proposed that GW-H1 exerts its cytolytic activity by folding into an amphipathic helix [Chen and colleagues, *supra*]. Therefore, this peptide is supposed to exert its cell lytic effects by two sequential events consisting on binding to cell membranes followed by permeabilization.

15 In a preferred embodiment of the invention, the polycationic peptide of the invention is the GW-H1 peptide, which has the SEQ ID NO:46.

 (iv) *A CD44 ligand*

CD44 is a cell-surface transmembrane glycoprotein involved in cell-cell and cell-matrix
20 interactions, cell adhesion and migration. CD44 has been implicated in inflammation and in diseases such as cancer [Bajorath, J. 2000. *Proteins*. 39:103-111]. Many isoforms are known, which are expressed in a cell-specific manner and also differentially glycosylated.

Accordingly, a “CD44 ligand” will be a molecule capable of binding to CD44. CD44 is
25 the major surface receptor for Hyaluronan, a component of the extracellular matrix, but it has other ligands, such as chondroitin sulfate, the heparin-binding domain of fibronectin, osteopontin, serglycin, collagen and laminin. Besides, CD44 can also interact with metalloproteinases and selectins.

30 In an embodiment of the invention, the polycationic peptide of the invention is a CD44 ligand. In a preferred embodiment of the invention, the CD44 ligand is selected from the group consisting of A5G27 (SEQ ID NO:34) and FNI/II/V (SEQ ID NO:35).

The peptide FNI/II/V corresponds to the HBFN-fragment V of Fibronectin. The peptide A5G27 corresponds to a peptide of the $\alpha 5$ chain of Laminin [Pesarrodon, M. *et al.* 2014. Int. J. of Pharmaceutics. 473:286-295].

5

(v) *Peptide capable of crossing the blood-brain barrier*

It is well known in the art that one major obstacle for the development of therapeutic approaches for brain pathologies is the blood-brain barrier (BBB). The brain is shielded against potentially toxic substances by the presence of two barrier systems: the blood-
10 brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is considered to be the major route for the uptake of serum ligands since its surface area is approximately 5000-fold greater than that of BCSFB. The brain endothelium, which constitutes the BBB, represents the major obstacle for the use of potential drugs against many disorders of the CNS. As a general rule, only small lipophilic molecules may pass
15 across the BBB, i.e., from circulating systemic blood to brain. Many drugs that have a larger size or higher hydrophobicity show promising results in animal studies for treating CNS disorders.

Therefore, a “peptide capable of crossing the blood-brain barrier” will be a peptide
20 capable of transporting itself as well as any molecule it is bound to, preferably a protein, from the blood torrent to the CNS.

In 1983 it was reported that a peptide, β -Casomorphin-5 could overcome the BBB [Ermisch, A. *et al.* 1983. J. of Neurochemistry. 41:1229-1233]. Since then, many other
25 peptides with BBB-permeating properties have been identified, characterized and catalogued, and in 2012 a comprehensive database was established, as reported by Van Dorpe *et al.* [Van Dorpe, S. *et al.* 2012. Brain Struct. Funct. 217:687-718]. Most of the peptides listed in the aforementioned database are suitable for the conjugate of the invention.

30

In an embodiment of the invention, the polycationic peptide of the invention is a peptide capable of crossing the blood-brain barrier. In a preferred embodiment of the invention,

the peptide capable of crossing the blood-brain barrier is a selected from the group consisting of Seq-1-7 (SEQ ID NO:36), Seq-1-8 (SEQ ID NO:37), and Angiopep-2-7 (SEQ ID NO:38).

5 (vi) *Cell penetrating peptide (CPP)*

The terms "cell-penetrating peptide" (CPP) refers to a peptide, typically of about 5-60 amino acid residues in length, that can facilitate cellular uptake of molecular cargo, particularly proteins they are a part of. Proteins can present one or more CPPs. CPPs can also be characterized as being able to facilitate the movement or traversal of molecular
10 cargo across/through one or more of a lipid bilayer, cell membrane, organelle membrane, vesicle membrane, or cell wall. A CPP herein will be polycationic.

Examples of CPPs useful herein, and further description of CPPs in general, are disclosed in Schmidt et al. [2010. FEBS Lett. 584:1806-1813], Holm et al. [2006. Nature Protocols
15 1:1001-1005], Yandek et al, [2007. Biophys. J. 92:2434-2444], Morris et al. [2001. Nat. Biotechnol. 19:1173-1176]. and U.S. Patent Application Publication No. 2014/0068797. CPPs do not depend on transporters or receptors, facilitating the traffic of the proteins they are part of directly through the lipid bilayer without the need of participation by any other cell components.

20

 (vii) *Nucleolin-binding peptide*

Accordingly, a "nucleolin-binding peptide" is a peptide capable of binding to the nucleolin protein in a cell, preferably to the cell-surface expressed fraction of nucleolin.
25 In an embodiment of the invention, the polycationic peptide of the invention is a nucleolin-binding peptide.

The International Patent Application Publication with number WO 2011/031477 A2 offers numerous examples of nucleolin-binding peptides that would be suitable for use in
30 the conjugate of the invention.

In a preferred embodiment of the invention, the nucleolin-binding peptide of the invention is the peptide of sequence SEQ ID NO:47 or the peptide of sequence SEQ ID NO:48.

IV- C. The third polypeptide region of the conjugate

5

In a particular embodiment, the polypeptide of the conjugate of the sixth aspect of the invention further comprises a third polypeptide region which is a positively charged amino acid-rich region.

- 10 The third polypeptide region which is a positively charged amino acid-rich region and comprised in the polypeptide of the conjugate is also referred to as the “third polypeptide of the conjugate”, “third polypeptide region of the conjugate”, “the third polypeptide region” or “third polypeptide region comprised in the polypeptide of the conjugate”. As understood by a skilled person, the expressions “third polypeptide of the conjugate”,
15 “third polypeptide region of the conjugate” or “third polypeptide region comprised in the polypeptide of the conjugate” are interchangeable with “positively charged amino acid-rich region”.

- The term “positively charged amino acid”, “positively charged amino-acid rich region”
20 or “third polypeptide region which is a positively charged amino acid-rich region”, as used herein, refers to the polypeptide sequence of the third polypeptide of the conjugate, different from the second polypeptide, region of the conjugate, characterized in that it contains multiple positively charged amino acids. In addition, the positively charged amino acid-rich region may be formed exclusively by positively charged amino acids or
25 may contain other amino acids provided that the overall net charge of the region at pH 7 is positive. Thus, the positively charged amino acid-rich region sequence may comprise 33%, preferably 40%, preferably 45%, preferably 50%, preferably 55%, preferably 60%, preferably 65%, preferably 70%, preferably 75%, preferably 80%, preferably 85%, more preferably 90%, more preferably 95%, even more preferably 99%, yet even more
30 preferably 100% of the amino acid residues of its complete sequence as positively charged amino acids residues.

The positively charged amino acid-rich region may contain only one type of positively charged amino acid or may contain more than one type of positively charged amino acid. In one embodiment, the positively charged amino acid-rich region is a polyhistidine region. In one embodiment, the positively charged amino acid-rich region is a polyarginine region. In one embodiment, the positively charged amino acid-rich region is a polyhistidine region. In one embodiment, the positively charged amino acid-rich region comprises lysine and arginine residues. In one embodiment, the positively charged amino acid-rich region comprises lysine and histidine residues. In one embodiment, the positively charged amino acid-rich region comprises arginine and histidine residues. In one embodiment, the positively charged amino acid-rich region comprises lysine, arginine and histidine residues

In some embodiments, the positively charged amino acid-rich region comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 positively charged amino acids residues, wherein the positively charged amino acid can be histidine, lysine, arginine or combinations thereof.

In some embodiments, the positively charged amino acid-rich region comprises fewer than 100, fewer than 90, fewer than 80, fewer than 70, fewer than 60, fewer than 50, fewer than 40, fewer than 30, fewer than 29, fewer than 28, fewer than 27, fewer than 26, fewer than 25, fewer than 24, fewer than 23, fewer than 22, fewer than 21, fewer than 20, fewer than 19, fewer than 18, fewer than 17, fewer than 16, fewer than 15, fewer than 14, fewer than 13, fewer than 12, fewer than 11, fewer than 10 or less positively charged amino acids residues, wherein the positively charged amino acid can be histidine, lysine, arginine or combinations thereof.

In some embodiments, the positively charged amino acid-rich region comprises between 2 and 50 amino acids, between 2 and 40 amino acids, between 2 and 30 amino acids, between 2 and 25 amino acids, between 2 and 20 amino acids, between 2 and 10 amino acids or between 2 and 8 amino acids.

In some embodiments, the positively charged amino acid-rich region comprises between 3 and 50 amino acids, between 3 and 40 amino acids, between 3 and 30 amino acids, between 3 and 25 amino acids, between 3 and 20 amino acids, between 3 and 10 amino acids or between 3 and 8 amino acids. In some embodiments, the positively charged amino acid-rich region comprises between 4 and 50 amino acids, between 4 and 40 amino acids, between 4 and 30 amino acids, between 4 and 25 amino acids, between 4 and 20 amino acids, between 4 and 10 amino acids or between 4 and 8 amino acids. In some embodiments, the positively charged amino acid-rich region comprises between 5 and 50 amino acids, between 5 and 40 amino acids, between 5 and 30 amino acids, between 5 and 25 amino acids, between 5 and 20 amino acids, between 5 and 10 amino acids or between 5 and 8 amino acids.

In an embodiment of the invention, the positively charged amino acid-rich region of the conjugate of the invention is a polyhistidine region. In a preferred embodiment of the invention, the polyhistidine region comprises between 2 and 10, preferably 6, contiguous histidine residues.

In an embodiment of the invention, the positively charged amino acid-rich region of the conjugate of the invention is a polyarginine region. In a preferred embodiment of the invention, the polyarginine region comprises between 2 and 10, preferably 6, contiguous arginine residues.

In an embodiment of the invention, the positively charged amino acid-rich region of the fusion protein of the invention is a polylysine region. In a preferred embodiment of the invention, the polylysine region comprises between 2 and 10, preferably 6, contiguous polylysine residues.

In a particular embodiment, the positively charged peptide sequence is RKRKRK (SEQ ID NO.77), RRRRRR (SEQ ID NO. 78), KKKKKK (SEQ ID NO:79), HHHHHH (SEQ ID NO. 80), RHRHRH (SEQ ID NO. 81), RKRKRKRK (SEQ ID NO. 82), RKRHRK (SEQ ID NO. 83), RKRHRH (SEQ ID NO. 84), RHRHRH (SEQ ID NO. 85) or RKRKRKR (SEQ ID NO: 86).

IV-D. Relative positions of the elements of the polypeptide of the conjugate and linking elements

5 The different elements of the polypeptide of the conjugate of the invention (first, second and third polypeptides of the conjugate) can be placed in any relative order provided that the second polypeptide, wherein said polypeptide is preferably a polycationic peptide, and the third polypeptide (or the positively charged amino acid-rich region) are functional on any position of the conjugate and also the first polypeptide remains functional totally
10 or in part (i.e. it substantially maintains the structure of the G2 domain of nidogen-1).

As used herein, the terms “N-terminal end”, “N-terminus”, and “amino-terminal end” of a polypeptide are indistinct. Equally, the terms “C-terminal end”, “C-terminus”, and “carboxy-terminal end” are considered equivalent. The terms are of common usage for
15 the person skilled in the art regarding the free moieties of the amino acids at the ends of the polypeptide chains comprised by a protein.

Thus, in an embodiment of the invention, the second polypeptide of the conjugate is located at the N-terminal end of the polypeptide of the conjugate, while the positively charged amino acid-rich region (i.e. the third polypeptide of the conjugate) of the
20 polypeptide is located at the C-terminal end of the polypeptide. In another embodiment of the invention, the positively charged amino acid-rich region of the polypeptide of the conjugate is located at the N-terminal end of the polypeptide, while the second polypeptide region is located at the C-terminal end of the polypeptide. In another
25 embodiment of the invention, the first polypeptide region can be located at either the C-terminal end or the N-terminal end of the polypeptide of the conjugate, while the second polypeptide is in the middle position of the polypeptide and the positively charged amino acid-rich region is at the end of the polypeptide opposite the first polypeptide region, or the positively charged amino acid-rich region is in the middle position of the polypeptide
30 and the second polypeptide is located at the end of the polypeptide opposite the first polypeptide region.

Accordingly, the relative order of the elements of the polypeptide of the conjugate according to the invention, can be:

- N-second polypeptide region-first polypeptide region - positively charged amino acid-rich region-C;
- 5 ▪ N- positively charged amino acid-rich region-first polypeptide region -second polypeptide region -C;
- N-second polypeptide region - positively charged amino acid-rich region-first polypeptide region -C;
- 10 ▪ N- positively charged amino acid-rich region-second polypeptide region -first polypeptide region -C;
- N-first polypeptide region -second polypeptide region - positively charged amino acid-rich region-C; or
- N-first polypeptide region - positively charged amino acid-rich region-second polypeptide region -C

15

In a particular embodiment, the order of the elements in the polypeptide of the conjugate of the sixth aspect of the invention is any of those indicated above.

20 In a preferred embodiment, the order of the elements in the polypeptide of the conjugate of the sixth aspect of the invention is N-second polypeptide region - first polypeptide region - positively charged amino acid-rich region -C.

25 The terms “N-terminal end” and “C-terminal end” do not mean that the components need to be directly conjugated end-to-end, but that they maintain that relative order of positions regardless of the presence of additional elements at the end of either component or intercalated between them, such as linkers/spacers.

Therefore, the polypeptide of the conjugate of the invention comprises the aforementioned elements ((1) second polypeptide region, (2) first polypeptide region and 30 (3) positively charged amino acid-rich region) and these can be conjugated end-to-end but also may include one or more optional peptide or polypeptide “linkers” or “spacers” intercalated between them, linked, preferably by peptidic bond.

According to the invention, the spacer or linker amino acid sequences can act as a hinge region between components (1) and (2) and (2) and (3), allowing them to move independently from one another while maintaining the three-dimensional form of the individual domains, such that the presence of peptide spacers or linkers does not alter the functionality of any of the components (1), (2) and (3). In this sense, a preferred intermediate amino acid sequence according to the invention would be a hinge region characterized by a structural ductility allowing this movement. In a particular embodiment, said intermediate amino acid sequence is a flexible linker. The effect of the linker region is to provide space between the components (1) and (2) and (2) and (3). It is thus assured that the secondary and tertiary structure of component (1), (2) or (3) is not affected by the presence of either of the others. The spacer is of a polypeptide nature. The linker peptide preferably comprises at least 2 amino acids, at least 3 amino acids, at least 5 amino acids, at least 10 amino acids, at least 15 amino acids, at least 20 amino acids, at least 30 amino acids, at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 70 amino acids, at least 80 amino acids, at least 90 amino acids or approximately 100 amino acids.

The spacer or linker can be bound to components flanking the two components of the polypeptide of the conjugates of the invention by means of covalent bonds, preferably by peptide bonds; and also preferably the spacer is essentially afunctional, and/or is not prone to proteolytic cleavage, and/or does not comprise any cysteine residue. Similarly, the three-dimensional structure of the spacer is preferably linear or substantially linear.

The preferred examples of spacer or linker peptides include those that have been used to bind proteins without substantially deteriorating the function of the bound peptides or at least without substantially deteriorating the function of one of the bound peptides. More preferably the spacers or linkers used to bind peptides comprise coiled coil structures.

Preferred examples of linker peptides comprise 2 or more amino acids selected from the group consisting of glycine, serine, alanine and threonine. A preferred example of a flexible linker is a polyglycine linker. The possible examples of linker/spacer sequences

include GGSSRSS (SEQ ID NO:39), GGSSRSSS (SEQ ID NO:76), SGGTSGSTSGTGST (SEQ ID NO:49), AGSSTGSSTGPGSTT (SEQ ID NO:50) or GGSGGAP (SEQ ID NO:51). These sequences have been used for binding designed coiled coils to other protein domains [Muller, K.M., Arndt, K.M. and Alber, T., Meth. Enzymology, 2000, 328: 261-281]. Further non-limiting examples of suitable linkers comprise the amino acid sequence GGGVEGGG (SEQ ID NO:52), the sequence of 10 amino acid residues of the upper hinge region of murine IgG3 (PKPSTPPGSS, SEQ ID NO:53), which has been used for the production of dimerized antibodies by means of a coiled coil [Pack, P. and Pluckthun, A., 1992, Biochemistry 31:1579-1584], the peptide of sequence APAETKAEPMT (SEQ ID NO:54), the peptide of sequence GAP, the peptide of sequence AAA and the peptide of sequence AAALE (SEQ ID NO:55). In another preferred embodiment, the linker is GGSSRSS (SEQ ID NO:39).

Alternatively, the components of the polypeptide of the conjugates of the invention can be connected by peptides the sequence of which contains a cleavage target for a protease, thus allowing the separation of any of the components. Protease cleavage sites suitable for their incorporation into the polypeptides of the conjugates of the invention include enterokinase (cleavage site DDDDK, SEQ ID NO:56), factor Xa (cleavage site IEDGR, SEQ ID NO:57), thrombin (cleavage site LVPRGS, SEQ ID NO:58), TEV protease (cleavage site ENLYFQG, SEQ ID NO:59), PreScission protease (cleavage site LEVLFQGP, SEQ ID NO:60), inteins and the like.

In a preferred embodiment, the polypeptide at the N-terminal position connects with the polypeptide at the middle position in the polypeptide of the conjugate by a linker, preferably a linker selected from any of the examples of linkers provided above. In another preferred embodiment, the polypeptide at the middle position connects with the polypeptide at the C-terminal position of the polypeptide of the conjugate by a linker, preferably a linker selected from any of the examples of linkers provided above. Thus, in an embodiment of the invention, the second polypeptide is connected to the first polypeptide region through a linker. In another embodiment of the invention, the first polypeptide region is connected to the positively charged amino acid-rich region through a linker. In yet another embodiment of the invention, the second polypeptide is connected

to the first polypeptide region through a linker and the first polypeptide region is bound to the positively charged amino acid-rich region through a linker also.

Thus in a particular embodiment, the second polypeptide region is connected to the first polypeptide region via a first peptide linker and/or the first polypeptide region is
5 connected to the third polypeptide region via a second peptide linker. In a particular embodiment, the first peptide linker comprises the GGSSRSS sequence (SEQ ID NO:39) sequence, the GGSSRSSS (SEQ ID NO:76), or the GGGNS sequence (SEQ ID NO:40). In a preferred embodiment, it comprises the GGSSRSS sequence (SEQ ID NO:39). In another preferred embodiment, it comprises GGSSRSS (SEQ ID NO:39).

10

As the person skilled in the art will acknowledge, the linkers connecting the second polypeptide to the first polypeptide region and the first polypeptide region to the positively charged amino acid-rich region may comprise the same sequence or different ones with the aforementioned limitation so that the presence and/or sequence of the
15 linkers does not result in functional alterations of the second polypeptide, the first polypeptide region, and/or the positively charged amino acid-rich region (for instance, but not limited to, due to secondary or tertiary structure modifications of the polypeptide of the conjugate or formation of disulfide bonds).

20 The aforementioned considerations regarding the relative positions from the N-terminal end to the C-terminal end of the elements of the polypeptide of the conjugate apply also in the presence of linkers between them, independently of the number of them or what elements are placed in-between. Therefore, the possible combinations and relative orders of elements will be the following (wherein the numbering stated above for the elements
25 is retained: (1) second polypeptide, (2) first polypeptide, (3) positively charged amino acid-rich region):

- N-(1)-(2)-(3)-C
- N-(1)-linker-(2)-(3)-C
- N-(1)-(2)-linker-(3)-C
- 30 ▪ N-(1)-linker-(2)-linker-(3)-C
- N-(3)-(2)-(1)-C
- N-(3)-linker-(2)-(1)-C

- N-(3)-(2)-linker-(1)-C
 - N-(3)-linker-(2)-linker-(1)-C
 - N-(2)-(1)-(3)-C
 - N-(2)-linker-(1)-(3)-C
 - 5 ▪ N-(2)-(1)-linker-(3)-C
 - N-(2)-linker-(1)-linker-(3)-C
 - N-(2)-(3)-(1)-C
 - N-(2)-linker-(3)-(1)-C
 - N-(2)-(3)-linker-(1)-C
 - 10 ▪ N-(2)-linker-(3)-linker-(1)-C
 - N-(1)-(3)-(2)-C
 - N-(1)-(3)-linker-(2)-C
 - N-(1)-linker-(3)-(2)-C
 - N-(1)-linker-(3)-linker-(2)-C
 - 15 ▪ N-(3)-(1)-(2)-C
 - N-(3)-linker-(1)-(2)-C
 - N-(3)-(1)-linker-(2)-C
 - N-(3)-linker-(1)-linker-(2)-C
- 20 In a preferred embodiment of the invention, the linkers of the polypeptide of the conjugate of the invention comprise the sequence GGSSRSS (SEQ ID NO:39) or the GGGNS sequence (SEQ ID NO:40).
- In a preferred embodiment, the polypeptide at the N-terminal position connects with the
- 25 polypeptide at the middle position of the polypeptide of the conjugate through a protease cleavage site, preferably through a protease cleavage site selected from any of the examples of protease cleavage sites provided above. In another preferred embodiment, the polypeptide at the middle position connects with the polypeptide at the C-terminal position of the conjugate through a protease cleavage site, preferably through a protease
- 30 cleavage site from any of the examples of cleavage sites provided above.

In another embodiment, the second polypeptide is connected to the first polypeptide region through a protease cleavage site. In another embodiment of the invention, the first polypeptide region is connected to the positively charged amino acid-rich region through a protease cleavage site. In yet another embodiment of the invention, the second
 5 polypeptide is connected to the first polypeptide region through a protease cleavage site and the first polypeptide region is bound to the positively charged amino acid-rich region through a protease cleavage site also.

As the person skilled in the art will acknowledge, the protease cleavage sites connecting
 10 the second polypeptide to the first polypeptide region and the first polypeptide region to the positively charged amino acid-rich region may comprise the same sequence or different ones with the aforementioned limitation so that the presence and/or sequence of the protease cleavage site does not result in functional alterations of the second polypeptide, the first polypeptide region, and/or the positively charged amino acid-rich
 15 region (for instance, but not limited to, due to secondary or tertiary structure modifications of the polypeptide of the conjugate or formation of disulfide bonds).

The aforementioned considerations regarding the relative positions from the N-terminal end to the C-terminal end of the elements of the polypeptide of the conjugate apply also
 20 in the presence of protease cleavage sites between them, independently of the number of them or what elements are placed in-between. Therefore, the possible combinations and relative orders of elements will be the following (wherein the numbering stated above for the elements is retained: (1) second polypeptide, (2) first polypeptide, (3) positively charged amino acid-rich region):

- 25 ▪ N-(1)-(2)-(3)-C
- N-(1)-protease cleavage site-(2)-(3)-C
- N-(1)-(2)- protease cleavage site -(3)-C
- N-(1)-protease cleavage site -(2)- protease cleavage site -(3)-C
- N-(3)-(2)-(1)-C
- 30 ▪ N-(3)- protease cleavage site -(2)-(1)-C
- N-(3)-(2)- protease cleavage site -(1)-C
- N-(3)- protease cleavage site -(2)- protease cleavage site -(1)-C

- N-(2)-(1)-(3)-C
- N-(2)- protease cleavage site -(1)-(3)-C
- N-(2)-(1)- protease cleavage site -(3)-C
- N-(2)- protease cleavage site -(1)- protease cleavage site -(3)-C
- 5 ▪ N-(2)-(3)-(1)-C
- N-(2)- protease cleavage site -(3)-(1)-C
- N-(2)-(3)- protease cleavage site-(1)-C
- N-(2)- protease cleavage site -(3)- protease cleavage site -(1)-C
- N-(1)-(3)-(2)-C
- 10 ▪ N-(1)-(3)- protease cleavage site -(2)-C
- N-(1)- protease cleavage site -(3)-(2)-C
- N-(1)- protease cleavage site -(3)- protease cleavage site -(2)-C
- N-(3)-(1)-(2)-C
- N-(3)- protease cleavage site -(1)-(2)-C
- 15 ▪ N-(3)-(1)- protease cleavage site -(2)-C
- N-(3)- protease cleavage site -(1)- protease cleavage site -(2)-C

In a particular embodiment, the conjugate comprises a linker connecting two polypeptides of the conjugate, and a protease cleavage site connected the other two polypeptides of the conjugate. In this case, the aforementioned considerations regarding the relative positions from the N-terminal end to the C-terminal end of the elements of the conjugate apply also in the presence of linkers and protease cleavage sites between them, independently of the number of them or what elements are placed in-between. Therefore, the possible combinations and relative orders of elements will be the following (wherein the numbering stated above for the elements is retained: (1) second polypeptide, (2) first polypeptide, (3) positively charged amino acid-rich region):

- N-(1)-linker -(2)- protease cleavage site -(3)-C
- N-(1)-protease cleavage site -(2)- linker -(3)-C
- N-(1)- linker -(2)- protease cleavage site -(3)-C
- 30 ▪ N-(1)- protease cleavage site -(2)- linker -(3)-C
- N-(2)- linker -(1)- protease cleavage site -(3)-C
- N-(2)- protease cleavage site -(1)- linker -(3)-C

- N-(2)- linker -(3)- protease cleavage site -(1)-C
- N-(2)- protease cleavage site -(3)- linker -(1)-C
- N-(1)- linker -(3)- protease cleavage site -(2)-C
- N-(1)- protease cleavage site -(3)- linker -(2)-C
- 5 ▪ N-(3)- linker -(1)- protease cleavage site -(2)-C
- N-(3)- protease cleavage site -(1)- linker -(2)-C

In a preferred embodiment, the combinations and relative orders of elements in the polypeptide of the conjugate is N-(1)-linker -(2)- protease cleavage site -(3)-C. Thus, in
 10 a preferred embodiment, the second polypeptide is connected to the first polypeptide region through a linker, and the first polypeptide is connected to the third polypeptide region through a protease cleavage site.

In another preferred embodiment, said linker comprises the sequence GGSSRSS (SEQ ID NO:39), GGSSRSSS (SEQ ID NO:76), or the GGGNS sequence (SEQ ID NO:40),
 15 preferably the sequence GGSSRSS (SEQ ID NO:39).

In preferred embodiments. the conjugates of the sixth aspect of the invention comprise a polypeptide which comprises the following elements:

20

Second polypeptide	first peptide linker	First polypeptide	Third polypeptide
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	Nid (238 amino acids) (SEQ ID NO:62)	Hexahistidine (SEQ ID NO:73)
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	Nid (236 amino acids) SEQ ID NO:63	Hexahistidine (SEQ ID NO:73)
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	Nidomut2 (238 amino acids) as defined in SEQ ID NO:64	Hexahistidine (SEQ ID NO:73)
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	NidoMut2 (236 amino acids) as defined in SEQ ID NO:65	Hexahistidine (SEQ ID NO:73)
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	NidoMut3 or any of SEQ ID NO:88-91 or 95-102	Hexahistidine (SEQ ID NO:73)
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	NidoMut4	Hexahistidine (SEQ ID NO:73)
T22 (SEQ ID NO:27)	GGSSRSS (SEQ ID NO:39)	NidoMut5 or any of SEQ ID NO: 102-105	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 (SEQ ID NO. 29)	GGSSRSS (SEQ ID NO:39)	Nidomut2 (238 amino acids) as defined in SEQ ID NO:64	Hexahistidine (SEQ ID NO:73)

Second polypeptide	first peptide linker	First polypeptide	Third polypeptide
Optimized EPI-X4 to the positively charged region of sequence RKRKRK (SEQ ID NO:133)	GGSSRSSH (SEQ ID NO:39)	NidoMut2 (236 amino acids) as defined in SEQ ID NO:65	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 to the positively charged region of sequence RKRKRK (SEQ ID NO:133)	GGSSRSSH (SEQ ID NO:39)	NidoMut3 or any of SEQ ID NO:88-91 or 95-102	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 to the positively charged region of sequence RKRKRK (SEQ ID NO:133)	GGSSRSSH (SEQ ID NO:39)	NidoMut4	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 to the positively charged region of sequence RKRKRK (SEQ ID NO:133)	GGSSRSSH (SEQ ID NO:39)	NidoMut5 or any of SEQ ID NO: 102-105	Hexahistidine (SEQ ID NO:73)

Table 3: Preferred polypeptides forming part of the conjugates of the invention

In another particular embodiment, a positively charged amino acid, preferably an arginine or a lysine, more preferably a lysine, is comprised between the first polypeptide region and the third polypeptide region of the conjugate of the sixth aspect.

In another preferred embodiment, the polypeptide forming part of the conjugate of the invention comprises, essentially comprises or consists of the amino acid sequence SEQ ID NO:61, optionally containing a methionine at the amino terminus.

10

In some embodiments, the polypeptide forming part of the conjugate of the invention comprises, essentially comprise or consists of the amino acid sequence of any of SEQ ID NO: 61 or 106-124, optionally containing a methionine at the amino terminus.

15 In a particular embodiment, the agent of interest of the conjugate of the sixth aspect of the invention is a therapeutic agent or an imaging agent.

IV-E. The agent of interest

In a particular embodiment, the agent of interest of the conjugate of the sixth aspect of the invention is a therapeutic agent or an imaging agent.

IV-E.1 The therapeutic agent

5

The term “therapeutic agent”, as used herein, is drawn to any compound, without chemical structure limitations, suitable for therapy and/or treatment of a condition, disorder or disease.

- 10 The nature of the therapeutic agent is not particularly limiting for the present invention provided it remains active in the conjugate or can be activated once it is delivered to the inside of the cell. Accordingly, any therapeutic agent can be used in the conjugate provided that it shows an activity or can reach an activity once it is delivered to the inside of the cell of at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least
- 15 50% or less of the activity of the unconjugated therapeutic agent. Alternatively, since the purpose of the invention is to facilitate the action of the therapeutic agent by increasing its selectivity and reducing its off-target effects, it is contemplated that the effects of the therapeutic agent conjugated to the polypeptide of the conjugate may be synergic and exceed the parametrized values already known for the specific therapeutic agent.
- 20 Accordingly, it is intended that some embodiments of the therapeutic agent conjugated to the polypeptide of the conjugate of the invention also show at least 101%, at least 105%, at least 110%, at least 115%, at least 120%, at least 125%, at least 130%, at least 135%, at least 140%, at least 145%, at least 150%, at least 175%, at least 200%, at least 300%, at least 400%, at least 500%, at least 1000%, or more of the functionality of the
- 25 therapeutic agent alone.

In an embodiment of the invention, the therapeutic agent conjugated to the polypeptide of the conjugate of the invention is selected from the group consisting of

- 30 (i) a chemotherapy agent,
- (ii) a cytotoxic polypeptide,
- (ii) an antiangiogenic polypeptide,
- (iii) a polypeptide encoded by a tumor suppressor gene,

- (iv) a pro-apoptotic polypeptide,
- (v) a polypeptide having anti-metastatic activity,
- (vi) a polypeptide encoded by a polynucleotide which is capable of activating the immune response towards a tumor,
- 5 (vii) an antiangiogenic molecule, and
- (viii) a toxin.

In a particular embodiment, the polypeptide of the conjugate is conjugated to a plurality of therapeutic agents, wherein said plurality of therapeutic agents are the same or
10 different.

(i) *Chemotherapy agent*

In a particular embodiment, the therapeutic agent is a chemotherapy agent.
15

It will be understood that the term “chemotherapeutic agents” refers to anti-cancer agents.

As used herein, an anti-cancer agent is an agent that at least partially inhibits the development or progression of a cancer, including inhibiting in whole or in part symptoms
20 associated with the cancer even if only for the short term.

Several anti-cancer agents can be categorized as DNA damaging agents and these include topoisomerase inhibitors (e.g., etoposide, irinotecan, topotecan, teniposide, mitoxantrone), DNA alkylating agents (e.g., cisplatin, mechlorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, busulfan, thiotepa, carmustine, lomustine, carboplatin, dacarbazine, procarbazine), DNA strand break inducing agents (e.g., bleomycin, doxorubicin, daunorubicin, idarubicin, mitomycin C), anti-microtubule agents (e.g., vincristine, vinblastine), anti-metabolic agents (e.g., cytarabine, methotrexate, hydroxyurea, 5-fluorouracil, floxuridine, 6-thioguanine, 6-mercaptopurine, fludarabine, pentostatin, chlorodeoxyadenosine), anthracyclines, vinca alkaloids, or
30 epipodophyllotoxins.

Additional examples of anti-cancer agents include without limitation Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin;

5 Batimastat; Benzodepa; Bicalutamide; Bisantrone Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Bortezomib (VELCADE); Brequinar Sodium; Bropirimine; Busulfan; Cactinomycin; Calusterone; Caracemide; Carbetimer; Carboplatin (a platinum- containing regimen); Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cisplatin (a platinum-containing

10 regimen); Cladribine; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin; Decitabine; Dexormaplatin; Dezaguanine; Diaziquone; Docetaxel (TAXOTERE); Doxorubicin; Droloxifene; Dromostanolone; Duazomycin; Edatrexate; Eflornithine; Elsamitrucin; Enloplatin; Enpromate; Epiropidine; Epirubicin; Erbulozole; Erlotinib (TARCEVA), Esorubicin; Estramustine; Etanidazole; Etoposide;

15 Etoprine; Fadrozole; Fazarabine; Fenretinide; Floxuridine; Fludarabine; 5-Fluorouracil; Flurocitabine; Fosquidone; Fostriecin; Gefitinib (IRESSA), Gemcitabine; Hydroxyurea; Idarubicin; Ifosfamide; Ilmofosine; Imatinib mesylate (GLEEVAC); Interferon alpha-2a; Interferon alpha-2b; Interferon alpha-n1; Interferon alpha-n3; Interferon beta-I a; Interferon gamma-I b; Iproplatin; Irinotecan; Lanreotide; Lenalidomide (REVLIMID,

20 REVIMID); Letrozole; Leuprolide; Liarozole; Lometrexol; Lomustine; Losoxantrone; Masoprocol; Maytansine; Mechlorethamine; Megestrol; Melengestrol; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Metoprine; Meturedopa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone; Mycophenolic Acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran;

25 Paclitaxel; Pemetrexed (ALIMTA), Pegaspargase; Peliomycin; Pentamustine; Pentomone; Peplomycin; Perfosfamide; Pipobroman; Pipsulfan; Piritrexim Isethionate; Piroxantrone; Plicamycin; Plomestane; Porfimer; Porfiromycin; Prednimustine; Procarbazine; Puromycin; Pyrazofurin; Riboprine; Rogletimide; Safingol; Semustine; Simtrazene; Sitogluside; Sparfosate; Sparsomycin; Spirogermanium; Spiromustine;

30 Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Tamsulosin; Taxol; Taxotere; Tecogalan; Tegafur; Teloxantrone; Temoporfin; Temozolomide (TEMODAR); Teniposide; Teroxirone; Testolactone; Thalidomide (THALOMID) and

derivatives thereof; Thiamiprine; Thioguanine; Thiotepa; Tiazofurin; Tirapazamine; Topotecan; Toremifene; Trestolone; Triciribine; Trimetrexate; Triptorelin; Tubulozole; Uracil; Mustard; Uredepa; Vapreotide; Verteporfin; Vinblastine; Vincristine; Vindesine; Vinepidine; Vinglycinate; Vinleurosine; Vinorelbine; Vinrosidine; Vinzolidine;
 5 Vorozole; Zeniplatin; Zinostatin; Zorubicin.

In one embodiment, the anti-cancer agent is provided as an oligomer containing several units of the anti-cancer molecule. In one embodiment, the anti-cancer agent is a floxuridine poly- or oligonucleotide, which comprises several floxuridine molecules. The
 10 floxuridine poly- or oligonucleotide contains at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more floxuridine molecules. In a preferred embodiment the floxuridine polynucleotide is a floxuridine pentanucleotide, i.e. an oligonucleotide containing 5 floxuridine molecules.

15 The anti-cancer agent may be an enzyme inhibitor including without limitation tyrosine kinase inhibitor, a CDK inhibitor, a MAP kinase inhibitor, or an EGFR inhibitor. The tyrosine kinase inhibitor may be without limitation Genistein (4', 5, 7-trihydroxyisoflavone), Tyrphostin 25 (3,4,5-trihydroxyphenyl), methylene]-propanedinitrile, Herbimycin A, Daidzein (4',7-dihydroxyisoflavone), AG-126, trans-1-
 20 (3'-carboxy-4'-hydroxyphenyl)-2-(2'',5''-dihydroxy-phenyl)ethane, or HDBA (2-Hydroxy5-(2,5-Dihydroxybenzylamino)-2-hydroxybenzoic acid. The CDK inhibitor may be without limitation p21, p27, p57, p15, p16, p18, or p19. The MAP kinase inhibitor may be without limitation KY12420 (C₂₃H₂₄O₈), CNI-1493, PD98059, or 4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole. The EGFR
 25 inhibitor may be without limitation erlotinib (TARCEVA), gefitinib (IRESSA), WHI-P97 (quinazoline derivative), LFM-A12 (leflunomide metabolite analog), ABX-EGF, lapatinib, canertinib, ZD-6474 (ZACTIMA), AEE788, and AG1458.

The anti-cancer agent may be a VEGF inhibitor including without limitation bevacizumab
 30 (AVASTIN), ranibizumab (LUCENTIS), pegaptanib (MACUGEN), sorafenib, sunitinib (SUTENT), vatalanib, ZD-6474 (ZACTIMA), anecortave (RETAANE), squalamine lactate, and semaphorin. The anti-cancer agent may be an antibody or an antibody

fragment including without limitation an antibody or an antibody fragment including but not limited to bevacizumab (AVASTIN), trastuzumab (HERCEPTIN), alemtuzumab (CAMPATH, indicated for B cell chronic lymphocytic leukemia), gemtuzumab (MYLOTARG, hP67.6, anti-CD33, indicated for leukemia such as acute myeloid leukemia), rituximab (RITUXAN), tositumomab (BEXXAR, anti-CD20, indicated for B cell malignancy), MDX-210 (bispecific antibody that binds simultaneously to HER-2/neu oncogene protein product and type I Fc receptors for immunoglobulin G (IgG) (Fc gamma RI)), oregovomab (OVAREX, indicated for ovarian cancer), edrecolomab (PANOREX), daclizumab (ZENAPAX), palivizumab (SYNAGIS, indicated for respiratory conditions such as RSV infection), ibritumomab tiuxetan (ZEVALIN, indicated for Non-Hodgkin's lymphoma), cetuximab (ERBITUX), MDX-447, MDX-22, MDX-220 (anti-TAG-72), IOR-C5, 10R-T6 (anti-CD 1), IOR EGF/R3, celogovab (ONCOSCINT OV 103), epratuzumab (LYMPHOCIDE), pemtumomab (THERAGYN), and Gliomab-H (indicated for brain cancer, melanoma).

15

It is contemplated that in certain embodiments of the invention a protein that acts as an angiogenesis inhibitor is targeted to a tumor. These agents include, in addition to the anti-angiogenic polypeptides mentioned above, Marimastat; AG3340; COL-3, BMS-275291, Thalidomide, Endostatin, SU5416, SU6668, EMD121974, 2-methoxyoestradiol, carboxiamidotriazole, CMIOI, pentosan polysulphate, angiopoietin 2 (Regeneron), herbimycin A, PNU145156E, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline, genistein, TNP470, endostatin, paclitaxel, accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM- 1470, platelet factor 4 or minocycline.

Other suitable active agents are DNA cleaving agents. Examples of DNA cleaving agents suitable for inclusion as the cell toxin in the conjugates used in practicing the methods include, but are not limited to, anthraquinone-oligopyrrol-carboxamide, benzimidazole, leinamycin; dynemycin A; enediyne; as well as biologically active analogs or derivatives thereof (i.e., those having a substantially equivalent biological activity). Known analogs and derivatives are disclosed, for examples in Islam et al., J. Med. Chem. 34 2954-61, 1991; Skibo et al., J. Med. Chem. 37:78-92, 1994; Behroozi et al., Biochemistry 35:1568-74, 1996; Helissey et al., Anticancer Drug Res. 11:527-51, 1996; Unno et al., Chem.

30

Pharm. Bull. 45:125-33, 1997; Unno et al., Bioorg. Med. Chem., 5:903-19, 1997; Unno et al., Bioorg. Med. Chem., 5: 883-901, 1997; and Xu et al., Biochemistry 37:1890-7, 1998). Other examples include, but are not limited to, endiynes quinone imines (U.S. Pat. No. 5,622,958); 2,2'-bis (2-aminoethyl)-4,4'-bithiazole [Lee et al., Biochem. Mol. Biol. Int. 40:151-7, 1996]; epilliticine-salen.copper conjugates [Routier et al., Bioconjug. Chem., 8: 789-92, 1997].

Some of the aforementioned chemotherapy agents can be grouped together under a common category as antimetabolites. "Antimetabolite" as used herein, refers to the compounds which inhibit the use of a metabolite that is part of normal metabolism. Antimetabolites are often similar in structure to the metabolite that they interfere with, such as the antifolates that interfere with the use of folic acid. Non-limiting examples of antimetabolites include the following compounds: bleomycin, busulfan, capecitabine, carmustine, carboplatin, chlorodeoxyadenosine, cisplatin, cyclophosphamide, cytarabine, dacarbazine, daunorubicin, docetaxel, doxorubicin, etoposide, fludarabine, fluorouracil, gemcitabine, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine, melphalan, mercaptopurine, methotrexate, mitomycin, mitoxantrone, oxaliplatin, paclitaxel, procarbazine, SN-38, thioguanine, thiotepa, teniposide, vinblastine, vincristine, and vinorelbine.

20

In a particular embodiment, the anti-cancer agent is an antimetabolite. In another particular embodiment, the antimetabolite is a pyrimidine analogue, or an oligomeric form thereof. In another particular embodiment, the pyrimidine analogue is floxuridine, or a pentameric form thereof.

25

The term "pyrimidine analog", as used herein, refers to nucleoside analog antimetabolites which mimic the structure of pyrimidines. The pyrimidine analogues interfere with nucleic acid synthesis. Their antiproliferative effect is achieved through incorporation into DNA, causing chain termination and inhibition of DNA synthesis. They can also interfere with enzymes involved in nucleic acid synthesis, such as DNA polymerases and ribonucleotide reductase. Non-limiting examples of pyrimidine analogs include:

30

Azacitidine, 6-Azauracil, Cytarabine, Decitabine, Gemcitabine, Troxacitabine, Floxuridine, Fluorouracil, Capecitabine, Tegafur-uracil.

The term “floxuridine”, as used herein, refers to an anti-cancer agent classified as
5 antimetabolite, which is a pyrimidine analog, classified as a deoxyuridine. The IUPAC
name of said anti-cancer agent is 5-Fluoro-1-[4-hydroxy-5-
(hydroxymethyl)tetrahydrofuran-2-yl]-1H-pyrimidine-2,4-dione.

The expression “oligomeric form thereof”, as used herein, refers to a molecule formed by
10 a few repeating units, as opposed to a polymer which is not limited to specific number of
units, wherein each unit is termed a monomer. Generally, the number of monomers in an
oligomer is between 5 and 100. Thus, an oligomeric form of pyrimidine analogue, refers
herein to a molecule formed by a sequence of a few pyrimidine analogues. In a particular
embodiment, the oligomeric or polymeric form of pyrimidine analogues refers to a
15 molecule comprising a sequence of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40,
45, 50 or more pyrimidine analogs. In another particular embodiment, it refers to a
molecule consisting of a sequence of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35,
40, 45, 50 or more pyrimidine analogs.

20 In a particular embodiment, the oligomeric or polymeric form of pyrimidine analogue is
a molecule comprising a sequence of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35,
40, 45, 50 or more floxuridines. In another particular embodiment, the oligomeric form
of pyrimidine analogue is a molecule consisting of a sequence of at least 2, 3, 4, 5, 6, 7,
8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more floxuridines.

25

The expression “pentameric form thereof”, when referring to a floxuridine analogue, is
understood as molecule comprising, or consisting of, a sequence of 5 floxuridines.

(ii) *Cytotoxic polypeptides*

30 As used herein, the term “cytotoxic polypeptide” refers to an agent that is capable of
inhibiting cell function. The agent may inhibit proliferation or may be toxic to cells. Any
polypeptides that when internalized by a cell interfere with or detrimentally alter cellular

metabolism or in any manner inhibit cell growth or proliferation are included within the ambit of this term, including, but not limited to, agents whose toxic effects are mediated when transported into the cell and also those whose toxic effects are mediated at the cell surface. Useful cytotoxic polypeptides include proteinaceous toxins such as bacterial
5 toxins.

Examples of proteinaceous cell toxins useful for incorporation into the conjugates according to the invention include, but are not limited to, type one and type two ribosome inactivating proteins (RIP). Useful type one plant RIPs include, but are not limited to,
10 dianthin 30, dianthin 32, lychnin, saporins 1-9, pokeweed activated protein (PAP), PAP II, PAP-R, PAP-S, PAP-C, mapalmin, dodecandrin, bryodin-L, bryodin, Colicin 1 and 2, luffin-A, luffin-B, luffin-S, 19K-protein synthesis inhibitory protein (PSI), 15K-PSI, 9K-PSI, alpha-kirilowin, beta-kirilowin, gelonin, momordin, momordin-II, momordin-Ic, MAP-30, alpha-momorcharin, beta-momorcharin, trichosanthin, TAP-29, trichokirin;
15 barley RIP; flax RIP, tritin, corn RIP, Asparin 1 and 2 [Stirpe *et al.*, 1992. Bio/Technology 10:405-12]. Useful type two RIPs include, but are not limited to, volkensin, ricin, nigrin-b, CIP-29, abrin, modeccin, ebullitin-[alpha], ebullitin-[beta], ebullitin-[gamma], vircumin, porrectin, as well as the biologically active enzymatic subunits thereof [Stirpe *et al.*, 1992. Bio/Technology 10:405-12; Pastan *et al.*, 1992. Annu. Rev. Biochem. 61:331-54;
20 Brinkmann and Pastan, 1994. Biochim. et Biophys. Acta 1198:27-45; and Sandvig and Van Deurs, 1996. Physiol. Rev. 76:949-66].

Examples of bacterial toxins useful as cell toxins include, but are not limited to, shiga toxin and shiga-like toxins (i.e., toxins that have the same activity or structure), as well as the catalytic subunits and biologically functional fragments thereof. These bacterial
25 toxins are also type two RIPs [Sandvig and Van Deurs, 1996. Physiol. Rev. 76:949-66; Armstrong, 1995. J. Infect. Dis., 171:1042-5; Kim *et al.*, 1997. Microbiol. Immunol. 41:805-8; and Skinner *et al.*, 1998. Microb. Pathog. 24:117-22]. Additional examples of useful bacterial toxins include, but are not limited to, *Pseudomonas* exotoxin and Diphtheria toxin [Pastan *et al.*, 1992. Annu. Rev. Biochem. 61:331-54; and Brinkmann
30 and Pastan, 1994. Biochim. et Biophys. Acta 1198:27-45]. Truncated forms and mutants of the toxin enzymatic subunits also can be used as a cell toxin moiety (Pastan *et al.*, Annu. Rev. Biochem. 61:331-54; Brinkmann and Pastan, Biochim. et Biophys. Acta

1198:27-45, 1994; Mesri *et al.*, J. Biol. Chem. 268:4852-62, 1993; Skinner *et al.*, Microb. Pathog. 24:117-22, 1998; and U.S. Pat. No. 5,082,927). Other targeted agents include, but are not limited to the more than 34 described Colicin family of RNase toxins which include colicins A, B, D, E1-9, cloacin DF13 and the fungal RNase, [alpha]-sarcin
 5 [Ogawa *et al.* 1999. Science 283: 2097-100.; Smarda *et al.*, 1998. Folia Microbiol (Praha) 43:563-82; Wool *et al.*, 1992. Trends Biochem. Sci., 17: 266-69].

(iii) *Antiangiogenic polypeptides*

Proliferation of tumor cells relies heavily on extensive tumor vascularization, which
 10 accompanies cancer progression. Thus, inhibition of new blood vessel formation with anti-angiogenic agents and targeted destruction of existing blood vessels have been introduced as effective and relatively non-toxic approaches to tumor treatment.

The term "anti-angiogenic polypeptide", as used herein, denotes a polypeptide capable of
 15 inhibiting angiogenesis. Suitable antiangiogenic polypeptides include, without limitation, angiostatin, endostatin, anti-angiogenic anti-thrombin III, sFRP-4 as described in WO2007115376, and an anti-VEGF antibody such as anibizumab, bevacizumab (avastin), Fab IMC 1121 and F200 Fab.

20 (iv) *Polypeptides encoded by a tumor suppressor gene*

As used herein, a "tumor suppressor" is a gene or gene product that has a normal biological role of restraining unregulated growth of a cell. The functional counterpart to a tumor suppressor is an oncogene—genes that promote normal cell growth may be known as "proto-oncogenes" A mutation that activates such a gene or gene product
 25 further converts it to an "oncogene", which continues the cell growth activity, but in a dysregulated manner Examples of tumor suppressor genes and gene products are well known in the literature and may include PTC, BRCA1, BRCA2, p16, APC, RB, WT1, EXT1, p53, NF1, TSC2, NF2, VHL, ST7, ST14, PTEN, APC, CD95 or SPARC.

30 (v) *Pro-apoptotic polypeptides*

The term "pro-apoptotic polypeptides", as used herein, refers to a protein which is capable of inducing cell death in a cell or cell population. The overexpression of these proteins

involved in apoptosis displaces the careful balance between anti-apoptotic and pro-apoptotic factors towards an apoptotic outcome. Suitable pro-apoptotic polypeptides include, without limitation, pro-apoptotic members of the BCL-2 family of proteins such as BAX, BAK, BOK/MTD, BID, BAD, BIK/NBK, BLK, HRK, BIM/BOD, BNIP3, NIX,
 5 NOXA, PUMA, BMF, EGL-I, and viral homologs, caspases such as caspase-8, the adenovirus E4orf4 gene, p53 pathway genes, pro-apoptotic ligands such as TNF, FasL, TRAIL and/or their receptors, such as TNFR, Fas, TRAIL-R1 and TRAIL-R2.

(vi) *Polypeptides with anti-metastatic activity*

10 The term “metastasis suppressor” as used herein, refers to a protein that acts to slow or prevent metastases (secondary tumors) from spreading in the body of an organism with cancer. Suitable metastasis suppressors include, without limitation, proteins such as BRMS 1, CRSP3, DRG1, KAI1, KISS-1, NM23, a TIMP-family protein and uteroglobin.

15 (vii) *Polypeptides encoded by a polynucleotide capable of activating the immune response towards a tumor*

As used herein, an immunostimulatory polypeptide agent is a polypeptide encoded by a polynucleotide which is capable of activating or stimulating the immune response (including enhancing a pre-existing immune response) in a subject to whom it is
 20 administered, whether alone or in combination with another agent. Suitable non-limiting examples of immunostimulatory peptides include flagellin, muramyl dipeptide), cytokines including interleukins (e.g., IL-2, IL-7, IL- 15 (or superagonist/mutant forms of these cytokines), IL-12, IFN-gamma, IFN-alpha, GM-CSF, FLT3-ligand, etc.), immunostimulatory antibodies (e.g., anti-CTLA-4, anti-CD28, anti-CD3, or single
 25 chain/antibody fragments of these molecules), and the like.

(viii) *Antiangiogenic molecules*

It is also contemplated that in certain embodiments the intervening region of the fusion protein of the invention corresponds to a protein that acts as an angiogenesis inhibitor is
 30 targeted to a tumor. These agents include, in addition to the anti-angiogenic polypeptides mentioned above, Marimastat; AG3340; COL-3, BMS-275291, Thalidomide, Endostatin, SU5416, SU6668, EMD121974, 2-methoxyoestradiol, carboxiamidotriazole, CM101,

pentosan polysulphate, angiopoietin 2 (Regeneron), herbimycin A, PNU145156E, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline, genistein, TNP470, endostatin, paclitaxel, accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline. Also included are VEGF inhibitors including
5 without limitation bevacizumab (AVASTIN), ranibizumab (LUCENTIS), pegaptanib (MACUGEN), sorafenib, sunitinib (SUTENT), vatalanib, ZD-6474 (ZACTIMA), anecortave (RETAANE), squalamine lactate, and semaphorin.

(ix) Toxins

10 As used herein, the term “toxins” refers to non-proteinaceous/non-polypeptidic cytotoxic compounds obtained from different organisms, as well as chemically modified derivatives of those same compounds and compounds obtained through chemical synthesis. The compounds of this category with biological origin may be obtained from
15 microorganisms (whether bacteria, archaea, protozoa or unicellular fungi) or pluricellular organisms (pluricellular fungi, plants, or animals, like mollusks). It is intended that the chemical composition and structure of these toxins is not limited in any way beyond their non-polypeptidic nature, therefore one or more amino acids may be part of their structure, whether as part of their basic composition or as result of chemical derivation, as long as all the amino acids participating in the structure are not bound together by peptide bonds.

20

Examples of toxins suitable for the invention are calicheamycin γ 1, dolastatin 10, maytansinoid (DM1) and pyrrolbenzodiazepine dimer (PBD).

(x) Additional therapeutic agents

25

In a particular embodiment of the conjugate of the invention, the therapeutic agent is selected from the therapeutic agents indicated in the third column of table 4 below.

Ligand	Cell receptor/cell type or microorganism	Therapeutic agent	Disease or disorder
Folic acid or folic acid targeting ligand, including methotrexate or aminopterin.	Folate receptor in leucocyte	Cytotoxic agent	Leukemia
Hyalurinic acid (HA)	CD44		
Xanthine scaffold, including lingalipitin	fibroblast activation protein (FAP) on wound fibroblast cells	Growth factor	Wounds
IgG Fc	Protein A in <i>S. aureus</i>	antimicrobial agent (antibacterial, antifungal, antiprotozoal, or antiviral)	Infections/sepsis
Ligands of Her 2 are well-known in the art, and can be any of those described in Wikman M et al., Protein Eng Des Sel 17: 455-62 (2004); Orlova A et al. Cancer Res 66: 4339-8 (2006); Ahlgren S et al., Bioconj Chem 19: 235-43 (2008); Feldwisch J et al., J Mol Biol 398: 232-47 (2010); US patents with patent number: 5,578,482; 5,856,110; 5,869,445; 5,985,553; 6,333,169; 6,987,088; 7,019,017; 7,282,365; 7,306,801; 7,435,797; 7,446,185; 7,449,480; 7,560,111; 7,674,460; 7,815,906; 7,879,325; 7,884,194; 7,993,650; 8,241,630; 8,349,585; 8,389,227; 8,501,909; 8,512,967; 8,652,474; and U.S. patent application US20110059090A1	HER2	Cytotoxic agent	Breast cancer
CRPPR peptide	Heart endothelium	NAP -2, TGF- α , ErbB3, VEGF, IGF-1, FGF-2, PDGF, IL-2, CD 19, CD20, and/or CD80/86, prostaglandins (PGE2, PGI2)	Cardiac disease
Angiotensin	angiotensin II type 1 (AT1) receptor	NAP -2, TGF- α , ErbB3, VEGF, IGF-1, FGF-2, PDGF, IL-2, CD 19, CD20, and/or CD80/86,	Cardiac disease

		prostaglandins (PGE2, PGI2)	
Ligand for interleukin-6 (IL-6) such as: - The S7 peptide described in SU JL et al. Cancer Res. 2019 Jul 15;79(14):3791, and with SEQ ID NO:66 (LSLITRL) - the IL6 ligand described in Weiergräber O et al. FEBS Lett. 1996 Jan 29;379(2):122-6 and with SEQ ID NO:67 (WQDPHSWNSSFYRLRFELRYRAERSKTFTTW); - interleukin-6 receptor (IL-6R); PDGF; angiopoietin; angiopoietin 2; Tie2; S1P; integrins av beta 3, av beta 5, and a5 beta 1; betacellulin; apelin/API; erythropoietin; complement factor D; TNFa; HtrA1; a VEGF receptor; ST-2 receptor; and proteins genetically linked to age-related macular degeneration (AMD) risk such as complement pathway components C2, factor B, factor H, CFHR3, C3b, C5, C5a, C3a, HtrA1, ARMS2, TIMP3, HLA, interleukin-8 (IL-8), CX3CR1, TLR3, TLR4, CETP, LPC, COL10A1, and TNFRSF10A	Cells from the eye, preferably cells from the retinal pigment epithelium.	Anti-VEGF (Pegaptanib Ranibizumab Bevacizumab Aflibercept or conbercept)	intraocular neovascular disease selected from the group consisting of proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusion (RVO), including Central Retinal Vein Occlusion (CRVO) and branched retinal vein occlusion (BRVO), corneal neovascularization, retinal neovascularization,

				and retinopathy of prematurity (ROP).
Opsonins, including vitronectin, fibronectin, complement components such as C1q (including any of its component polypeptide chains A, B and C), complement fragments such as C3d, C3b and C4b, mannose-binding protein, conglutinin, surfactant proteins A and D, C-reactive protein (CRP), alpha2-macroglobulin, and immunoglobulins, for example, the Fc portion of an immunoglobulin	Microbe (virus, bacterial cell, fungal cell, protozoa)	antimicrobial agent (antibacterial, antifungal, antiprotozoal, or antiviral)	Infections/sepsis	
Mannose-binding lectin (MBL)	Microbe (virus, bacterial cell, fungal cell, protozoa)	antimicrobial agent (antibacterial, antifungal, antiprotozoal, or antiviral)	Infections/sepsis	

Table 4 Ligands, and receptors, cells or microorganisms, to which said ligands bind are provided in columns 1 and 2, as in table 1 above. In addition, therapeutic agents to be associated to said ligands for the treatment of a certain disease or disorder are provided in the third column, and the disease or disorder to be treated in the fourth column. The agents to be associated to a certain ligand or group of ligands is provided in the same row as said ligand or group of ligands. The disease or disorder to be treated by the association of a ligand, or group of ligands, and a therapeutic agent, or a group of therapeutic agents, are provided in the same row as said ligand/s and therapeutic agent/s.

In a particular embodiment of the conjugate of the invention, the second polypeptide region is a ligand selected from the ligands provided in Table 4, and the agent of interest is selected from the therapeutic agents provided in the same row as said ligand in Table

5 4. Another particular embodiment refers to the conjugate of the invention as defined in the previous embodiments of this section, for use in the treatment of a disease or disorder, wherein said disease or disorder is selected from those provided in Table 4. Another particular embodiment refers to the conjugate of the invention as defined in the previous

10 from those provided in the row of table 4, corresponding to the row where the second polypeptide region of the conjugate is provided.

IV-E.2 Imaging agent

15 The term "imaging agent" is used herein to refer to a biocompatible compound, the use of which facilitates the differentiation of different parts of an image, by increasing the contrast between those different regions of the image. The term "imaging agent" thus encompasses agents that are used to enhance the quality of an image that may nonetheless be generated in the absence of such an agent (as is the case, for instance, in MRI), as well

20 as agents that are prerequisites for the generation of an image (as is the case, for instance, in nuclear imaging). Suitable imaging agents include, without limitation, imaging agents for Radionuclide imaging, for computerized tomography, for Raman spectroscopy, for Magnetic resonance imaging (MRI) and for optical imaging.

25 Imaging agents for radionuclide imaging include iodine 123, technetium 99, indium 111, rhenium 188, rhenium 186, copper 67, iodine 131, yttrium 90, iodine 125, astatine 211, gallium 67, iridium 192, cobalt 60, radium 226, gold 198, cesium 137 and phosphorus 32 ions. Examples of fluorogenic agents include gadolinium and renographin. Examples of paramagnetic ions include chromium (III), manganese (II), iron (III), iron (II), cobalt (II),

30 nickel (H)³ copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III) ions.

Imaging agents for optical imaging include, for example, fluorescein, a fluorescein derivative, indocyanine green, Oregon green, a derivative of Oregon green derivative, rhodamine green, a derivative of rhodamine green, an eosin, an erythrosin, Texas red, a derivative of Texas red, malachite green, nanogold sulfosuccinimidyl ester, cascade blue, a coumarin derivative, a naphthalene, a pyridyloxazole derivative, cascade yellow dye, dapoxyl dye. It also includes fluorescent proteins, that refer herein to proteins whose atomic structure allows them to present fluorescence, which is a phenomenon well-known in the art. Non-limiting examples of commonly used fluorescent proteins suitable for the conjugate of the invention, are the green fluorescent protein (GFP, first discovered in *Aequorea victoria*), the red fluorescent protein (RFP), the yellow fluorescent protein (YFP), the blue fluorescent protein (BFP), the cyan fluorescent protein, or any other variant, examples of which can be found in Kremers et al. [Kremers, G-J- et al. 2011. J.Cell Sci. 124:157-160].

Additional non-limiting examples of fluorescent proteins suitable for the conjugate of the invention are the enhanced green fluorescent protein (eGFP), enhanced cyan fluorescent protein CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz (TYFP), Venus, Citrine, mCitrine, GFPuv, destabilized EGFP (dEGFP), destabilised ECFP (dECFP), destabilized EYFP (dEYFP), mCFPm, Cerulean, T-Sapphire, CyPet, YPet, mKO, HcRed, t-HcRed, DsRed, DsRed2, DsRed- monomer, J-Red, dimer2, t-dimer2(12), mRFPI, pocilloporin, Renilla GFP, Monster GFP, paGFP, Kaede protein and kindling protein, Phycobiliproteins and Phycobiliprotein conjugates including B-Phycoerythrin, R-Phycoerythrin and Allophycocyanin. In other embodiments, the imaging agent is a fluorescent protein selected from the group consisting of the mHoneydew, mBanana, mOrange, dTomato, tdTomato, mTangerine, mStrawberry, mCherry, mGrapel, mRaspberry, mGrape2, mPlum [Shaner et al. (2005) Nat. Methods 2:905-909], and the like.

Imaging agent for magnetic resonance imaging apparatus gadolinium chelates, manganese chelates, chromium chelates, ^{19}F and iron particles.

MRI imaging agents include complexes of metals selected from the group consisting of chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III).

5

IV-E.3 Linkage of the agent of interest to the polypeptide of the conjugate

The polypeptide of the conjugate can be conjugated to a single agent of interest or to a plurality of agents. When a plurality of agents are conjugated to the polypeptide of the conjugate, said agents can be either the same or different. In a particular embodiment, said plurality of agents of interest are therapeutic agents, and they are the same or different therapeutic agents, as defined above. In another particular embodiment, said plurality of agents of interest are imaging agents, and they are the same or different imaging agents, as defined above.

15

The following applies to any agent of interest, i.e. to a therapeutic, to an imaging agent, to a plurality of therapeutic agents, and to a plurality of imaging agents as just defined. Thus in a particular embodiment, any of the following embodiment addressed to an agent apply to a therapeutic agent by substituting the term “agent” by “therapeutic agent”. In another particular embodiment, any of the following embodiments addressed to an agent apply to an imaging agent by substituting the term “agent” by “imaging agent”. In another particular embodiment, any of the following embodiments apply to a plurality of therapeutic agents, by substituting the expression “plurality of agents” by “plurality of therapeutic agents”, where said plurality of therapeutic agents is as just defined above. In another particular embodiment, any of the following embodiments apply to a plurality of imaging agents, by substituting the expression “plurality of agents” by “plurality of imaging agents”, where said plurality of imaging agents is as just defined above.

25

The agent or agents of interest can be conjugated to any sequence of the polypeptide of the conjugate.

30

Therefore, in a particular embodiment, the agent of interest is conjugated to the first polypeptide region. In a particular embodiment, it is conjugated to the second polypeptide region of the conjugate. In another particular embodiment, it is conjugated to the third polypeptide region of the conjugate. In another particular embodiment, it is conjugated to
5 any of the linking region between the first and the second polypeptide. In another particular embodiment, it is conjugated to the linking region between the second and the third polypeptide region. In another particular embodiment, it is conjugated to the linking region between the second and the third polypeptide region. In another particular embodiment, it is conjugated to the protease cleavage site between the first and the second
10 polypeptide regions. In another particular embodiment, it is conjugated to the protease cleavage site between the first and the third polypeptide regions. In another particular embodiment, it is conjugated to the protease cleavage site between the second and the third polypeptide regions.

15 In a particular embodiment, the plurality of agents of interest are conjugated the same polypeptide region of the conjugate. In a preferred embodiment, they are conjugated to the first polypeptide region. In a particular embodiment, they are conjugated to the second polypeptide region of the conjugate. In another particular embodiment, they are conjugated to the third polypeptide region of the conjugate. In another particular
20 embodiment, they are conjugated to all three polypeptide regions of the polypeptide of the conjugate. In another particular embodiment, they are conjugated to the first and the second polypeptide regions of the polypeptide. In another particular embodiment, they are conjugated to the first and third polypeptide regions of the conjugate. In another particular embodiment, they are conjugated to the second and third polypeptide regions
25 of the conjugate.

In another particular embodiment, they are conjugated to the linking regions of the polypeptide of the conjugate. In another particular embodiment, they are conjugated to the linking region between the second and the first polypeptide regions. In another
30 particular embodiment, they are conjugated to the linking region between the first and the third polypeptide regions. In another particular embodiment, they are conjugated to the linking region between the second and the third polypeptide regions. In another particular

embodiment, they are conjugated to the protease cleavage sites of the polypeptide of the conjugate. In another particular embodiment, they are conjugated to the protease cleavage site between the second and the first polypeptide regions. In another particular embodiment, they are conjugated to the protease cleavage site between the first and the
5 third polypeptide regions. In another particular embodiment, they are conjugated to the protease cleavage site between the second and the third polypeptide regions. In another particular embodiment, they are conjugated to all the protease cleavage sites of the polypeptide.

10 In another particular embodiment, they are conjugated to any of the polypeptide regions indicated above and to the linking regions of the polypeptide of the conjugate. In another particular embodiment, they are conjugated to any of the polypeptide regions indicated above and to the linking region between the second and the first polypeptide regions. In another particular embodiment, they are conjugated to any of the polypeptide regions
15 indicated above and to the linking region between the first and the third polypeptide regions. In another particular embodiment, they are conjugated to any of the polypeptide regions indicated above and to the linking region between the second and the third polypeptide regions. In another particular embodiment, they are conjugated to any of the polypeptide regions of the polypeptide indicated above and to all the linking regions of
20 the polypeptide.

In another particular embodiment, they are conjugated to any of the polypeptide regions indicated above and to the protease cleavage sites of the polypeptide of the conjugate. In another particular embodiment, they are conjugated to any of the polypeptide regions
25 indicated above and to the protease cleavage site between the first polypeptide region and the second polypeptide region. In another particular embodiment, they are conjugated to any of the polypeptide regions of the polypeptide and to the protease cleavage site between the first and the third polypeptide region. In another particular embodiment, they are conjugated to any of the polypeptide regions of the polypeptide and to the protease
30 cleavage site between the second and the third polypeptide region. In another particular embodiment, they are conjugated to the polypeptide regions indicated above and to all the cleavage sites of the polypeptide.

In another particular embodiment, they are conjugated to the linking region of the polypeptide and to the protease cleavage site of the polypeptide. In another particular embodiment, they are conjugated to any of the polypeptide regions indicated above, to
5 the linking region and to the protease cleavage site of the polypeptide of the conjugate.

It is intended that the agent of interest, as aforementioned, is conjugated to the polypeptide of the conjugate, without limitation of the position of the conjugation inside the polypeptide with regards to its N-terminal and C-terminal ends. Accordingly, the agent
10 of interest can be conjugated to the polypeptide of the conjugate in an equidistant position with respect to the N-terminal and C-terminal ends or it can be closer to either of them. Hence, the agent of interest can be conjugated to the polypeptide region at a distance of 500, 450, 400, 350, 325, 300, 275, 250, 236, 230, 220, 210, 200, 190, 180, 170, 160, 100, 90, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 30, 25, 20, 15, 20, 10, 5, or less amino
15 acid residues from the N-terminal or C-terminal end of said polypeptide, or at the same residue of the N-terminal or C-terminal end of said polypeptide. This paragraph applies to each agent conjugated to the polypeptide, where a plurality of agents are conjugated to the polypeptide of the conjugate.

20 The only intended limitation in the conjugation position/s of the agent/s of interest is that the agent/s and the elements of the polypeptide are functional and the conjugation of the agent/s does not interfere with the activity of either the agent, the polypeptide or the conjugate.

25 So, the agent of interest, the second polypeptide, the first polypeptide region and the positively charged amino acid-rich region conserve at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, preferably 95%, more preferably 99%, even more preferably 100% of their functionality with respect to their non-conjugated forms. This also applies regardless of
30 the position of the conjugation in the polypeptide of the conjugate. This paragraph also applies to each of the agents conjugated to the polypeptide where a plurality of agents are conjugated to the polypeptide of the conjugate. It is intended that the agent can either be

conjugated directly to a residue of the polypeptide of the conjugate or indirectly through a linking moiety.

Thus, in a particular embodiment, the agent of interest is conjugated to the polypeptide
5 of the conjugate directly. In another particular embodiment, the agent of interest is conjugated to the polypeptide of the conjugate through a linking moiety.

In another particular embodiment, where a plurality of agents are conjugated to the polypeptide of the conjugate, all of them are conjugated directly to a residue of the
10 polypeptide. In another particular embodiment, where a plurality of agents are conjugated to the polypeptide of the conjugate, part of them are conjugated directly to a residue of the polypeptide and the rest are conjugated indirectly through a linking moiety. In another particular embodiment, all the agents are conjugated through a linking moiety.

15 The expression “linking moiety”, or “linker” has already been defined in the second aspect of the invention.

The person skilled in the art will acknowledge that the previous provisions regarding the functionality of the elements of the polypeptide of the conjugate and the agent apply also
20 whenever a coupler mediates the conjugation between an agent of interest and the polypeptide of the conjugate. Therefore, whenever an agent of interest is conjugated to the polypeptide of the conjugate through a linking moiety, said agent of interest, the second polypeptide region, the first polypeptide region and the positively charged amino acid-rich region conserve at least 45%, at least 50%, at least 55%, at least 60%, at least
25 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, preferably 95%, more preferably 99%, even more preferably 100% of their functionality with respect to their non-conjugated forms, regardless of the position of the conjugation in the polypeptide of the conjugate, the chemical composition or structure of the linking moiety and the chemical nature of the bond/s between the linking moiety and the agent and
30 between the linking moiety and the polypeptide of the conjugate. This applies to all the agents of interest when a plurality of agents are conjugated to the polypeptide of the conjugate.

- In a preferred embodiment of the invention, the linking moiety that mediates the attachment between the agent and the polypeptide of the conjugate is a 6-Maleimido hexanoic acid N-hydroxysuccinimide ester or a 4-maleimido hexanoic acid N-hydroxysuccinimide ester. In another particular embodiment, where a plurality of agents are conjugated to the polypeptide of the conjugate, all the linking moieties that mediate the attachment of the agent/s to the polypeptide of the conjugate are 6-Maleimido hexanoic acid N-hydroxysuccinimide ester or a 4-maleimido hexanoic acid N-hydroxysuccinimide ester. In another particular embodiment, where a plurality of agents are conjugated to the polypeptide of the conjugate, part of the linking moieties that mediate the attachment of the agent/s to the polypeptide of the conjugate are 6-Maleimido hexanoic acid N-hydroxysuccinimide ester or a 4-maleimido hexanoic acid N-hydroxysuccinimide ester.
- 15 In another preferred embodiment, the linking moiety that mediates the attachment between the agent and the polypeptide of a conjugate is a moiety which is capable of reacting with the sulfhydryl groups present in the side chain of the polypeptide and an active group in the agent of interest. Suitable linking groups capable of reacting with sulfhydryl groups present in the side chain of the polypeptide include, without limitation, 20 maleimido reagents, haloacetyls, aziridines, acryloyls, arylating agents, vinylsulfones, pyridyl disulfides, TNB-thiols and disulfide reducing agents. Most of these groups conjugate to sulfhydryls by either alkylation (usually the formation of a thioether bond) or disulfide exchange (formation of a disulfide bond).
- 25 In some embodiments, the linking moiety contains a spacer region which connects the part of the linking moiety that is connected to the polypeptide forming part of the conjugate and the part of the linking moiety that is connected to the agent of interest. In some embodiments, the linking moiety is connected with the agent of interest by the spacer and the linking moiety connects the spacer-agent of interest with the polypeptide.
- 30 In one embodiment, the linking moiety is connected with the polypeptide and the linking moiety connects the spacer-polypeptide with the agent of interest.

As used herein the term "spacer" refers to a moiety that connects at least two other moieties with each other. In some embodiments, the spacer is a polymer.

As used herein, the term "polymer" means a molecule comprising repeating structural units, i.e. the monomers, connected by chemical bonds in a linear, circular, branched, crosslinked or dendrimeric way or a combination thereof, which may be of synthetic or biological origin or a combination of both. The monomers may be identical, in which case the polymer is a homopolymer, or may be different, in which case the polymer is a heteropolymer. A heteropolymer may also be referred to as a "copolymer" and includes, for example, alternating copolymers in which monomers of different types alternate, periodic copolymers, in which monomers of different types are arranged in a repeating sequence; statistical copolymers, in which monomers of different types are arranged randomly; block copolymers, in which blocks of different homopolymers consisting of only one type of monomers are linked by a covalent bond; and gradient copolymers, in which the composition of different monomers changes gradually along a polymer chain. In some embodiments, the polymer comprise one or more other moieties, which in certain embodiments are selected from the group consisting of C₁₋₅₀ alkyl, C₂₋₅₀ alkenyl, C₂₋₅₀ alkynyl, C₃₋₁₀ cycloalkyl, 3- to 10-membered heterocyclyl, 8- to 11-membered heterobicyclyl, phenyl, naphthyl, indenyl, indanyl, and tetralinyl. In some embodiments, the spacer is a PEG-based spacer.

As used herein, the term "PEG-based" in relation to a spacer means that said spacer comprises PEG. Such PEG-based moiety or reagent comprises at least 10 percent (w/w) PEG, such as at least 20 percent (w/w) PEG, such as at least 30 percent (w/w) PEG, such as at least 40 percent (w/w) PEG, such as at least 50 percent (w/w), such as at least 60 (w/w) PEG, such as at least 70 percent (w/w) PEG, such as at least 80 percent (w/w) PEG, such as at least 90 percent (w/w) PEG, or such as at least 95 percent (w/w) PEG. The remaining weight percentage of the PEG-based moiety or reagent may be other moieties, such as those selected from the group consisting of:

[0079] • C₁₋₅₀ alkyl, C₂₋₅₀ alkenyl, C₂₋₅₀ alkynyl, C₃₋₁₀ cycloalkyl, 3- to 10-membered heterocyclyl, 8- to 11-membered heterobicyclyl, phenyl, naphthyl, indenyl, indanyl, and tetralinyl; branching points, such as -CR<, >C< or -N<; and linkages selected from the

group consisting of wherein dashed lines indicate attachment to the remainder of the moiety or reagent, and -R and -R^a are independently of each other selected from the group consisting of -H, and C₁₋₆ alkyl; and which moieties and linkages are optionally further substituted.

5

In some embodiments of the invention, the linking moiety binding agent/s to the polypeptide of a conjugate is susceptible to be processed by enzymes present in the cytoplasm, releasing the therapeutic agent from the fusion protein once the therapy agent conjugated to the fusion protein has been internalized in a cell.

10

Besides, some agents may be polymerized in such a way that multiple copies of the same molecule may be bound together, forming polymers where each monomer of the polymer is one of said molecules. A non-limiting example of such polymers is 5-Fluoro-2'-deoxyUridine (FdU), which result in oligo-FdU. It is intended that some embodiments of the invention may comprise such polymers. Also, it is intended that some other embodiments of the invention may comprise polymers of 2 or more different molecules of agents provided that the agents do not interfere with the physiological or biological effects of each other. The person skilled in the art will recognize that those embodiments of the invention featuring polymers of agents of interest may feature 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50 or more molecules polymerized together of 1 or more different agents of interest in a proportion of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, or more.

20

IV-F. Reporter proteins

In another embodiment of the invention, the polypeptide of the conjugate of the invention further comprises a reporter protein.

25

The person skilled in the art will acknowledge the term "reporter protein" as referring to a protein resulting from the expression of a "reporter gene". Reporter proteins are well known and commonly used in the art as markers suitable for multiple purposes, such as location of the expression of the reporter genes in tissues, cells or subcellular locations, protein-protein interactions, transport across the plasmatic membranes or endomembranes, vesicular traffic, ligand-receptor interactions, etcetera.

30

Useful reporter proteins in the context of the present invention include luciferase-4-monooxygenase from *Photinus pyralis*, β -galactosidase, thymidine kinase, and the like. The reporter proteins also include fluorescent proteins, which have already been
 5 discussed.

The reporter protein comprised by the polypeptide of the conjugate of the invention is directly adjacent to the positively charged amino acid-rich region or separated by a linker. The relative position of the positively charged amino acid-rich region, however, remains
 10 as per the aforementioned considerations about the relative position of the elements of the fusion protein. Hence, independently of the position of the positively charged amino acid-rich region in the fusion protein, the fluorescent protein is always adjacent to it, either directly or separated by a linker.

15 Accordingly, in the embodiments of the invention comprising a fluorescent protein, the possible relative positions of the elements of the polypeptide of the conjugate of the invention would fit the following scheme (wherein RP refers to a reporter protein and the numbering stated above for the elements is retained: (1) second polypeptide region, (2) first polypeptide region, (3) positively charged amino acid region):

20

- N-(1)-(2)-RP-(3)-C
- N-(1)-linker-(2)-RP-(3)-C
- N-(1)-protease cleavage site-(2)-RP-(3)-C
- N-(1)-(2)-linker-RP-(3)-C
- 25 ▪ N-(1)-(2)- protease cleavage site -RP-(3)-C
- N-(1)-linker-(2)-linker-RP-(3)-C
- N-(1)- protease cleavage site-(2)- protease cleavage site -RP-(3)-C
- N-(1)- linker -(2)- protease cleavage site -RP-(3)-C
- N-(1)- protease cleavage site-(2)- linker -RP-(3)-C
- 30 ▪ N-(3)-RP-(2)-(1)-C
- N-(3)-RP-linker-(2)-(1)-C
- N-(3)-RP- protease cleavage site -(2)-(1)-C

- N-(3)-RP-(2)-linker-(1)-C
- N-(3)-RP-(2)-linker-(1)-C
- N-(3)-RP-linker-(2)-linker-(3)-C
- N-(3)-RP- protease cleavage site -(2)- protease cleavage site -(3)-C
- 5 ▪ N-(3)-RP- linker -(2)- protease cleavage site -(3)-C
- N-(3)-RP- protease cleavage site -(2)- linker -(3)-C
- N-(1)-(2)-RP-linker-(3)-C
- N-(1)-(2)-RP- protease cleavage site -(3)-C
- N-(1)-linker-(2)-RP-linker-(3)-C
- 10 ▪ N-(1)- protease cleavage site -(2)-RP- protease cleavage site -(3)-C
- N-(1)- linker -(2)-RP- protease cleavage site -(3)-C
- N-(1)- protease cleavage site -(2)-RP- linker -(3)-C
- N-(1)-(2)-linker-RP-linker-(3)-C
- N-(1)-(2)- protease cleavage site -RP- protease cleavage site -(3)-C
- 15 ▪ N-(1)-(2)- linker -RP- protease cleavage site -(3)-C
- N-(1)-(2)- protease cleavage site -RP- linker -(3)-C
- N-(1)-linker-(2)-linker-RP-linker-(3)-C
- N-(1)- protease cleavage site -(2)- protease cleavage site -RP- protease cleavage site -(3)-C
- 20 ▪ N-(1)- protease cleavage site -(2)- linker -RP- protease cleavage site -(3)-C
- N-(1)- protease cleavage site -(2)- protease cleavage site -RP- linker -(3)-C
- N-(1)- linker -(2)- protease cleavage site -RP- protease cleavage site -(3)-C
- N-(1)- linker -(2)- linker -RP- protease cleavage site -(3)-C
- N-(1)- linker -(2)- protease cleavage site -RP- linker -(3)-C
- 25 ▪ N-(1)- protease cleavage site -(2)- linker -RP- linker -(3)-C
- N-(3)-linker-RP-(2)-(1)-C
- N-(3)- protease cleavage site -RP-(2)-(1)-C
- N-(3)-linker-RP-linker-(2)-(1)-C
- N-(3)- protease cleavage site -RP- protease cleavage site -(2)-(1)-C
- 30 ▪ N-(3)- protease cleavage site -RP- linker -(2)-(1)-C
- N-(3)- linker -RP- protease cleavage site -(2)-(1)-C
- N-(3)-linker-RP-(2)-linker-(1)-C

- N-(3)- protease cleavage site -RP-(2)- protease cleavage site -(1)-C
- N-(3)- linker -RP-(2)- protease cleavage site -(1)-C
- N-(3)- protease cleavage site -RP-(2)- linker -(1)-C
- N-(3)-linker-RP-linker-(2)-linker-(3)-C
- 5 ▪ N-(3)- protease cleavage site -RP- protease cleavage site -(2)- protease cleavage site -(3)-C
- N-(3)- linker -RP- protease cleavage site -(2)- protease cleavage site -(3)-C
- N-(3)- protease cleavage site -RP- linker -(2)- protease cleavage site -(3)-C
- N-(3)- protease cleavage site -RP- protease cleavage site -(2)- linker -(3)-C
- 10 ▪ N-(3)- linker -RP- linker -(2)- protease cleavage site -(3)-C
- N-(3)- linker -RP- protease cleavage site -(2)- linker -(3)-C
- N-(3)- protease cleavage site -RP- linker -(2)- linker -(3)-C
- N-(2)-(1)-RP-(3)-C
- N-(2)-linker-(1)-RP-(3)-C
- 15 ▪ N-(2)- protease cleavage site -(1)-RP-(3)-C
- N-(2)-(1)-linker-RP-(3)-C
- N-(2)-(1)- protease cleavage site -RP-(3)-C
- N-(2)-linker-(1)-linker-RP-(3)-C
- N-(2)- protease cleavage site -(1)- protease cleavage site -RP-(3)-C
- 20 ▪ N-(2)- linker -(1)- protease cleavage site -RP-(3)-C
- N-(2)- protease cleavage site -(1)- linker -RP-(3)-C
- N-(2)-RP-(3)-(1)-C
- N-(2)-(3)-RP-(1)-C
- N-(2)-linker-RP-(3)-(1)-C
- 25 ▪ N-(2)- protease cleavage site -RP-(3)-(1)-C
- N-(2)-linker-(3)-RP-(1)-C
- N-(2)- protease cleavage site -(3)-RP-(1)-C
- N-(2)-RP-(3)-linker-(1)-C
- N-(2)-RP-(3)- protease cleavage site -(1)-C
- 30 ▪ N-(2)-(3)-RP-linker-(1)-C
- N-(2)-(3)-RP- protease cleavage site -(1)-C
- N-(2)-linker-RP-(3)-linker-(1)-C

- N-(2)- protease cleavage site -RP-(3)- protease cleavage site -(1)-C
- N-(2)- linker -RP-(3)- protease cleavage site -(1)-C
- N-(2)- protease cleavage site -RP-(3)- linker -(1)-C
- N-(2)-linker-(3)RP--linker-(1)-C
- 5 ▪ N-(2)- protease cleavage site -(3)RP-protease cleavage site -(1)-C
- N-(2)- linker -(3)RP-protease cleavage site -(1)-C
- N-(2)- protease cleavage site -(3)RP-linker -(1)-C
- N-(1)-RP-(3)-(2)-C
- N-(1)-(3)-RP-(2)-C
- 10 ▪ N-(1)-RP-(3)-linker-(2)-C
- N-(1)-RP-(3)- protease cleavage site -(2)-C
- N-(1)-(3)-RP-linker-(2)-C
- N-(1)-(3)-RP- protease cleavage site -(2)-C
- N-(1)-linker-RP-(3)-(2)-C
- 15 ▪ N-(1)- protease cleavage site -RP-(3)-(2)-C
- N-(1)-linker-(3)-RP-(2)-C
- N-(1)- protease cleavage site -(3)-RP-(2)-C
- N-(1)-linker-RP-(3)-linker-(2)-C
- N-(1)- protease cleavage site -RP-(3)- protease cleavage site -(2)-C
- 20 ▪ N-(1)- linker -RP-(3)- protease cleavage site -(2)-C
- N-(1)- protease cleavage site -RP-(3)- linker -(2)-C
- N-(1)-linker-(3)-RP-linker-(2)-C
- N-(1)- protease cleavage site -(3)-RP- protease cleavage site -(2)-C
- N-(1)- linker -(3)-RP- protease cleavage site -(2)-C
- 25 ▪ N-(1)- protease cleavage site -(3)-RP- linker -(2)-C
- N-RP-(3)-(1)-(2)-C
- N-(3)-RP-(1)-(2)-C
- N-RP-(3)-linker-(1)-(2)-C
- N-RP-(3)- protease cleavage site -(1)-(2)-C
- 30 ▪ N-(3)-RP-linker-(1)-(2)-C
- N-(3)-RP- protease cleavage site -(1)-(2)-C
- N-RP-(3)-(1)-linker-(2)-C

- N-RP-(3)-(1)- protease cleavage site -(2)-C
- N-(3)-RP-(1)-linker-(2)-C
- N-(3)-RP-(1)- protease cleavage site -(2)-C
- N-RP-(3)-linker-(1)-linker-(2)-C
- 5 ▪ N-RP-(3)- protease cleavage site -(1)- protease cleavage site -(2)-C
- N-RP-(3)- linker -(1)- protease cleavage site -(2)-C
- N-RP-(3)- protease cleavage site -(1)- linker -(2)-C
- N-(3)-RP-linker-(1)-linker-(2)-C
- N-(3)-RP- protease cleavage site -(1)- protease cleavage site -(2)-C
- 10 ▪ N-(3)-RP- linker -(1)- protease cleavage site -(2)-C
- N-(3)-RP- protease cleavage site -(1)- linker -(2)-C

IV-G. Preferred conjugates of the invention

- 15 Preferred embodiments of the invention are conjugates in which the components are as defined in the Table 3 above and wherein the agent of interest is one or more copies of a floxuridine or floxuridine pentanucleotide. In a more preferred embodiment, the conjugates defined above result from linkages between an amino group or a thiol group in the side chain of the first region of the polypeptide forming the conjugate and a thiol
- 20 or hydroxy or phosphate group or amino or carboxy in the therapeutic agent connected or not by a 9 to 35-atom spacer-linker region.

IV-H Stoichiometry of the conjugates of the invention

- 25 The number of agents of interest which are conjugated to the fusion protein of the invention, while not being particularly limitative, will depend on the number of available residues in the polypeptide of the invention which are available for chemical conjugation with the agent of interest. Since most conjugations occur via amino- or sulfhydryl groups present in the side chains of the amino acids forming part of the polypeptide of the
- 30 conjugate, the number of agents conjugated to the polypeptide of the conjugate will depend on the number of lysine and arginine residues (for a conjugation via an amino groups in the side chains) or on the number of cysteine residues (for conjugation via

sulfhydryl groups in the side chains) as well as on the yield of the conjugation reaction. Thus, in a particular embodiment of the invention, the polypeptide of the conjugate of the invention is conjugated to at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, 25, 30 agents of interest.

5

It will be understood that, in the particular case wherein the agent is provided as a polymer, the number of agents will also depend on the number of the monomers in the polymer. In the particular case of a FdU oligomer, the number of agents of interest in a given conjugate will be the result of multiplying the number of oligomers attached to the polypeptide of the conjugate by the number of monomers. In the preferred case of a FdU
10 pentamer, preferred embodiments include conjugates comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 75, 85, 100, 125, 150 or more therapeutic agents per polypeptide of the invention, corresponding, respectively, to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, 25 or 30 FdU pentamers conjugated per molecule.

15

In addition, the nanoparticles according to the invention result from the assembly of multiple copies of the conjugates of the invention. In preferred embodiments, the nanoparticle comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 25, more preferably at least 15 monomers of the conjugate of the invention.

20

Accordingly, the total number of agents of interest attached to each nanoparticle will depend on (i) the number of agents conjugated to each polypeptide of the conjugate, (ii) the oligomerization state of the agents and (iii) the number of conjugates forming the nanoparticle. In preferred embodiments, the nanoparticle is conjugated to at least 30, 35,
25 40, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300 agents of interest. In a further preferred embodiment, the nanoparticle is conjugated to at least 30, 35, 40, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, 100, more preferably at least 60 molecules of FdU pentamer.

30 In a certain embodiment, all the terms and embodiments described in the first, second, third, fourth and fifth aspects of the invention are equally applicable to the sixth aspect of the invention

V- Method for preparing the conjugates of the invention

In a seventh aspect, the invention relates to a method to prepare the conjugate of the sixth
5 aspect of the invention comprising the steps of:

- (i) providing the polypeptide of the conjugate of the sixth aspect of the invention comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- (ii) contacting said polypeptide with an activated form of the agent of interest of
10 the conjugate according to the sixth aspect of the invention which is capable of reacting with at least one group in the polypeptide and wherein the contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the agent of interest and the group in the polypeptide

15

In another embodiment, the invention relates to a method to prepare the conjugates of the sixth aspect of the invention comprising the steps of:

- (i) providing the polypeptide of the conjugate of the sixth aspect of the invention comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof wherein the polypeptide is provided in an activated form and
- (ii) contacting said polypeptide with the agent of interest which is capable of
20 reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptide and the group in the agent of interest

25

The person skilled in the art will recognize that “reactive group”, as used herein, refers to any moiety of a molecule which is capable of chemically reacting with another moiety from another molecule in such a fashion so as to bind the two molecules together, usually with the release of one or more additional molecules. Many such reactions are known in
30 the art such as the formation of the peptide bond between a carboxyl and an amine group being one non-limiting example among them.

“Activated”, as used herein when referring to a molecule, refers to a modified version of the molecule which contains a chemical modification whereby said molecule is capable to chemically react in a manner not previously present in the molecule (for instance, the activation adds a reactive group not present previously, allowing for a bond that was
5 unfeasible before) or with an increased reactivity (meaning that the reaction of the molecule with another molecule requires a lower activation energy than in the inactivated state). The present invention contemplates the possibility of activating the agent of interest of the conjugate and then contacting the activated agent with the polypeptide of the conjugate or of activating the polypeptide of the conjugate and then contacting the
10 activated polypeptide with the agent of interest. In both cases, the activation of the polypeptide or of the agent of interest is usually carried out by reacting the molecule to be activated with a reagent that introduces the reactive group in the molecule to be activated. Examples of reactive groups that allow the agent of interest of the conjugate or the polypeptide of the conjugate to be activated include, but are not limited, to carboxyl,
15 amine, imine, thiol, sulfone, hydroxyl, sulfate, and phosphate moieties, among many others which are commonly known to the person skilled in the art. The activated form of the agent of interest of the conjugate is also herein referred to as the “activated agent of interest”. The activated form of the polypeptide of the conjugate is also herein referred to as the “activated polypeptide”. The reactive group or groups in the activated polypeptide
20 is or are located in the regions of the polypeptide where the agent of interest is/are to be conjugated, as described in the sixth aspect of the invention. Therefore, in a particular embodiment, the reactive group is located: in the first polypeptide region, in the second polypeptide region, in the third polypeptide region, in the linker/s between any of said polypeptide regions, or in the protease cleavage site/s between said polypeptide regions
25 of the conjugate. In another particular embodiment, the reactive groups are located: in the first polypeptide region, and/or in the second polypeptide region, and/or in the third polypeptide region, and/or in the linkers between any of said polypeptide regions, and/or in the protease cleavage sites between said polypeptide regions of the conjugate.

30 In a preferred embodiment, it is/they are located in the first polypeptide region comprised in the polypeptide of the conjugate. In a particular embodiment the reactive groups are

located in the first polypeptide region of the conjugate and also in other regions comprised in the polypeptide of the conjugate of the sixth aspect just mentioned.

In those embodiments of the invention wherein a linking moiety mediates the bond
5 between the polypeptide and the agent of interest of the conjugate, the linking moiety is a bifunctional cross-linker and, more preferably, a heterobifunctional cross-linker, that reacts with the groups in the agent of interest and in the polypeptide of the conjugate, either sequentially (either reacting with the activated agent first and then with the polypeptide, or first with the polypeptide and then with the activated agent) or
10 simultaneously, using among other linkages such as thioethers, amide bonds, carbon-nitrogen double bonds, or linkages generated by cycloaddition as disclosed in Kalia J et al. *Advances in bioconjugation. Curr Org Chem* 2010 January, 14(2):138-147). As a way of example typical thiol-reactive functional groups include iodoacetamides, maleimides, and disulfides. In addition, a protein can be treated with a small molecule or surface
15 displaying an activated ester (e.g., an N-hydroxysuccinimidyl ester) to form amide bonds with the amino groups on lysine side chains and the N terminus. In another embodiment, the linking moiety is a heterobifunctional cross-linker which contains reactive groups capable of reacting with a thiol group and with an amino group. In one embodiment, the heterobifunctional cross-linker is 6-maleimidohexanoic acid N-hydroxysuccinimide
20 ester.

In a preferred embodiment, the linking moiety reacts in a first step with the activated agent of interest and in a second step with the polypeptide of the conjugate. In another embodiment, the linking moiety reacts in a first step with the polypeptide of the conjugate
25 and, in a second step, with the agent of interest.

It is intended that the step of contacting the polypeptide of the conjugate of the sixth aspect of the invention with the activated form of the agent of interest is carried out in a medium which favors the reaction establishing the bond between them. Media suitable
30 for the reactions are commonly known to the person skilled in the art, including aqueous buffers and non-aqueous buffers. It is also intended that solid supports can be used in conjunction with the media for any of the reaction steps conducing to the synthesis of the

activated agent and the conjugation of the polypeptide of the conjugate, and the agent of interest, as well as the linking moiety in the embodiments that include one. Furthermore, it is intended that the method for the preparation of the conjugates between the polypeptide and the therapeutic agent is not limited to comprise the polypeptide, the
5 activated agent of interest, and the linking moiety, but that some embodiments include also the use of one or more catalysts and co-factors in the reaction.

Thus, in one embodiment of the invention, the activated form of the agent of interest contains a group which reacts with at least one of the side chains in the polypeptide of the
10 conjugate, preferably in the first polypeptide region comprised in the polypeptide of the conjugate. As a skilled person will understand “a side chain in the polypeptide” refers to a side chain in an amino acid residue of the polypeptide sequence.

In another preferred embodiment said residue is an external lysine. In a further preferred
15 embodiment of the invention, the group of the activated agent of interest, preferably the chemotherapeutic agent, which reacts with at least one of the side chains in the polypeptide of the conjugate is a thiol group.

In an even more preferred embodiment of the invention, the activated therapeutic agent
20 is an activated chemotherapeutic agent, more preferably a thiol-functionalized oligofloxuridine.

In a preferred embodiment, the linking moiety is 6-maleimidohexanoic acid N-hydroxysuccinimide ester, and mediates the conjugation between the activated agent and
25 the side chain in the polypeptide of the conjugate indicated in the previous embodiments of this section. In a yet more preferred embodiment, the linking moiety 6-maleimidohexanoic acid N-hydroxysuccinimide ester is bound in a first step to the agent of interest, preferably the activated FdU, yet more preferably FdU functionalized with a sulfhydryl, and in a second step to the side chain in the polypeptide of the conjugate, more
30 preferably to external lysines of the polypeptide of the conjugate, even more preferably to external lysines of the first polypeptide region of the conjugate of the invention.

In another preferred embodiment of the invention, the activated therapeutic agent is an activated chemotherapeutic agent, more preferably an amino-functionalized oligofloxuridine.

5 In a preferred embodiment, the linking moiety is 6-maleimidohexanoic acid N-hydroxysuccinimide ester, and mediates the conjugation between the activated agent and the side chain in the polypeptide of the conjugate indicated in the previous embodiments of this section. In a yet more preferred embodiment, the linking moiety 6-maleimidohexanoic acid N-hydroxysuccinimide ester is bound in a first step to the agent
10 of interest, preferably the activated FdU, yet more preferably FdU functionalized with a amino, and in a second step to the side chain in the polypeptide of the conjugate, more preferably to external cysteines of the polypeptide of the conjugate, even more preferably to external cysteines of the first polypeptide region of the conjugate of the invention.

15 In another preferred embodiment of the invention, the activated therapeutic agent is an activated chemotherapeutic agent, more preferably a carboxi-functionalized oligofloxuridine.

In a preferred embodiment, the activated FdU, yet more preferably FdU functionalized
20 with an activated form of carboxylic acid, reacts the carboxyl group in the agent of interest and with the reactive group in the polypeptide (e.g. an amino group which is capable of forming an amide group with the carboxyl group in the agent of interest) to the side chain in the polypeptide of the conjugate, more preferably to external lysines of the polypeptide of the conjugate, even more preferably to external lysines of the first polypeptide region
25 of the conjugate of the invention.

In a further preferred embodiment, the agent of interest and, more preferably, FdU or the pentameric form thereof is functionalized with an amino group and the linking moiety is a bifunctional reagent that reacts with the amino group in the agent of interest and with
30 the reactive group in the polypeptide. In some embodiments, the bifunctional reagent contains a moiety that reacts with an amino group (e.g. a carboxylate group which is capable of forming an amide group with the amino group in the agent of interest) and a moiety that reacts with a sulfhydryl group in the side chain of the protein (e.g. a maleimide

group, which is capable of forming a thioether with the sulfhydryl group in the side chain of the polypeptide).

In a further preferred embodiment, the agent of interest and, more preferably, FdU or the
5 pentameric form thereof is functionalized with a carboxyl group and the linking moiety is a bifunctional reagent that reacts with the carboxyl group in the agent of interest and with the reactive group in the polypeptide. In some embodiments, the bifunctional reagent contains a moiety that reacts with the carboxyl group (e.g. an amino group which is capable of forming an amide group with the carboxyl group in the agent of interest) and
10 a moiety that reacts with a sulfhydryl group in the side chain of the protein (e.g. a maleimidide group, which is capable of forming a thioether with the sulfhydryl group in the side chain of the polypeptide) or with an amino group in the protein.

Additional linkers that can be used in the context of the present invention between the
15 agent of interest and the polypeptide include those which are commonly used to prepare antibody-drug conjugates, such as those disclosed in Leung et al. (Antibodies 2020, 9, 2; doi:10.3390/antib9010002) (see Figure 6) as well as those disclosed in Bargh et al. (Chem. Soc. Rev., 2019, DOI: 10.1039/c8cs00676h), the contents of which are hereby incorporated by reference.

20 Thus, in one embodiment of the invention, the activated form of the polypeptide of the conjugate contains a group which reacts with at least one moiety in the agent of interest. In a further preferred embodiment of the invention, the group of the agent of interest, preferably the chemotherapeutic agent, which reacts with the activated polypeptide of the conjugate is a thiol group.

25

In an even more preferred embodiment of the invention, the activated polypeptide of the conjugate is obtained by reacting one or more amino groups in the side chains of the polypeptide with a bifunctional reagent comprising an activated carboxyl group, such as a N-hydroxysuccinimide group. In one embodiment, the bifunctional reagent contains a
30 second activated carboxyl group that can be reacted with an amino, thiol or hydroxyl group within the agent of interest.

In a further preferred embodiment, the linking moiety is 6-maleimido hexanoic acid N-hydroxysuccinimide ester and mediates the conjugation between an amino group in the polypeptide of the conjugate and a thiol group in the agent of interest. In a yet more preferred embodiment, the linking moiety 6-maleimido hexanoic acid N-hydroxysuccinimide ester is bound in a first step to the polypeptide of the conjugate, more preferably to external lysines of the polypeptide of the conjugate and in a second step to the agent side chain, preferably to the thiol group of the activated agent.

In a further preferred embodiment, the linking moiety is 6-maleimido hexanoic acid N-hydroxysuccinimide ester and mediates the conjugation between a thiol group in the polypeptide of the conjugate and an amino group in the agent of interest. In a yet more preferred embodiment, the linking moiety 6-maleimido hexanoic acid N-hydroxysuccinimide ester is bound in a first step to the polypeptide of the conjugate, more preferably to external Cysteines of the polypeptide of the conjugate and in a second step to the agent of interest, preferably to the amino group in the agent of interest.

In a further preferred embodiment, the linking moiety is 6-maleimido hexanoic acid N-hydroxysuccinimide ester and mediates the conjugation between a thiol group in the polypeptide of the conjugate and an amino group in the agent of interest. In a yet more preferred embodiment, the linking moiety 6-maleimido hexanoic acid N-hydroxysuccinimide ester is bound in a first step to the agent of interest, preferably to the amino group in the agent of interest. And in a second step to the polypeptide of the conjugate, more preferably to the thiol group of external Cysteines of the polypeptide of the conjugate.

In a certain embodiment, all the terms and embodiments described in the previous aspects of the invention are equally applicable to the seventh aspect of the invention

VI – Polypeptides of the invention containing antagonistic CXCR4 ligands

The authors of the present invention have observed that a fusion protein comprising an antagonistic CXCR4 ligand and the G2 domain of nidogen-1 or a variant form thereof are

capable of targeting and penetrating CXCR4-expressing cells. Moreover, when this fusion protein is further modified by the presence of a polycationic region, the fusion protein spontaneously assembles into nanoparticles that are also capable of targeting and penetrating CXCR4-expressing cells and inducing apoptosis in CXCR4-expressing cells.

5

Accordingly, in another aspect, the invention relates to a polypeptide (also known as second polypeptide of the invention, fusion protein of the invention, CXCR4-antagonistic polypeptide of the invention) which comprises

- (i) a first region comprising the G2 domain of nidogen-1 or a functionally equivalent
10 variant thereof and
- (ii) a second region which comprises an antagonistic CXCR4 ligand.

The first region has been defined above in the context of the first polypeptide of the invention and in the context of the first region of the polypeptides forming part of the
15 conjugates of the invention and applies equally to the second polypeptide of the invention. In some embodiments, the first region is a functionally equivalent variant of the G2 domain of nidogen-1, wherein said functionally equivalent variant of the G2 domain of nidogen-1 is any of the variants defined above in the first polypeptide of the invention. In some embodiments, the polypeptide according to claims 53 or 54 wherein the first region
20 comprises amino acids 430 to 667 with respect to the sequence of human nidogen-1 defined in the UniProt database with accession number P14543-1. In some embodiments, the functionally equivalent variant of the domain G2 of nidogen-1 forming part of the first region comprises a mutation in one or more amino acid residues at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 151, 604, 638, 641, 469 and 518 with
25 respect to the numbering of the sequence of human nidogen-1 defined under the UniProt database with accession number P14543-1 (version dated July 7, 2009). Thus, in another particular embodiment, the polypeptide of the conjugate of the sixth aspect of the invention is a functionally equivalent variant of the domain G2 of nidogen-1 comprising a mutation in one or more amino acid residues at positions 459, 468, 639, 650, 543, 545,
30 449, 525, 561, 618, 619, 151, 604, 638, 641, 469 and 518 with respect to the numbering of the sequence of human nidogen-1 defined under the UniProt database with accession number P14543-1 (version dated July 7, 2009). In some embodiments, the nidogen G2

domain variant that can be included in the first polypeptide region include, without limitation, any of the nidogen G2 domain variants defined above in the context of the first aspect of the invention including the variant carrying the NIDOMut2, NIDOMut3, the NIDOMut3-V45T, the NIDOMut3_V121Q, the NIDOMut3-F157E, the NIDOMut3-V215T, the NIDOMut4, the NIDOMut4_T215V, the NIDOMut5, NIDOMut3-V176T, the NIDOMut3-I200T, the NIDOMut3-V236Y, the NIDOMut3-L237T, the NIDOMut3-S65I, the NIDOMut3-R114I, the NIDOMut3-C214S, the NIDOMut3-S65I_R114I, the NIDOMut5-S65I_R114I, the NIDOMut3-S65I_R114I and the NIDOMut5-S65I_R114I as defined, respectively, as SEQ ID NO: 64, 65 and 87 to 104.

10

The second region of the second polypeptide of the invention comprises an antagonistic CXCR4 ligand. The term “antagonistic CXCR4 ligand”, as used herein, refers to any polypeptide, peptide or peptide mimetic that is capable of specifically binding to CXCR4 and diminishes, inhibits, or prevents biological activity of the said molecule in response to its interaction with an agonist. In one embodiment, the antagonistic CXCR4 ligand is a competitive antagonist, i.e. an antagonist that reversibly binds to CXCR4 at the same binding site (active site) as the endogenous ligand or agonist, without necessarily activating the receptor.

20 Suitable methods for determining whether a given peptide is capable of binding to CXCR4 have been defined above in the context of the conjugates of the invention and are equally applicable for the instant polypeptides. In some embodiments, the second region is capable of specifically binding to CXCR4 with a dissociation constant (K_D) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M. Methods to determine if a polypeptide is capable of binding to a target molecule, as well as to determine the dissociation constant of said binding are provided in the definition of “specifically binding” in the second aspect of the invention.

30 Suitable antagonists for use according to the present invention are characterized in that compete for binding to CXCR4 by its natural ligand CXCL12 with an IC_{50} of not more than 0.1 μ M, not more than 0.2 μ M, not more than 0.3 μ M, not more than 0.4 μ M, not

more than 0.5 μM , not more than 0.6 μM , not more than 0.7 μM , not more than 0.8 μM , not more than 0.9 μM , not more than 1 μM , not more than 2 μM , not more than 3 μM , not more than 4 μM , not more than 5 μM , not more than 6 μM , not more than 7 μM , not more than 8 μM , not more than 9 μM , not more than 10 μM , not more than 15 μM , not more than 20 μM , not more than 30 μM , not more than 40 μM , not more than 50 μM , not more than 60 μM , not more than 70 μM , not more than 80 μM , not more than 90 μM or not more than 100 μM .

Suitable methods for determining whether a molecule is an antagonistic CXCR4 ligand are, for example, the methods shown in Zirafi et al. (Cell Rep. 2015, 11, 737), the contents of which are hereby incorporated by reference. These methods include assays based on the detection of the ability of the antagonist to inhibit binding of CXCL12 to CXCR4 (for determination on whether the molecule is a ligand) as well as those methods based on the detection of the ability of the molecule to block Ca^{2+} release from CXCR4-expressing cells such as HEX293 cells, based on the ability of the molecule to block CXCL12-directed transwell migration of Jurkat T cells and/or based on the detection of the ability of the molecule to block CXCL12-induced migration of human CD34⁺ stem cells (for determination of whether the molecule act as an antagonist).

In some embodiments, the second region of the polypeptide carrying the antagonistic CXCR4 ligand according to the invention further comprises a positively-charged amino acid region.

The positively charged peptide sequence may contain only one type of positively charged amino acid or may contain more than one type of positively charged amino acid. In one embodiment, the positively charged peptide sequence is a polyarginine region. In one embodiment, the positively charged peptide sequence is a polylysine region. In one embodiment, the positively charged peptide sequence is a polyhistidine region. In one embodiment, the positively charged peptide sequence comprises lysine and arginine residues. In one embodiment, the positively charged peptide sequence comprises lysine and histidine residues. In one embodiment, the positively charged peptide sequence

comprises arginine and histidine residues. In one embodiment, the positively charged peptide sequence comprises lysine, arginine and histidine residues.

In some embodiments, the positively charged peptide sequence comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 positively charged amino acids residues, wherein the positively charged amino acids can be arginine, lysine, histidine, or combinations thereof.

In some embodiments, the positively charged peptide sequence comprises fewer than 100, fewer than 90, fewer than 80, fewer than 70, fewer than 60, fewer than 50, fewer than 40, fewer than 30, fewer than 29, fewer than 28, fewer than 27, fewer than 26, fewer than 25, fewer than 24, fewer than 23, fewer than 22, fewer than 21, fewer than 20, fewer than 19, fewer than 18, fewer than 17, fewer than 16, fewer than 15, fewer than 14, fewer than 13, fewer than 12, fewer than 11, fewer than 10, fewer than 9, fewer than 8, fewer than 7, fewer than 6, fewer than 5, fewer than 4, fewer than 3 or less positively charged amino acids residues, wherein the positively charged amino acids can be arginine, lysine, histidine, or combinations thereof.

In some embodiments, the positively charged peptide sequence comprises between 2 and 50 amino acids, between 2 and 40 amino acids, between 2 and 30 amino acids, between 2 and 25 amino acids, between 2 and 20 amino acids, between 2 and 10 amino acids, between 2 and 8 amino acids, between 3 and 7 amino acids, between 4 and 7 amino acids, or between 5 and 7 amino acids.

25

In some embodiments, the positively charged peptide sequence comprises between 3 and 50 amino acids, between 3 and 40 amino acids, between 3 and 30 amino acids, between 3 and 25 amino acids, between 3 and 20 amino acids, between 3 and 10 amino acids or between 3 and 8 amino acids. In some embodiments, the positively charged peptide sequence comprises between 4 and 50 amino acids, between 4 and 40 amino acids, between 4 and 30 amino acids, between 4 and 25 amino acids, between 4 and 20 amino acids, between 4 and 10 amino acids or between 4 and 8 amino acids. In some

30

embodiments, the positively charged peptide sequence comprises between 5 and 50 amino acids, between 5 and 40 amino acids, between 5 and 30 amino acids, between 5 and 25 amino acids, between 5 and 20 amino acids, between 5 and 10 amino acids or between 5 and 8 amino acids.

5

In another embodiment, the positively charged peptide sequence comprises 3, 4, 5, 6, 7, 8, 9, 10 amino acids, preferably 6 amino acids.

In an embodiment of the invention, the positively charged peptide sequence comprises arginine and lysine residues. In a preferred embodiment of the invention, the positively charged peptide sequence comprises between 1 and 5 arginines, preferably 3 arginines, and between 1 and 5 lysines, preferably 3 lysines.

In an embodiment of the invention, the positively charged peptide sequence comprises arginine and histidine residues. In a preferred embodiment of the invention, the positively charged peptide sequence comprises between 1 and 5 arginines, preferably 3 arginines, and between 1 and 5 histidines, preferably 3 histidines.

In an embodiment of the invention, the positively charged peptide sequence comprises lysine and histidine residues. In a preferred embodiment of the invention, the positively charged peptide sequence comprises between 1 and 5 lysines, preferably 3 lysines, and between 1 and 5 histidines, preferably 3 histidines.

In an embodiment of the invention, the positively charged peptide sequence of the conjugate of the invention is a polyarginine region. In a preferred embodiment of the invention, the polyarginine region comprises between 2 and 10, preferably 6, contiguous arginine residues.

In an embodiment of the invention, the positively charged amino acid-rich region of the conjugate of the invention is a polylysine region. In a preferred embodiment of the invention, the polylysine region comprises between 2 and 10, preferably 6, contiguous lysine residues.

In an embodiment of the invention, the positively charged amino acid-rich region of the fusion protein of the invention is a polyhistidine region. In a preferred embodiment of the invention, the polyhistidine region comprises between 2 and 10, preferably 6, contiguous
5 polyhistidine residues.

In a particular embodiment, the positively charged peptide sequence is RKRKRK (SEQ ID NO.77), RRRRRR (SEQ ID NO. 78), KKKKKK (SEQ ID NO:79), HHHHHH (SEQ ID NO. 80), RHRHRH (SEQ ID NO. 81), RKRKRKRK (SEQ ID NO. 82), RKRHRK
10 (SEQ ID NO. 83), RKRHRH (SEQ ID NO. 84) or RHRHRH (SEQ ID NO. 85)

In a preferred embodiment, the positively charged peptide sequence is attached to the N- or to the C-terminal end of the sequence of the optimized EPI-X4 (SEQ ID NO.29), preferably to the C-terminal end of the optimized EPI-X4 sequence (SEQ ID NO.29). In
15 a preferred embodiment, the positively charged peptide sequence is attached to the N- or to the C-terminal end of the sequence of the EPI-X4 (SEQ ID NO.132, preferably to the C-terminal end of the sequence EPI-X4 (SEQ ID NO.132).

In some embodiments, the second polypeptide of the invention further comprises a third
20 polypeptide region which is a positively charged amino acid-rich region. Suitable positively charged amino acid-rich region that can act as third region of the second polypeptide of the invention are as defined above in respect of the third polypeptide region of the conjugates according to the invention. In preferred embodiments, the positively charged amino acid-rich region is a polyhistidine region. In a preferred
25 embodiment of the invention, the polyhistidine region comprises between 2 and 10, preferably 6, contiguous histidine residues. In preferred embodiments, the positively charged amino acid-rich region is a polyarginine region. In a preferred embodiment of the invention, the polyarginine region comprises between 2 and 10, preferably 6, contiguous arginine residues. In preferred embodiments, the positively charged amino
30 acid-rich region is a polylysine region. In a preferred embodiment of the invention, the polyarginine region comprises between 2 and 10, preferably 6, contiguous lysine residues.

In preferred embodiments, the positively charged amino acid rich region comprises or consists of the sequences RKRKRK (SEQ ID NO.77), RRRRRR (SEQ ID NO. 78), KKKKKK (SEQ ID NO:79), HHHHHH (SEQ ID NO. 80), RHRHRH (SEQ ID NO. 81), RKRKRKRK (SEQ ID NO. 82), RKRHRK (SEQ ID NO. 83), RKRHRH (SEQ ID NO. 84) or RHRHRH (SEQ ID NO. 85).

In some embodiment, the first, second and third region of the second polypeptide of the invention are located within the second polypeptide of the invention in the following order:

- 10 ▪ N - First polypeptide region - Second polypeptide region- Third polypeptide region -C;
- N - First polypeptide region - Third polypeptide region- Second polypeptide region -C;
- N - Second polypeptide region – First polypeptide region - Third polypeptide region -C;
- 15 ▪ N - Second polypeptide region – Third polypeptide region - First polypeptide region -C
- N - Third polypeptide region – First polypeptide region - Second polypeptide region -C;
- 20 ▪ N - Third polypeptide region – Second polypeptide region- First polypeptide region –C.

Therefore, the elements of the second polypeptide of the invention can be connected end-to-end but also may include one or more optional peptide or polypeptide "linkers" or "spacers" intercalated between them, linked, preferably by peptidic bond. The linker peptide or peptides preferably comprise at least 2 amino acids, at least 3 amino acids, at least 5 amino acids, at least 10 amino acids, at least 15 amino acids, at least 20 amino acids, at least 30 amino acids, at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 70 amino acids, at least 80 amino acids, at least 90 amino acids or approximately 100 amino acids. Preferred examples of linker peptides comprise 2 or more amino acids selected from the group consisting of glycine, serine, alanine and threonine. A preferred example of a flexible linker is a polyglycine linker. The possible examples of

linker/spacer sequences include GGSSRSS (SEQ ID NO:39), GGSSRSSS (SEQ ID NO:76), SGGTSGSTSGTGST (SEQ ID NO:49), AGSSTGSSTGPGSTT (SEQ ID NO:50) or GGSGGAP (SEQ ID NO:51). These sequences have been used for binding designed coiled coils to other protein domains [Muller, K.M., Arndt, K.M. and Alber, T.,
 5 Meth. Enzymology, 2000, 328: 261-281]. Further non-limiting examples of suitable linkers comprise the amino acid sequence GGGVEGGG (SEQ ID NO:52), the sequence of 10 amino acid residues of the upper hinge region of murine IgG3 (PKPSTPPGSS, SEQ ID NO:53), which has been used for the production of dimerized antibodies by means of a coiled coil [Pack, P. and Pluckthun, A., 1992, Biochemistry 31:1579-1584], the peptide
 10 of sequence APAETKAEPMT (SEQ ID NO:54), the peptide of sequence GAP, the peptide of sequence AAA and the peptide of sequence AAAL (SEQ ID NO:55). In a preferred embodiment, the linker is GGSSRSS (SEQ ID NO:39).

In some embodiment, depending on the presence of a linker connecting the different
 15 regions of the second polypeptide of the invention and depending on the order of elements forming the second polypeptide of the invention, the second polypeptide of the invention can have the following arrangements of elements (wherein the numbering stated above for the elements is as follows: (1) first region polypeptide, (2) second polypeptide region, (3) third polypeptide region:

- 20 ▪ N-(1)-(2)-(3)-C
- N-(1)-linker-(2)-(3)-C
- N-(1)-(2)-linker-(3)-C
- N-(1)-linker-(2)-linker-(3)-C
- N-(3)-(2)-(1)-C
- 25 ▪ N-(3)-linker-(2)-(1)-C
- N-(3)-(2)-linker-(1)-C
- N-(3)-linker-(2)-linker-(1)-C
- N-(2)-(1)-(3)-C
- N-(2)-linker-(1)-(3)-C
- 30 ▪ N-(2)-(1)-linker-(3)-C
- N-(2)-linker-(1)-linker-(3)-C
- N-(2)-(3)-(1)-C

- N-(2)-linker-(3)-(1)-C
- N-(2)-(3)-linker-(1)-C
- N-(2)-linker-(3)-linker-(1)-C
- N-(1)-(3)-(2)-C
- 5 ▪ N-(1)-(3)-linker-(2)-C
- N-(1)-linker-(3)-(2)-C
- N-(1)-linker-(3)-linker-(2)-C
- N-(3)-(1)-(2)-C
- N-(3)-linker-(1)-(2)-C
- 10 ▪ N-(3)-(1)-linker-(2)-C
- N-(3)-linker-(1)-linker-(2)-C

Preferred fusion proteins having antagonistic CXCR4 ligands are as defined in the following Table

15

First region	first peptide linker	Second region	Third region
Optimized EPI-X4 (SEQ ID NO:29)	GGSSRSS (SEQ ID NO:39)	GFP	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 coupled to the positively charged region of sequence RKRKRK (SEQ ID NO:131)	GGSSRSS (SEQ ID NO:39)	GFP	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 (SEQ ID NO:29)	GGSSRSS (SEQ ID NO:39)	NIDOm2, NIDOm3, the NIDOm3-V45T, the NIDOm3_V121Q, the NIDOm3-F157E, the NIDOm3-V215T, the NIDOm4, the NIDOm4_T215V, the NIDOm5, NIDOm3- V176T, the NIDOm3- I200T, the NIDOm3- V236Y, the NIDOm3- L237T, the NIDOm3- S65I, the NIDOm3- R114I, the NIDOm3- C214S, the NIDOm3- S65I_R114I, the NIDOm5- S65I_R114I, the NIDOm3-S65I_R114I and the NIDOm5- S65I_R114I	Hexahistidine (SEQ ID NO:73)
Optimized EPI-X4 coupled to the positively	GGSSRSS (SEQ ID NO:39)	NIDOm2, NIDOm3, the NIDOm3-V45T,	Hexahistidine (SEQ ID NO:73)

First region	first peptide linker	Second region	Third region
charged region of sequence RKRKRK (SEQ ID NO:131)		the NIDOm3_V121Q, the NIDOm3-F157E, the NIDOm3-V215T, the NIDOm4, the NIDOm4_T215V, the NIDOm5, NIDOm3- V176T, the NIDOm3- I200T, the NIDOm3- V236Y, the NIDOm3- L237T, the NIDOm3- S65I, the NIDOm3- R114I, the NIDOm3- C214S, the NIDOm3- S65I_R114I, the NIDOm5- S65I_R114I, the NIDOm3-S65I_R114I and the NIDOm5- S65I_R114I	

Table 5: Preferred fusion proteins having antagonistic CXCR4 ligands*VII - Nanoparticles of the invention and methods for their preparation*

- 5 In an additional aspect, the invention relates to a method to prepare nanoparticles comprising multiple copies of the conjugate according to the sixth aspect of the invention comprising placing a preparation of said conjugate under conditions adequate for the assembly of a plurality of copies of the conjugate into a nanoparticle.
- 10 The term “conjugate” has been defined above in the context of the conjugates according to the invention and are equally applicable to the present method.

In another aspect, the invention relates to a method for preparing a nanoparticle comprising multiple copies of the conjugate according to the sixth aspect of the invention

15 comprises

- (i) placing a plurality of polypeptides each comprising
 1. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,
 2. a second polypeptide region which is capable of specifically binding to
- 20 a target of interest wherein said second polypeptide is a polycationic peptide and

3. a third polypeptide region which is a positively charged amino acid-rich region,

5 wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptide and wherein the polypeptide is provided in an activated form, wherein said activated form of the polypeptide contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of the polypeptide and

10 (ii) contacting the nanoparticle obtained in step (i) with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptide and the group in the agent of interest.

15 The terms “first polypeptide region”, “second polypeptide region” and “third polypeptide region” have been defined above in the context of the polypeptides forming the conjugates of the invention and are equally applicable to the method for obtaining nanoparticles comprising multiple copies of the conjugate.

20 The term “conditions adequate for the formation of a nanoparticle containing a plurality of copies of the polypeptide”, as used herein, refer to any conditions that are suitable for the incorporation of a substantial percentage of the polypeptides in the sample into nanoparticles. In one embodiment, the conditions involve incubating the conjugates or polypeptides to be assembled into a low salt buffer.

25

As the person skilled in the art will recognize, “nanoparticles” are microscopic particles whose size is measured in nanometers. The nanoparticles of the invention comprise the nanoparticles that result from the aggregation of multiple copies of the conjugate, or of the polypeptide of the conjugate, of the sixth aspect of the invention as defined in the
30 previous section. In the methods (i) and (ii) above, the conjugates or polypeptide of the conjugate of the sixth aspect of the invention used for the preparation of the nanoparticles are thermodynamically favored to form non-covalent electrostatic unions and

spontaneously aggregate in the conditions of the low salt buffer. In a particular embodiment, said thermodynamically favored conjugates or polypeptides of the conjugate of the sixth aspect of the invention, comprise the first, second and third polypeptide regions comprised in the polypeptide of the conjugate of the sixth aspect of the invention. In a particular embodiment, said polypeptides, or the polypeptides comprised in said conjugates, are as the polypeptides defined in the sixth aspect of the invention, wherein the second polypeptide region is a polycationic peptide, located at the N-terminus of the first polypeptide region, and the third polypeptide region is located at the C terminus of the first polypeptide region of the conjugate.

10

In a particular embodiment, the conditions adequate for the assembly of a plurality of copies of the conjugate into a nanoparticle in the method (i) of the eighth aspect of the invention, comprise an incubation in a low salt buffer. In another particular embodiment, the condition adequate for the assembly of a plurality of copies of the polypeptide into a nanoparticle in the method (ii) of the eighth aspect of the invention, comprise an incubation in a low salt buffer.

It will be understood that the expression “low salt buffer” comprises any buffer solution resulting from the dissolution of one or more salts in water with the capability to moderate changes in pH, wherein the amount of dissolved salt or salts results in an osmolarity lower or equal to that of the physiological fluids, such as the cytoplasm or the extracellular medium, for instance. Thus, the low salt buffer is understood to keep pH and osmolarity inside the range of physiological values and will be used inside the range of physiological temperatures.

25

The person skilled in the art will recognize that the range of physiological temperatures can oscillate between 15 and 45° C, more preferably between 20 and 40°C, even more preferably between 25 and 39°C, yet even more preferably between 30 and 37°C. The person skilled in the art will also acknowledge that the osmolarity of the low salt buffer will be in the range between 100 and 400 milli-osmoles/L (mOsm/L), preferably between 150 and 350 mOsm/L, more preferably between 200 and 300 mOsm/L, even more preferably between 225 and 275 mOsm/L.

30

In some embodiments, the low salt buffer has a pH of between pH 4 and pH 7, preferably between pH 5 and pH 6, more preferably is of about PH 5.3, PH 6.5 or PH 7.2.

- 5 In some embodiments, the low salt buffer is selected from the group consisting of a carbonate buffer, a citrate buffer, an acetate buffer, a Tris buffer and a phosphate buffer. Low salt buffers suitable for the invention, for instance, are the Tris-dextrose buffer (20 mM Tris +5% dextrose, pH 7.4), the Tris-NaCl buffer (20 mM Tris, 500 NaCl, pH 7.4), the PBS-glycerol buffer (phosphate buffered saline, PBS, pH 7.4, which is well known in
10 the art, +10% glycerol), Tris Buffered Saline (TBS)-dextrose (20 mM Tris-HCl buffer pH 7.5, well known in the art, 200NaCl, +5% dextrose), Tris Buffered Saline-Tween 20 (TBST) buffer (10 mM Tris-HCl pH 7.5, 200 mM NaCl, +0.01% Tween 20), or any physiological buffer known in the art with a pH not lower than 6.
- 15 In one embodiment, the low salt buffer further comprises Polysorbate 80 and/or sucrose. In yet another embodiment, the sucrose in the low salt buffer is found at a concentration of between 20 mg/ml and 100 mg/ml, more preferably of between 50 mg/ml and 90 mg/ml, preferably of 70 mg/ml.
- 20 In one embodiment, the low salt buffer is a citrate buffer that comprises Polysorbate 80 (0.4 mg/ml), sucrose (80 mg/ml), sodium citrate 2-hydrate (2.7 mg/ml) and citric acid anhydrous (0.146 mg/ml) and has a pH of about 6,5.

In one embodiment, the low salt buffer is an acetate buffer that comprises sucrose (70
25 mg/ml), glacial acetic acid (0.12 mg/ml), sodium acetate 3-hydrate (2.45 mg/ml) and has a pH of about 5.3.

In another embodiment, the low salt buffer is a phosphate buffer that comprises Polysorbate 80 (0.05 mg/ml), sucrose (50 mg/ml), sodium phosphate monobasic 1-
30 hydrate (0.22 mg/ml), sodium phosphate dibasic anhydrous (0.49 mg/ml) and has a pH of about 7.2.

In some embodiments, the low salt buffer suitable for carrying out the method for obtaining nanoparticles is the A9 buffer which comprises

- sucrose at a concentration between 40 mg/ml and 100 mg/ml, preferably of 80 mg/ml,
- 5 ○ polysorbate 80 at a concentration between 0.01 mg/ml and 10 mg/ml, preferably of 4 mg/ml,
- sodium citrate 2-hydrate at a concentration of 2.7 mg/ml,
- citric acid anhydrous at a concentration of 0.146 mg/ml

and wherein the pH of the buffer is between pH 5 and pH 9, preferably between pH 7 and
10 pH 8, more preferably is pH 6.5.

In some embodiments, the low salt buffer suitable for carrying out the method for obtaining nanoparticles is the B6 buffer that comprises:

- sucrose at a concentration between 50 and 90, preferably of 70 mg/ml,
- 15 - glacial acetic acid at a concentration between 0.05 mg/ml and 25 mg/ml, preferably of 0.12mg/ml,
- sodium acetate 3-hydrate at a concentration between 1mg/ml and 4 mg/ml, preferably of 2.45 mg/ml,

wherein the pH of the B6 buffer is between pH4 and pH7, preferably between pH5 and
20 pH6, more preferably is pH5.3.

In some embodiments, the low salt buffer suitable for carrying out the method for obtaining nanoparticles is the D1 buffer that comprises:

- sucrose at a concentration between 40 mg/ml and 60 mg/ml, preferably of
25 50mg/ml,
- polysorbate 80 at a concentration between 0.01 mg/ml and 1mg/ml, preferably of 0.05 mg/ml,
- sodium phosphate monobasic 1-hydrate at a concentration between 0.1 mg/ml and 0.5 mg/ml, preferably of 0.22 mg/ml
- 30 - sodium phosphate dibasic anhydrous at a concentration between 0.2 mg/ml and 1 mg/ml, preferably of 0.49 mg/ml,

wherein the pH of the sucrose buffer D1 is between pH5 and pH9, preferably between pH7 and pH8, more preferably is pH7.2

The inventors additionally produced biparatopic nanoparticles by allowing the
5 oligomerization of two types fusion proteins: a first type of fusion proteins comprising the T22 CXCR4 ligand and a scaffold protein and a second type of fusion proteins comprising the EPI-X4 antagonistic CXCR4 ligand and the same scaffold protein. These biparatopic nanoparticles showed higher levels of internalization in CXCR4+ cells *in vitro* than nanoparticles formed by a single type of fusion protein, being either the fusion
10 protein comprising the T22 CXCR4 ligand or the fusion protein comprising the EPI-X4 CXCR4 ligand alone. Upon administration to the mouse model, the biparatopic nanoparticles also showed higher levels of internalization in the tumor cells and a higher number of apoptotic bodies in the tumoral cells than the any of the two types of monoparatopic nanoparticles comprising a single type of fusion protein.

15

In another aspect, the invention relates to a method to prepare biparatopic nanoparticles comprising multiple copies of two different types of conjugates according to the sixth aspect of the invention, wherein the sequence of the second polypeptide of the first type of conjugate is different from the sequence of the second polypeptide of the second type
20 of conjugate, said method comprising placing a preparation of said two types of conjugates in a low salt buffer.

In a ninth aspect, the invention relates to a method for preparing a biparatopic nanoparticle comprising multiple copies of a first type of conjugate and multiple copies of a second
25 type of conjugate, wherein the first and second types of conjugates of the invention and wherein the first and the second type of conjugates differ in the sequence of the polycationic peptide, said method comprising contacting a preparation of said first type of conjugate with a preparation of said second type of conjugate under conditions adequate for the assembly of a plurality of copies of the two types of conjugates into a
30 nanoparticle.

In another embodiment, the invention relates to a method for preparing a biparatopic nanoparticle comprising multiple copies of a first type of conjugate and multiple copies of a second type of conjugates according to the invention, wherein the sequence of the polycationic peptide of the first type of conjugate is different from the sequence of the polycationic peptide of the second type of conjugate, said method comprising

- i. contacting a preparation of a first polypeptide with a preparation of a second polypeptide wherein the first type and second polypeptides comprise

- (i) a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,

- (ii) a second polypeptide region which is capable of specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide and the sequence of the polycationic peptide of one polypeptide is different from the sequence of the polycationic peptide of the other polypeptide,

- (iii) a third polypeptide region which is a positively charged amino acid-rich region,

wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptides,

wherein the polypeptides are provided in an activated form, wherein said activated form of the polypeptides contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of polypeptides and

- ii. contacting the nanoparticle obtained in step I with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in each polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptides and the group in the agent of interest.

As used herein, the term “biparatopic nanoparticle”, refers to nanoparticles which are formed by at least two types of conjugates, wherein the two types of conjugates contain polypeptides that differ in the nature of the sequence which is specifically binding to a target of interest, being these sequences either ligands for different receptors or, more preferably, two different ligands for the same receptor.

Suitable conjugates that can be used as first type or second types of conjugates in the above methods are as defined above in the context of the conjugates according to the invention. In some embodiments, the first region of the polypeptide forming the conjugate is selected from the group consisting of NIDOMut2, NIDOMut3, the NIDOMut3-V45T, the NIDOMut3_V121Q, the NIDOMut3-F157E, the NIDOMut3-V215T, the NIDOMut4, the NIDOMut4_T215V, the NIDOMut5, NIDOMut3-V176T, the NIDOMut3-I200T, the NIDOMut3-V236Y, the NIDOMut3-L237T, the NIDOMut3-S65I, the NIDOMut3-R114I, the NIDOMut3-C214S, the NIDOMut3-S65I_R114I, the NIDOMut5-S65I_R114I, the NIDOMut3-S65I_R114I and the NIDOMut5-S65I_R114I as defined, respectively, as SEQ ID NO: 64, 65 and 87 to 104. In some embodiments, the second region of the polypeptide forming the conjugate is a CXCR4 ligand, more preferably the T22 peptide having the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 peptide (SEQ ID NO:27), the vCCL2 peptide (SEQ ID NO:28) and the optimized EPI-X4 sequence (SEQ ID NO:29) or the EPI-X4 sequence (SEQ ID NO:132), wherein the EPI-X4 sequence or the optimized EPI-X4 sequence may be provided as a fusion with a polycationic peptide, preferably the peptide having the sequence RKRKRK (SEQ ID NO.77). In some embodiments, the third region of the polypeptide forming part of the conjugates is a positively charged amino acid-rich region selected from the group consisting of RKRKRK (SEQ ID NO.77), RRRRRR (SEQ ID NO. 78), KKKKKK (SEQ ID NO:79), HHHHHH (SEQ ID NO. 80), RHRHRH (SEQ ID NO. 81), RKRKRKRK (SEQ ID NO. 82), RKRHRK (SEQ ID NO. 83), RKRHRH (SEQ ID NO. 84) or RHRHRH (SEQ ID NO. 85).

In some embodiments, both the first and second type of conjugates used in the methods for preparing biparatopic nanoparticules contain CXCR4 ligands, being said ligands the T22 peptide having the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25) and

the and the optimized EPI-X4 sequence (SEQ ID NO:29) or the EPI-X4 sequence (SEQ ID NO: 132), wherein the optimized EPI-X4 sequence or the EPI-X4 sequence may be provided as a fusion with a polycationic peptide, preferably the peptide having the sequence RKRKRK (SEQ ID NO:77).

5

In the methods for preparing biparatopic nanoparticles described above, the conjugates or polypeptide of the two types of conjugates used for the preparation of the nanoparticles are thermodynamically favored to form non-covalent electrostatic unions and spontaneously aggregate in the conditions of the low salt buffer. Suitable conditions for the assembly into a biparatopic nanoparticle of the plurality of copies of the two conjugates in the methods for the preparation of biparatopic nanoparticles are similar to the conditions adequate for the assembly of a plurality of copies of the conjugate into a nanoparticle in the methods according to the eighth aspect of the invention.

15 In one embodiment, the low salt buffer further comprises Polysorbate 80 and/or sucrose. In yet another embodiment, the sucrose in the low salt buffer is found at a concentration of between 20 mg/ml and 100 mg/ml, more preferably of between 50 mg/ml and 90 mg/ml, preferably of 70 mg/ml.

20 In one embodiment, the low salt buffer is a citrate buffer that comprises Polysorbate 80 (0.4 mg/ml), sucrose (80 mg/ml), sodium citrate dihydrate (2.7 mg/ml) and citric acid anhydrous (0.146 mg/ml) and has a pH of about 6,5.

In one embodiment, the low salt buffer is an acetate buffer that comprises sucrose (70 mg/ml), glacial acetic acid (0.12 mg/ml), sodium acetate 3-hydrate (2.45mg/ml) and has a pH of about 5.3.

In another embodiment, the low salt buffer is a phosphate buffer that comprises Polysorbate 80 (0.05 mg/ml), sucrose (50 mg/ml), sodium phosphate monobasic 1-hydrate (0.22 mg/ml), sodium phosphate dibasic anhydrous (0.49 mg/ml) and has a pH of about 7.2.

30

In preferred embodiment, the buffer used in the methods for obtaining the biparatopic nanoparticles is selected from the group comprising the A9 buffer, the B6 buffer and the D1 buffer, the composition of which has been described above.

In a preferred embodiment of the invention, the low salt buffer adequate for the assembly
5 of a plurality of copies of the polypeptides of the conjugate into a nanoparticle in the method (ii) of the eighth aspect of the invention is selected from the group consisting of a carbonate buffer, a Tris buffer and a phosphate buffer.

In a particularly preferred embodiment of the invention, the low salt buffer of the method
10 (ii) of the eighth aspect of the invention is a carbonate buffer that comprises sodium bicarbonate at a concentration between 100 and 300 nM. In another particularly preferred embodiment of the invention, the low salt buffer of the method (ii) eighth aspect of the invention is a Tris buffer that comprises Tris at a concentration of between 10 and 30 nM. In another particularly preferred embodiment of the method (ii) eighth aspect of the
15 invention, the low salt buffer of the invention is a phosphate buffer that comprises Na_2HPO_4 and NaH_2PO_4 at a total concentration of between 5 mM and 20 mM.

In an even more preferred embodiment of the invention, the low salt buffer of the method
(ii) eighth aspect of the invention further comprises dextrose and/or glycerol.

20

In a yet more preferred embodiment of the invention, the low salt buffer of the method
(ii) eighth aspect of the invention has a pH between 6.5 and 8.5.

In a tenth aspect, the invention relates to nanoparticles comprising multiple copies of a
25 conjugate according to the invention or multiple copies of a polypeptide comprising an antagonistic CXCR4 ligand according to the invention or a nanoparticle that has been obtained by any of the methods explained above. It will be understood that these nanoparticles are formed by a single type of conjugate or by a single type of polypeptide and that, accordingly, they are monospecific (i.e. all the conjugates or polypeptides
30 forming the nanoparticle bind to a single type of target molecule) and monoparatopic (i.e. all the conjugates or polypeptides forming the nanoparticle bind to the same region in the target molecule).

Thus, the nanoparticles of the invention comprise aggregates of multiple copies of the conjugates of the sixth aspect of the invention, which result from the electrostatic interaction between regions in their structures favoring their non-covalent binding and coupling in physiological conditions. Since the method of the invention for the preparation of nanoparticles comprises placing a preparation of the conjugates, or of polypeptides of the conjugates, of the sixth aspect of the invention in a low salt buffer, it is understood that the nanoparticles thus formed comprise also an aggregate of multiple copies of the conjugates of the sixth aspect of the invention.

10

In a particular embodiment, the conjugates are as the conjugates defined in the sixth aspect of the invention, wherein the second polypeptide region of the conjugate is a polycationic peptide, located at the N-terminus of the first polypeptide region of the conjugate, and the third polypeptide region of the conjugate is located at the C terminus of the first polypeptide region of the conjugate.

15

In some embodiments, the nanoparticles according to the invention are characterized in that the polycationic peptide of the conjugates forming the nanoparticles is a CXCR4 ligand. In some embodiments, the polycationic region of the conjugate or of the polypeptide forming the nanoparticle is selected from the group consisting of the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 (SEQ ID NO:27) peptide, the vCCL2 (SEQ ID NO:28) and functionally equivalent variant thereof. In some embodiments, wherein the nanoparticles result from the assembly of multiple copies of polypeptides comprising an antagonistic CXCR4 ligand according to the invention, the antagonistic CXCR4 ligand is the optimized EPI-X4 sequence (SEQ ID NO:29), the EPI-X4 sequence (SEQ ID N:132) or a functionally equivalent variant thereof. In some embodiments, the EPI-X4 sequence (SEQ ID NO:29) or the optimized EPI-X4 sequence is joined to a RKRKRK (SEQ ID NO:77) sequence.

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30

In an eleventh aspect, the invention relates to a biparatopic nanoparticle that comprises multiple copies of a first type of conjugates and of a second type of conjugates according

to the invention wherein the first and second types of conjugates differ in the polycationic peptide. In some embodiments, the nanoparticles according to the invention are characterized in that the polycationic peptide of the first type of conjugates and of the second type of conjugates forming the nanoparticles is a CXCR4 ligand. In some
5 embodiments, the polycationic region of the conjugate or of the polypeptide forming the nanoparticle is selected from the group consisting of the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 (SEQ ID NO:27) peptide, the vCCL2 (SEQ ID NO:28) and functionally equivalent variant thereof. In some embodiments, wherein the nanoparticles result from
10 the assembly of multiple copies of polypeptides comprising an antagonistic CXCR4 ligand according to the invention, the antagonistic CXCR4 ligand is the optimized EPI-X4 sequence (SEQ ID NO:29), the EPI-X4 sequence (SEQ ID NO:132) or a functionally equivalent variant thereof. In some embodiments, the optimized EPI-X4 sequence (SEQ ID NO:29) or the EPI-X4 sequence I(SEQ ID NO:132) is joined to a RKRKRK (SEQ ID
15 NO:77) sequence.

In a twelfth aspect, the invention relates to a biparatopic nanoparticle that comprises multiple copies of a conjugate according to the invention wherein the polycationic region is a CXCR4 ligand and multiple copies of a polypeptide comprising an antagonistic
20 CXCR4 ligand according to the invention. It will be understood that the conjugate and the polypeptide comprising an antagonistic CXCR4 ligand bind to different regions within CXCR4 and hence, despite being monospecific, they are biparatopic.

In some embodiments, the CXCR4 ligand included in the conjugates of the biparatopic
25 nanoparticles is selected from the group consisting of the peptide that comprises the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26) the CXCL12 peptide (SEQ ID NO:27), the vCCL2 peptide (SEQ ID NO:28), the optimized EPI-X4 sequence (SEQ ID NO:29), the EPI-X4 sequence (SEQ ID N:132) and a functionally equivalent variant thereof.

30

In some embodiments, the CXCR4 antagonistic ligand included in the polypeptides forming the biparatopic nanoparticles is the optimized EPI-X4 sequence (SEQ ID NO:29)

and a functionally equivalent variant thereof. In some embodiments, the optimized EPI-X4 sequence (SEQ ID NO:29) is provided forming part of a polycationic region resulting from the fusion of the EPI-X4 sequence to the RKRKRK (SEQ ID NO:77) sequence.

- 5 In one embodiment, the biparatopic nanoparticle according to the invention is formed by conjugates according to the invention in which the polycationic peptide comprises the sequence RRWCYRKCYKGYCYRKCR and by polypeptides comprising an CXCR4 antagonistic ligand in which the CXCR4 antagonistic ligand is the optimized EPI-X4 sequence (SEQ ID NO:29), optionally forming part of a fusion peptide with a polycationic
10 sequence, preferably, the RKRKRK (SEQ ID NO:77) sequence.

- Preferred forms of the biparatopic nanoparticles according to the present invention are formed by any of the preferred conjugates defined in Table 3 and any of the preferred fusion proteins comprising an antagonistic ligand as defined in Table 5. In some
15 embodiments, the first type of conjugates forming part of the biparatopic nanoparticles (those exemplified in Table 3) do not contain an agent of interest as defined above. In some embodiments, the first type of conjugates forming part of the biparatopic nanoparticles contain an agent of interest, which is preferably any of those defined above in the context of the conjugates of the invention, more preferably floxuridine and even
20 more preferably a floxuridine pentamer.

- In a particular embodiment, the expressions “polypeptide of the nanoparticle”, “first polypeptide of the nanoparticle”, “second polypeptide of the nanoparticle”, “polycationic peptide of the nanoparticle”, “CXCR4 ligand of the nanoparticle”, “third polypeptide of
25 the nanoparticle”, “polypeptide regions of the nanoparticle”, “agent of interest of the nanoparticle”, “linking moiety between the agent of interest and the nanoparticle”, or “positively charged peptide sequence”, refer to the corresponding component of the nanoparticle that is part of the conjugates assembled in the nanoparticles of the invention. Thus, said components are as defined above in the context of the conjugates of the
30 invention, and thus, are as the corresponding parts of the conjugates of the invention.

The person skilled in the art will acknowledge that the size of the nanoparticles of the invention, including the biparatopic nanoparticles of the invention, can be in the range between 1 and 1000 nm, more preferably between 2,5 and 500 nm, even more preferably between 5 and 250 nm, and yet even more preferably between 10 and 100 nm.

5

In a preferred embodiment of the invention, the nanoparticles of the invention have a diameter between 10 and 100 nm. In another preferred embodiment, the biparatopic nanoparticles of the invention have a diameter between 10 and 100 nm.

10 As it will be noted by a person skilled in the art, the biparatopic nanoparticles of the invention are considered one of the nanoparticles of the invention. Therefore, in a preferred embodiment, when referring to "the nanoparticle of the invention", or to "the nanoparticle", the biparatopic nanoparticles as defined herein are also designated.

15 In a certain embodiment, all the terms and embodiments described in the first, second, third, fourth, fifth, sixth, and seventh aspects of the invention, are equally applicable to the eighth aspect of the invention. In another embodiment, all the terms and embodiments described in the first, second, third, fourth, fifth, sixth, seventh and eighth aspects of the invention, are equally applicable to the ninth aspect of the invention.

20

VIII - Medical uses of the conjugates and the nanoparticles of the invention

In a twelfth aspect, the invention relates to a conjugate or a nanoparticle according to the invention for use in medicine. In another aspect, the invention relates to the use of a
25 conjugate or a nanoparticle according to the invention for the treatment of a patient suffering from a disease that responds to the therapeutic agent forming part of the conjugate of the invention.

As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or
30 amelioration of the progression, severity and/or duration of a condition, disorder or disease, or the amelioration of one or more symptoms (preferably, one or more discernible symptoms) of a condition, disorder or disease. The terms "treat", "treatment" and

"treating" also refer to the amelioration of at least one measurable physical parameter of a condition, disorder or disease not necessarily discernible by the patient. Furthermore, "treat", "treatment" and "treating" refer also to the inhibition of the progression of a condition, disorder or disease, either physically by, e.g., stabilization of a discernible
5 symptom, physiologically by, e.g., stabilization of a physical parameter, or both. "Treat", "treatment" and "treating" may refer, too, to the reduction or stabilization of the condition, disorder or disease.

It will be understood by the person skilled in the art that by use in medicine, the conjugate
10 or nanoparticle of the invention can be administered to a patient in order to induce a therapeutic response.

The therapeutic response comprises the suppression, reduction or arrest of the causes of the pathological condition or the disease suffered by a patient; the elimination, reduction,
15 arrest or amelioration of the symptoms of the condition or disease; or the extinction, arrest or slowing down of the progression of the condition or disease in the patient.

The person skilled in the art will acknowledge that the conjugate or nanoparticle of the invention suitable for use in medicine may be presented accompanied by a
20 pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" means a non- toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Remington's Pharmaceutical Sciences. Ed. by Gennaro, Mack Publishing, Easton, Pa., 1995 discloses various carriers used in formulating pharmaceutical compositions and known techniques for the
25 preparation thereof.

Accordingly, the compositions comprising the conjugate or nanoparticle of the invention and a pharmaceutically acceptable carrier are pharmaceutical compositions.

30 The pharmaceutical compositions of this invention can be administered to a patient by any means known in the art including oral and parenteral routes. According to such embodiments, inventive compositions may be administered by injection (e.g.,

intravenous, subcutaneous or intramuscular, intraperitoneal injection), rectally, vaginally, topically (as by powders, creams, ointments, or drops), or by inhalation (as by sprays).

5 *VII.A-Use of the conjugate or the nanoparticle of the invention in the treatment of cancer.*

Another embodiment of the invention relates to the conjugate or the nanoparticle of the invention, or their corresponding pharmaceutical compositions, wherein the polypeptide of the conjugate, or at least a polypeptide of the nanoparticle, comprises a sequence which
10 is capable of specifically interacting with a receptor on a cell surface and promoting the internalization of the conjugate, or the nanoparticle, into the cell, wherein said cell expressing the receptor is a tumor cell present in cancer for use in the treatment of cancer.

In a particular embodiment, the agent of interest of said conjugates for use, or of said
15 nanoparticles for use, or of the conjugates or nanoparticles of the corresponding pharmaceutical compositions for use, is a therapeutic agent selected from the group consisting of

- (i) A chemotherapy agent,
- (ii) a cytotoxic polypeptide,
- 20 (iii) an antiangiogenic polypeptide,
- (iv) a polypeptide encoded by a tumor suppressor gene,
- (v) a pro-apoptotic polypeptide,
- (vi) a polypeptide having anti-metastatic activity,
- (vii) a polypeptide encoded by a polynucleotide which is capable of activating
25 the immune response towards a tumor and
- (viii) an antiangiogenic molecule.
- (ix) a toxin.

As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or
30 amelioration of the progression, severity and/or duration of cancer, or the amelioration of one or more symptoms (preferably, one or more discernible symptoms) of cancer. The terms "treat", "treatment" and "treating" also refer to the amelioration of at least one

measurable physical parameter of cancer, such as growth of a tumor, not necessarily discernible by the patient. Furthermore, "treat", "treatment" and "treating" refer also to the inhibition of the progression of cancer, either physically by, e.g., stabilization of a discernible symptom, physiologically by, e.g., stabilization of a physical parameter, or
5 both. "Treat", "treatment" and "treating" may refer, too, to the reduction or stabilization of tumor size or cancerous cell count.

The term "cancer" refers to a group of diseases involving abnormal, uncontrolled cell growth and proliferation (neoplasia) with the potential to invade or spread (metastasize)
10 to other tissues, organs or, in general, distant parts of the organism; metastasis is one of the hallmarks of the malignancy of cancer and cancerous tumors. The abnormal growth and/or proliferation of cancerous cells is the result of a combination of genetic and environmental factors that alter their normal physiology. The growth and/or proliferation abnormalities of cancerous cells result in physiological disorders and, in many cases,
15 death of the individual, due to the dysfunctionality or loss of functionality of the cell types, tissues and organs affected.

The term "cancer" includes, but is not restricted to, cancer of the breast, prostate, lung, ovarian, colon, colorectal, pancreatic, kidney, brain, non-Hodgkin's lymphoma, chronic
20 lymphocytic leukemia. heart, small intestine, spleen, kidney, bladder, head, neck, skin, bone, bone marrow, blood, thymus, womb, testicles, hepatobiliary system and liver; in addition to tumors such as, but not limited to, adenoma, angiosarcoma, astrocytoma, epithelial carcinoma, germinoma, glioblastoma, glioma, hemangioendothelioma, hemangiosarcoma, hematoma, hepatoblastoma, leukemia, lymphoma, medulloblastoma,
25 melanoma, neuroblastoma, hepatobiliary cancer, osteosarcoma, retinoblastoma, rhabdomyosarcoma, sarcoma and teratoma. Furthermore, this term includes acrolentiginous melanoma, actinic keratosis adenocarcinoma, adenoid cystic carcinoma, adenomas, adenocarcinoma, adenosquamous carcinoma, astrocytic tumors, Bartholin gland carcinoma, basal cell carcinoma, bronchial gland carcinoma, capillary carcinoid,
30 carcinoma, carcinosarcoma, cholangiocarcinoma, cystadenoma, endodermal sinus tumor, endometrial hyperplasia, endometrial stromal sarcoma, endometrioid adenocarcinoma, ependymal sarcoma, Ewing sarcoma, focal nodular hyperplasia, germ cell tumors,

glioblastoma, glucagonoma, hemangioblastoma, hemagioendothelioma, hemangioma, hepatic adenoma, hepatic adenomastosis, hepatocellular carcinoma, hepatobiliary cancer, insulinoma, intraepithelial neoplasia, squamous cell intraepithelial neoplasia, invasive squamous-cell carcinoma, large cell carcinoma, leiomyosarcoma, melanoma, malignant melanoma, malignant mesothelial tumor, meduloblastoma, medulloepithelioma, mucoepidermoid carcinoma, neuroblastoma, neuroepithelial adenocarcinoma, nodular melanoma, osteosarcoma, papillary serous adenocarcinoma, pituitary tumors, plasmacytoma, pseudosarcoma, pulmonary blastoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, sarcoma, serous carcinoma, microcytic carcinoma, soft tissue carcinoma, somatostatin secreting tumor, squamous carcinoma, squamous cell carcinoma, undifferentiated carcinoma, uveal melanoma, verrucous carcinoma, vipoma, Wilm tumor, intracerebral cancer, head and neck cancer, rectal cancer, astrocytoma, glioblastoma, microcytic cancer and non-microcytic cancer, metastatic melanoma, androgen-independent metastatic prostate cancer, androgen-dependent metastatic prostate cancer and breast cancer.

Thus, in a preferred embodiment of the invention, the therapeutic agent is selected from the group consisting of

- (i) A chemotherapy agent,
- (ii) a cytotoxic polypeptide,
- (iii) an antiangiogenic polypeptide,
- (iv) a polypeptide encoded by a tumor suppressor gene,
- (v) a pro-apoptotic polypeptide,
- (vi) a polypeptide having anti-metastatic activity,
- (vii) a polypeptide encoded by a polynucleotide which is capable of activating the immune response towards a tumor and
- (viii) an antiangiogenic molecule.
- (ix) a toxin

In a particular embodiment of the invention, the therapeutic agent is an antitumor peptide selected from the group consisting of the BH3 domain of BAK, PUMA, GW-H1, and the active segment of diphtheria toxin I, and the *Pseudomonas aeruginosa* exotoxin A.

As used herein “BAK” refers to the well-known pro-apoptotic factor belonging to the Bcl-2 protein family that triggers programmed cell death by caspase-dependent apoptotic pathway through inactivating anti-apoptotic proteins, permeabilizing the mitochondrial
5 membrane, and consequently, releasing cytochrome C and other mitochondrial cell death factors. [as seen in Llambi, F. et al. 2011. Mol. Cell, 44:517-31]. In one embodiment, BAK refers to full length BAK (SEQ ID NO:67). In other embodiment, BAK refers to any truncated form thereof containing the functional BH3 domain (SEQ ID NO:68).

10 As used herein, “PUMA” refers to a protein characterized by a full sequence corresponding to SEQ ID NO:69) which is a (Bcl-2 homology 3) BH3-only protein that triggers cell death by interacting with pro and antiapoptotic proteins of the Bcl-2 family.

As used herein, GW-H1 refers to a polypeptide having the sequence of SEQ ID NO:46
15 which exerts its cytolytic activity by folding into an amphipathic helix.

.
Diphtheria toxin I (produced by the bacteria of the species *Corynebacterium diphtheriae*) (SEQ ID NO:70) and the exotoxin of *P. aeruginosa* (SEQ ID NO:71) belong to the family of ADP-ribosylating toxins. Both toxins are proteins that act on eukaryotic Elongation
20 Factor-2 (eEF-2), basically inhibiting the translational activity of the cell that incorporates them and inducing apoptosis. The structure of both toxins presents a receptor-binding domain (that binds to a surface receptor of the cell and induces endocytosis; heparin binding epidermal growth factor precursor in the case of diphtheria toxin, CD91 in the case of the exotoxin A), a translocation domain, and a catalytic domain that performs the
25 action on eEF-2 (an overview is provided in Shapira, A. & Benhar, I., 2010, Toxins, 2:2519-2583).

In an even more preferred embodiment of the invention, the polycationic peptide of the conjugate of the invention or of the nanoparticle of the invention is a CXCR4 ligand, and
30 the cancer targeted to be treated with the conjugate or the nanoparticle of the invention is characterized in that it comprises cells which express the CXCR4 receptor. In a more preferred embodiment, the cells cancer cells that express or overexpress CXCR4 are

metastatic stem cells. The term "metastatic stem cells", as used herein, refers to cells that are responsible for metastasis initiation and metastasis maintenance

In a yet more preferred embodiment of the invention, the CXCR4 ligand of the conjugate
5 or of the nanoparticle of the invention is selected from the group comprising the T22 peptide (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 peptide (SEQ ID NO:27), the vCCL2 peptide (SEQ ID NO:28), the optimized EPI-X4 sequence (SEQ ID NO:29), the EPI-X4 sequence (SEQ ID NO:132) or a functionally equivalent variant thereof.

10

In another more preferred embodiment of the invention, the cancer to be treated with the conjugate or the nanoparticle of the invention is selected from the group consisting of pancreatic and colorectal cancer.

15 In another preferred embodiment of the invention, the conjugate and the nanoparticle of the invention are used for the treatment of cancerous tumor, wherein the cancerous tumor is a primary tumor or a metastasis.

In a certain embodiment, all the terms and embodiments described in the previous aspects
20 of the invention, are equally applicable to the twelfth aspect of the invention.

VII.B - Use of the conjugate or of the nanoparticle of the invention in the treatment of bacterial infections

25 Another embodiment of the invention relates to the conjugate or of the nanoparticle of the invention for use in the treatment of a disease caused by a bacterial infection.

As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or amelioration of the progression, severity and/or duration of a bacterial infection, or the
30 amelioration of one or more symptoms (preferably, one or more discernible symptoms) of a bacterial infection. The terms "treat", "treatment" and "treating" also refer to the amelioration of at least one measurable physical parameter of a bacterial infection, such

as presence of bacterial toxins, not necessarily discernible by the patient. Furthermore, "treat", "treatment" and "treating" refer also to the inhibition of the progression of a bacterial infection, either physically by, e.g., stabilization of a discernible symptom, physiologically by, e.g., stabilization of a physical parameter, or both. "Treat",
 5 "treatment" and "treating" may refer, too, to the reduction or stabilization of the bacterial cell count.

The term "bacteria", as used herein, refers to Prokaryotes of the domain *Bacteria*. Non-limiting examples of bacterial genera that may be used in the method of the present
 10 invention include: *Actinomyces*, *Bacillus*, *Bacteroides*, *Bartonella*, *Bordetella*, *Borrelia*, *Brucella*, *Burkholderia*, *Campylobacter*, *Chlamydia*, *Clostridium*, *Corynebacterium*, *Coxiella*, *Ehrlichia*, *Enterococcus*, *Eschericia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Klebsiella*, *Legionella*, *Leptospira*, *Listeria*, *Moraxella*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Nocardia*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*,
 15 *Streptobacillus*, *Streptococcus*, *Treponema*, *Ureaplasma*, *Vibrio* and *Yersinia*. Individual Prokaryotes of the domain *Bacteria* are denominated bacterium.

The invention contemplates the suitability of the fusion protein, the polynucleotide, the vector, the host cell, or the nanoparticle for the treatment of infections of bacteria such as
 20 *Neisseria spp.*, including *N. gonorrhea* and *N. meningitides*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mutans*; *Haemophilus ducreyi*; *Moraxella spp.*, including *M. catarrhalis*, also known as *Branhamella catarrhalis* *Bordetella spp.*, including *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, *Mycobacterium spp.*, including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. avium*, *M. paratuberculosis*, *M.*
 25 *smegmatis*; *Legionella spp.*, including *L. pneumophila*, *Escherichia spp.*, including enterotoxigenic *E. coli*, enterohemorrhagic *E. coli* and enteropathogenic *E. coli*, *Vibrio spp.*, including *V. cholera*, *Shigella spp.*, including *S. sonnei*, *S. dysenteriae*, *S. flexnerii*; *Yersinia spp.*, including *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*; *Campylobacter spp.*, including *C. jejuni*, *Salmonella spp.*, including *S. typhi*, *S. enterica*
 30 and *S. bongori*; *Listeria spp.*, including *L. monocytogenes*; *Helicobacter spp.*, including *H. pylori*, *Pseudomonas spp.*, including *P. aeruginosa*; *Staphylococcus spp.*, including *S. aureus*, *S. epidermidis*; *Enterococcus spp.*, including *E. faecalis*, *E. faecium*; *Clostridium*

spp., including *C. tetani*, *C. botulinum*, *C. difficile*, *Bacillus* spp., including *B. anthracis*; *Corynebacterium* spp., including *C. diphtheria*, *Borrelia* spp., including *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. andersonfi*, *B. hermsii*; *Ehrlichia* spp., including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia* spp., including *R. rickettsii*;
 5 *Chlamydia* spp., including *C. trachomatis*, *Chlamydia pneumoniae*, *C. psittaci*; *Leptospira* spp., including *L. interrogans*; *Treponema* spp., including *T. pallidum*, *T. denticola*, *T. hyodysenteriae*, *Mycobacterium tuberculosis*, *Streptococcus* spp., including *S. pneumoniae*, *Haemophilus* spp., including *H. influenzae* type B, and non typeable *H. influenzae*, among others and without limitation.

10

VII.C - Use of the conjugate or of the nanoparticle of the invention in the treatment of viral infections

Another embodiment of the invention, relates to the conjugate or of the nanoparticle of
 15 the invention, wherein the polycationic peptide is capable of specifically interacting with a receptor on the cell surface of a cell infected by a virus causing an infection; and wherein the intervening polypeptide region is an antiviral agent, for use in the treatment of a disease caused by a viral infection.

20 As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or amelioration of the progression, severity and/or duration of a viral infection, or the amelioration of one or more symptoms (preferably, one or more discernible symptoms) of a viral infection. The terms "treat", "treatment" and "treating" also refer to the amelioration of at least one measurable physical parameter of a bacterial infection, such
 25 as viral titer, not necessarily discernible by the patient. Furthermore, "treat", "treatment" and "treating" refer also to the inhibition of the progression of a viral infection, either physically by, e.g., stabilization of a discernible symptom, physiologically by, e.g., stabilization of a physical parameter, or both. "Treat", "treatment" and "treating" may refer, too, to the reduction or stabilization of the viral titer.

30

The term "virus", as used herein, refers to a small infectious agent that can replicate only inside the living cells of organisms. Non-limiting examples of viral families that may be

used in the method of the present invention include *Adenoviridae*, African swine fever-like viruses, *Arenaviridae*, *Arteriviridae*, *Astroviridae*, *Baculoviridae*, *Birnaviridae*, *Bunyaviridae*, *Caliciviridae*, *Circoviridae*, *Coronaviridae*, *Deltavirus*, *Filoviridae*, *Flaviviridae*, *Hepadnaviridae*, *Hepeviridae*, *Herpesviridae*, *Orthomyxoviridae*,
 5 *Paramyxoviridae*, *Picomaviridae*, *Poxviridae*, *Reoviridae*, *Retroviridae* and *Rhabdoviridae*.

Examples of viral infections that the fusion protein, the polynucleotide, the vector, the host cell, or the nanoparticle of the invention are suitable to treat include those of Human
 10 Immunodeficiency Virus (HIV-1), human herpes viruses, like HSV1 or HSV2, cytomegalovirus, especially Human, Epstein Barr virus, Varicella Zoster Virus, hepatitis virus such as hepatitis B virus, hepatitis C virus, paramyxoviruses such as Respiratory Syncytial virus, parainfluenza virus, rubella virus, measles virus, mumps virus, human papilloma viruses, flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne
 15 encephalitis virus, Japanese Encephalitis Virus), Influenza virus, rotavirus, and the like.

In an even more preferred embodiment of the invention, the antiviral agent of the fusion protein, the polynucleotide, the vector, the host cell, or the nanoparticle of the invention is selected from the group consisting of

- 20 (i) A cytotoxic polypeptide,
 - (ii) A pro-apoptotic polypeptide,
 - (iii) A polypeptide encoded by a suicide gene; and
 - (iv) An antiretroviral polypeptide
- 25 Cytotoxic polypeptides (i), pro-apoptotic polypeptides (ii) and polypeptides encoded by a suicide gene have already been discussed in the section corresponding to the fusion protein.

Antiretroviral agents are one subtype of the antiviral class of antimicrobials.
 30 Antiretroviral agents are used specifically for treating viral infections caused by retroviruses. Retroviruses comprise the *Retroviridae* family of viruses, which includes genera such as *Alpharetrovirus*, *Betaretrovirus*, and *Lentivirus*, to name a few. They are

characterized by being single-stranded, positive-sense RNA-genome viruses. Retroviruses generate, through their own reverse transcriptase, a double stranded DNA copy of their genome that integrates in the genome of their host cell. The person skilled in the art will recognize that “antiretroviral agents” comprises any molecules or
5 compounds capable of interfering with the normal replication cycle of a retrovirus at any of its stages. Thus, an antiretroviral polypeptide (iv), as used herein refers to a polypeptide with antiretroviral properties.

Antiretroviral polypeptides suitable for the invention are, for instance, “entry inhibitors”,
10 also known as “fusion inhibitors”, peptides which interfere with the binding, fusion and entry of the retrovirus to the host cell. Examples of this group are efavirtide, a biomimetic peptide that competes with the fusion machinery of HIV-1, and peptide T, a peptide that blocks chemokine receptors CCR2 and CCR5.

15 Also comprised as entry inhibitors are antibodies specific against the receptors used by retroviruses to fuse with the cell. Non-limiting examples of these receptors suitable to be blocked with antibodies, are CD4, CCR2, CCR5, and CXCR4.

The term “antibody”, as used herein, refers to a glycoprotein that exhibits specific binding
20 activity for a particular protein, which is referred to as “antigen”. The term “antibody” comprises whole monoclonal antibodies or polyclonal antibodies, or fragments thereof, and includes human antibodies, humanised antibodies, chimeric antibodies and antibodies of a non-human origin. “Monoclonal antibodies” are homogenous, highly specific antibody populations directed against a single site or antigenic “determinant”.
25 “Polyclonal antibodies” include heterogeneous antibody populations directed against different antigenic determinants.

As used herein, the antibodies suitable for the invention encompass not only full length antibodies (e.g., IgG), but also antigen-binding fragments thereof, for example, Fab, Fab’,
30 F(ab')₂, Fv fragments, human antibodies, humanised antibodies, chimeric antibodies, antibodies of a non-human origin, recombinant antibodies, and polypeptides derived from immunoglobulins produced by means of genetic engineering techniques, for example,

single chain Fv (scFv), diabodies, heavy chain or fragments thereof, light chain or fragment thereof, VH or dimers thereof, VL or dimers thereof, Fv fragments stabilized by means of disulfide bridges (dsFv), molecules with single chain variable region domains (Abs), minibodies, scFv-Fc, and fusion proteins comprising an antibody, or any
5 other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of a desired specificity. The antibody of the invention may also be a bispecific antibody. An antibody fragment may refer to an antigen binding fragment. An antibody includes an antibody of any class, namely IgA, IgD, IgE, IgG (or sub-classes thereof), and IgM, and the antibody need not be of any particular class.

10

Thus, a yet more preferred embodiment of the invention relates to the fusion protein, polynucleotide, vector, host cell, or nanoparticle of the invention, wherein the polycationic peptide is a CXCR4 ligand, and wherein the cell is an HIV-infected cell, for use in the treatment of HIV infection.

15

A yet even more preferred embodiment of the invention relates to the fusion protein, the polynucleotide, the vector, the host cell, or nanoparticle of the invention, wherein the CXCR4 ligand is selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8 or a functionally equivalent variant thereof for use in
20 the treatment of a viral infection.

VII.D - Use of the conjugate or of the nanoparticle of the invention in the treatment of neurodegenerative diseases

25 In another embodiment of the invention relates to the conjugate or of the nanoparticle of the invention, wherein the polycationic peptide is a peptide capable of crossing the blood-brain barrier, and wherein the intervening polypeptide region is a chaperone or an inhibitor of protein aggregation, for use in the treatment of a neurodegenerative disease.

30 Protein aggregation is a biological phenomenon which results from the accumulation of misfolded proteins, whether intra- or extracellularly. The resulting protein aggregates can originate diseases and, in fact, it has been found their involvement in a wide range of

diseases known as amyloidoses. The amyloidoses comprise several well-studied neurodegenerative diseases, like ALS, Alzheimer's, Parkinson's and prion disease.

Suitable chaperones or inhibitors of protein aggregation are as defined above. Diseases
 5 that can be treated using the fusion proteins, nanoparticles, vectors or host cells according to the invention include Alzheimer's disease, Pick's disease, Alpha1-antitrypsin deficiency, Parkinson's disease and other synucleinopathies, Creutzfeldt–Jakob disease, Retinal ganglion cell degeneration in glaucoma, Cerebral β -amyloid angiopathy, Prion diseases, Tauopathies, Frontotemporal lobar degeneration, Type II diabetes,
 10 Amyotrophic lateral sclerosis, Huntington's disease and other trinucleotide repeat disorders, Familial Danish dementia, Familial English dementia, Hereditary cerebral hemorrhage with amyloidosis, Alexander disease, Seipinopathies, Familial amyloidotic neuropathy, Senile systemic amyloidosis, Lysozyme amyloidosis, Fibrinogen amyloidosis, Dialysis amyloidosis, Inclusion body myositis/myopathy, Cataracts,
 15 Retinitis pigmentosa with rhodopsin mutations, Medullary thyroid carcinoma, Cardiac atrial amyloidosis, Pituitary prolactinoma, Hereditary lattice corneal dystrophy, Cutaneous lichen amyloidosis, Mallory bodies, Corneal lactoferrin amyloidosis, Pulmonary alveolar proteinosis, Odontogenic tumor amyloid, Seminal vesicle amyloid, Apolipoprotein C2 amyloidosis, Apolipoprotein C3 amyloidosis, Lect2 amyloidosis,
 20 Insulin amyloidosis, Galectin-7 amyloidosis (primary localized cutaneous amyloidosis), Corneodesmosin amyloidosis, Enfuvirtide amyloidosis, Cystic Fibrosis, Sickle cell disease, Hereditary cerebral hemorrhage with amyloidosis, AL amyloidosis AH amyloidosis, AA amyloidosis, Aortic medial amyloidosis, ApoAI amyloidosis, ApoAII amyloidosis, ApoAIV amyloidosis and Familial amyloidosis of the Finnish type.
 25

Accordingly, a preferred embodiment of the invention relates to the conjugate or to the nanoparticle of the invention, wherein the intervening polypeptide region is a chaperone or an inhibitor of protein aggregation, for use in the treatment of a neurodegenerative disease, wherein the polycationic peptide capable of crossing the blood-brain barrier is
 30 selected from the group consisting of Seq-1-7, Seq-1-8, and Angiopep-2-7.

IX - Method for imaging a target cell

In another aspect, the invention is addressed to a method allowing detecting the presence of a specific cell, or group of cells, within a cell sample, by means of the use of the conjugates or the nanoparticles of the invention.

5

Therefore, in an thirteenth aspect, the invention relates to a method for the imaging of a target cell which comprises specific binding sites for one or more components of the conjugate according to the sixth aspect of the invention, or of the nanoparticle according to the ninth aspect of the invention, the method comprising

- 10 (i) contacting a sample containing said cell with a conjugate according to the sixth aspect of the invention or with a nanoparticle according to the tenth or to the eleventh aspect of the invention under conditions adequate for the binding of the conjugate or of the nanoparticle to the cell and wherein the agent of interest is an imaging agent and
- 15 (ii) Imaging the cell by detecting the signal provided by the imaging agent.

The expression “method for the imaging”, as used herein, refers to any method allowing to visualize a target cell within a cell sample, by increasing the contrast between the parts of an image of the sample that comprise the target cell/s and the parts of the image that
20 do not comprise said cell/s. This increase in the contrast is generally mediated by means of an imaging agent, as it has been defined in section IV-E.2 above. In a particular embodiment, the imaging agent is any of those specified in said section.

The term “target cell”, as used herein, refers to a cell showing at least one characteristic
25 of interest associated to the presence of a specific binding site in the surface of said target cells. In a particular embodiment, said cell is any of the cells comprising the cell receptors specified in the sixth aspect of the invention.

In a preferred embodiment, the cell expresses or overexpresses the CXCR4 receptor. In
30 another preferred embodiment, the target cell is a cancer cell from any of the cancers specified in the definition of “cancer” in the sixth aspect of the invention. Preferably, said cell is a cell from breast cancer, prostate cancer, lung cancer, ovarian cancer, colon cancer,

colorectal cancer, pancreatic cancer, kidney cancer, brain cancer, non-Hodgkin's lymphoma and chronic lymphocytic leukemia.

The term “binding site”, as used herein, refers to any pair of a binding pair, wherein the
5 other part of the binding pair is one of the components of the conjugate or nanoparticles of the invention, preferably the second polypeptide of the conjugate or of the nanoparticle of the invention. The expression “binding pair” refers to a molecule that can interact and bind to another molecule resulting in a binding complex, wherein the first and second
10 components are bound to each other by means of non-covalent bonds, such as hydrogen bonds, hydrophobic interactions, van der Waals bonds, ionic bonds or a combination thereof. A binding pair can be part of any type of interaction such as protein/protein interaction, peptide/protein interaction, protein region/protein interaction, antigen/antibody, antigen/antibody fragment or hapten/anti-hapten. The terms “binding”, or “bound” have been defined in the second aspect of the invention.

15 In a particular embodiment, the binding site is any of the targets of interest specified in section IV- B above. In another particular embodiment, it is a cell receptor. In a particular embodiment, it is a cell receptor selected from those specified in section IV-B above. In particular embodiment, it is the CXCR4 receptor. As understood by a skilled person, said
20 targets of interest, as well as the cell receptors, are not necessarily specific for the binding of the second polypeptide of the conjugate of the invention, or of the nanoparticles of the invention. Said targets of interest can be specific for the binding of any component of the conjugate or of the nanoparticle of the invention, or even for the binding of more than one component of the conjugates or nanoparticles.

25 In a particular embodiment, the target cell comprises more than one binding site, wherein some of the binding sites are specific for the binding of one component of the conjugate, and the rest of binding sites are specific for the binding of at least another component of the conjugate of the invention, or of the nanoparticles of the invention.

30 As understood by a skilled person, the components of the conjugate or of the nanoparticle of the invention can be: the first, second, and third polypeptide regions of the conjugate

of the invention, or of the nanoparticle of the invention, the linking moieties between said polypeptide regions, the protease cleavage sites between said polypeptide regions, the agent of interest of the polypeptides or the nanoparticles of the invention, or the linking moieties between the agent of interest and the polypeptide of the conjugate of the invention, or of the nanoparticle of the invention.

In a particular embodiment, the one or more components of the conjugate or of the nanoparticle that bind to the binding site in the target cell is the second polypeptide of the conjugate, or of the nanoparticle of the invention, wherein said polypeptide is as defined in the sixth aspect of the invention. In a particular embodiment, said second polypeptide is a polycationic peptide. In a preferred embodiment, the polycationic peptide is a CXCR4 ligand. In a more preferred embodiment, it is a CXCR4 ligand selected from the group of CXCR4 ligands specified in section IV-B.(i).

In a preferred embodiment of the imaging method of the invention, the target cell expresses or overexpressed CXCR4 and the one or more components of the conjugate or of the nanoparticle of the invention is a polycationic peptide and is a ligand of CXCR4. In a preferred embodiment, said ligand is selected from the CXCR4 ligands specified in section IV-B (i).

As it will be understood by a skilled person, when the nanoparticle is a biparatopic nanoparticle, the one or more components of the two different conjugates of the nanoparticle that bind to the binding site in the target cell is the second polypeptide of each of the conjugates forming the biparatopic nanoparticle of the invention, wherein said polypeptides are different between the different conjugates of the biparatopic nanoparticle and selected from those defined in the sixth aspect of the invention.

In a particular embodiment, the second polypeptide region of one of the two conjugates of the biparatopic nanoparticle is a polycationic peptide. In a preferred embodiment, the polycationic peptide is a CXCR4 ligand. In a more preferred embodiment, it is a CXCR4 ligand selected from the group of CXCR4 ligands specified in section IV-B.(i). In another particular embodiment, the second polypeptide region of both conjugates of the

biparatopic nanoparticle is a polycationic peptide. In a preferred embodiment, the polycationic peptide of both conjugates is a CXCR4 ligand. In a more preferred embodiment, the polycationic peptide of both conjugates is a CXCR4 ligand selected from the group of CXCR4 ligands specified in section IV-B.(i), wherein each of the

5 polycationic peptides is different. IN a preferred embodiment, the polycationic peptide of one of the conjugates of the biparatopic nanoparticles is the T22 peptide (SEQ ID NO. 25, RRWCYRKCYKGYCYRKCR). In another preferred embodiment, the polycationic peptide of one of the conjugates of the biparatopic nanoparticle is the optimized EPI-X4 sequence (SEQ ID NO. 29). In a more preferred embodiment, the polycationic peptide of

10 one of the conjugates of the biparatopic nanoparticles is the T22 peptide (SEQ ID NO. 25, RRWCYRKCYKGYCYRKCR), and the polycationic peptide of the other conjugate of the biparatopic nanoparticle is the optimized EPI-X4 sequence (SEQ ID NO. 29).

The term “sample” or “biological sample”, as used herein, refers to biological material

15 isolated from an organism, preferably a subject. The biological sample of the thirteenth aspect of the invention contains any biological material suitable for detecting target cells present in the sample. The biological sample can comprise cell and/or non-cell material of the subject. The sample can be isolated from any suitable tissue or biological fluid such as, for example a tissue biopsy, solid tumor biopsy, blood, saliva, cerebrospinal fluid,

20 urine, stool, bone marrow, a nipple aspirate, a solid tumor biopsy, plasma, serum, cerebrospinal liquid (CSF), feces, a buccal or buccal-pharyngeal swab, semen, a surgical specimen, a specimen obtained from a biopsy, and a tissue sample embedded in paraffin.

The sample is contacted with the conjugates of the invention or the nanoparticles of the

25 invention under conditions adequate for the binding of the conjugate or the nanoparticle to the binding site of the cell. The expression “under conditions adequate for binding” means that the conditions preferably include diluting the conjugates or the nanoconjugates with solutions that are not toxic for the cells, such as BSA or phosphate buffered saline (PBS). These added agents also tend to assist in the reduction of

30 nonspecific background.

The “suitable” or “adequate” conditions also mean that the incubation is at a temperature or for a period of time sufficient to allow effective binding. The conjugates or nanoparticles of the invention are incubated for any suitable time, for instance at least 5 min, at least 15 min, at least 30min, at least 1 hour, at least 2 hours, at least 4 hours. They
5 are performed at temperatures allowing the survival of the cells and the binding of the conjugates or nanoparticles to the binding sites. Preferably, said temperature is in the order of 15-45°C, preferably 25°C, more preferably 37°C, or at room temperature.

After said incubation, the excess of conjugates and/or nanoparticles in the sample are
10 removed by methods well-known by an expert in the field and include the use of a suitable medium, such as PBS. The medium may contain a detergent such as Tween20. It may be washed for any suitable time, e.g. 1 to 30 minutes or 3 to 10 minutes for each wash. Washing may include gentle shaking or rocking of the carrier of said cell. The washing temperature is such that the cell can survive and binding is not disrupted. For example, it
15 can be between 15-45°C, preferably 25°C, more preferably 37°C, or at room temperature. Suitable protocols for washing complexes formed by a polypeptide bound to a binding site in a cell, i.e formed by the conjugate or the nanoparticle of the invention bound to the binding site of the target cell, are well known in the art.

20 Imaging the cell in the sample involves detecting the signal emitted by the imaging agent present in the conjugates or nanoconjugates present in the sample. As understood by a skilled person, after the washing step, said conjugates and nanoconjugates are primarily those bound to the binding site of the target cells present in the sample. Thus, detecting the signal emitted by the imaging agents in the sample indirectly allows detecting the
25 target cells present in the sample. The technique used to detect the imaging agent will depend on the imaging agent used. Said techniques are well-known by an expert in the field, and may comprise the use of a microscope coupled to a camera capable to detect the specific imaging agent. For instance, if the imaging agent is fluorescent agent, it might comprise the use of a fluorescent microscope, if the imaging agent is a radionucleotide, it
30 might comprise the use a of a radioluminescence microscope, or a single-cell radioluminescence microscope. Adequate methods to detect a specific type of imaging

agent are well known in the art and are of the type indicated in section IV-B.1 for each group of imaging agent specified in this section.

In a particular embodiment, the imaging method allows to determine the target cells
5 present in the sample and also the amount of said target cells in the sample. As understood by a skilled person, it allows to compare the amount of target cells present in sample with the amount of cells present in a control sample.

In a certain embodiment, all the terms and embodiments described in the previous aspects
10 of the invention, are equally applicable to the thirteenth aspect of the invention.

X - Methods for identifying a polypeptide that binds to a target peptide.

The polypeptide according to the first aspect of the invention is capable of presenting
15 heterologous peptides which are inserted in one or more of the loop regions AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK. These variant polypeptides can be provided as a peptide library wherein different polypeptides are presented by different members of the library. These libraries can be used for the identification of peptides which are capable of binding to a target polypeptide by selecting those members of the library which bind to the target
20 polypeptide and determining the sequence of the peptide within the member of the polypeptide library which is responsible for the binding. Thus, in another aspect, the invention is addressed to a method for the identification of a polypeptide that binds to a target peptide.

25 In a fourteenth aspect, the invention relates to a method for identifying a polypeptide that binds to a target peptide, said method comprising:

- i) contacting a target peptide with the polypeptide display library according to the second aspect of the invention under conditions that allow a polypeptide to interact with the target peptide,
- 30 ii) recovering those members of the library that have specifically interacted with the target peptide, and
- iii) identifying the sequence of the polypeptide that interacts with the target peptide.

The term “target peptide”, as used herein, refers to any peptide of interest, with no function, sequence or structure limitation.

- 5 The expression “under conditions that allow a polypeptide to interact with the target peptide” refers to conditions allowing the target peptide and the members of the polypeptide library to maintain their integrity, so that at least the tertiary structure of the polypeptides of the library and of the target peptides is maintained and thus, the polypeptide of the library that has binding affinity for the target peptide specifically binds
- 10 to the target peptide. Said conditions involves diluting the members of the library in a certain buffer allowing the polypeptide to maintain its integrity. Said buffers are well-known by an expert in the field and can include BSA, bovine gamma globulin (BGG), phosphate buffered saline (PBS), or Tris-buffered saline (TBS). Said conditions also imply that the polypeptide and the target peptide are incubated for a period of time and a
- 15 temperature adequate for said specific binding to take place effectively. Said conditions are well-known by an expert in the field, and typically include 1 to 4 hours of incubation, at temperatures preferably in the order of 15-45°C, preferably 25°C, more preferably 37°C, or at room temperature or may be overnight at 4°C.
- 20 The term “member of the polypeptide library” has been defined in the first aspect of the invention. Thus, in a particular embodiment, the member of the library is the polypeptide of the library as defined in the second aspect of the invention. In another particular embodiment, it is the complex comprising a polypeptide of the first aspect of the invention, directly linked to a nucleic acid encoding it, as defined in the second aspect of
- 25 the invention. In another particular embodiment, it is a microorganism comprising the polypeptide of the library as defined in the second aspect of the invention.

- The terms “binding” and “specifically binding” have been defined in the first aspect of the invention. In a particular embodiment, the term “binds” and “interacts” in the context
- 30 in the present aspect are interchangeable. In another particular embodiment, the method is addressed to identify a polypeptide that specifically binds to a target peptide, wherein the polypeptide is considered to specifically bind a target peptide if the binding affinity

between said polypeptide and target peptide is of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M.

- 5 In a particular embodiment, the target peptide is immobilized in a solid support.

Non-limiting exemplary solid supports include polymers (such as agarose, sepharose, cellulose, nitrocellulose, alginate, Teflon, latex, acrylamide, nylon, plastic, polystyrene, silicone, etc.), glass, silica, ceramics, and metals. Such solid supports may take any form,
10 such as particles (including microparticles), sheets, dip-sticks, gels, filters, membranes, microfiber strips, tubes, wells, plates (such as microplates, including 6-well plates, 24-well plates, 96-well plates, 384-well plates, etc.), fibers, capillaries, combs, pipette tips, microarray chips, etc. In some embodiments, the biotin-binding moiety is associated with the surface of a solid support. In some embodiments, the surface of the solid support
15 comprises an irregular surface, such as a porous, particulate, fibrous, webbed, or sintered surface.

In some embodiments, a solid support is selected from a microplate, a microarray chip, and a microparticle. In some embodiments, a solid support is at least partially composed
20 of a polymer. In some embodiments, a microparticle solid support comprises monodisperse or polydisperse spherical beads. Monodisperse microparticles are substantially uniform in size (i.e., they have a diameter standard deviation of less than 5 percent), while polydisperse microparticles vary in size. In some embodiments, microparticles are composed of the same polymer throughout, or are core-shell polymers,
25 in which the core of the microparticle is composed of one polymer, and the outer layer (or "shell") is composed of another. In some embodiments, microparticles are magnetic.

In some embodiments, the target peptide is attached to a solid support through a linker moiety. In a particular embodiment, said linker comprises a protease cleavage site.
30

In a second step of the method, the member or members of the library which are specifically bound to the target peptide are recovered. It will be understood that, in order

to identify in step (ii) those polypeptides which bind specifically to the target polypeptide, a separate binding reaction (hereinafter referred to as “negative selection step”) should be carried out in parallel in which the target polypeptide is contacted with the non-variant or naturally occurring form of the polypeptide which is found in the library. Preferably, said

5 non-variant or naturally occurring form is identical to the member of the library but wherein the loop region has not been modified by the insertion of an heterologous peptide or polypeptide. Only if the target polypeptide binds to the member of the polypeptide display library but not to the non-variant or naturally occurring form thereof, then the member of the polypeptide library will be selected as capable of specifically binding to

10 the target polypeptide. Thus, by way of illustration, if the polypeptide library is formed by variegated forms of the human nidogen G2 domain as defined in SEQ ID NO:62 or 63 in which one or more of the loop regions AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK are modified by the insertion of an heterologous peptide, then the negative selection step used to exclude peptides which do not bind specifically to the heterologous peptide will

15 be carried out using the polypeptides of SEQ ID NO:62 or 63 respectively. The polypeptide library may be formed by variegated forms of the human nidogen G2 domain containing one or more mutations at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 151, 604, 638, 641, 469 and 518 wherein the numbering corresponds to that of the full-length human nidogen-1 defined under the UniProt database with

20 accession number P14543-1 (version dated July 7, 2009). In some embodiments, the polypeptide library is formed by variegated forms of the nidogen G2 domain containing one or more of mutations H459A, R468N, F639S, R650A, H543K, H545N, V449T, V525Q, V619T, F561E, C618S, S469I, R518I as defined above. Suitable nidogen G2 domain variants that can be used in the polypeptide library include, without limitation,

25 any of the nidogen G2 domain variants defined above in the context of the previous aspects of the invention including the variant carrying the NIDOMut2, NIDOMut3, the NIDOMut3-V45T, the NIDOMut3_V121Q, the NIDOMut3-F157E, the NIDOMut3-V215T, the NIDOMut4, the NIDOMut4_T215V, the NIDOMut5, NIDOMut3-V176T, the NIDOMut3-I200T, the NIDOMut3-V236Y, the NIDOMut3-L237T, the NIDOMut3-S65I,

30 the NIDOMut3-R114I, the NIDOMut3-C214S, the NIDOMut3-S65I_R114I, the NIDOMut5-S65I_R114I, the NIDOMut3-S65I_R114I and the NIDOMut5-S65I_R114I as defined, respectively, as SEQ ID NO: 64, 65 and 87 to 104 and in which one or more of

the loop regions AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK are modified by the insertion of an heterologous peptide, then the negative selection step used to exclude peptides which do not bind specifically to the heterologous peptide will be carried out using the polypeptides defined above but lacking the heterologous peptide.

5

For that purpose, the target peptide is first washed to remove the members of the library that have not bound to the target peptide. Washing conditions are well-known by an expert in the field and include the use of a suitable medium, such as PBS. The medium may contain a detergent such as Tween20. It may be washed for any suitable time, e.g. 1 to 30
10 minutes or 3 to 10 minutes for each wash. Washing may include gentle shaking or rocking of the carrier of said target peptide. The washing temperature is such that the binding between the target peptide and the polypeptide of the library is not disrupted. For example, it can be between 15 and 45°C or between 30 and 40°C. Typically, it is about 37°C or room temperature.

15

Once the target peptide has been washed, the members of the library bound to the target peptide are recovered. Said recovery may consist on eluting the polypeptide of the library bound to the target peptides. Different techniques can be used for said elution. For instance, elution can be carried by incubating the complex “target peptide-member of the
20 library” under low pH conditions that disrupt the binding between both, such as at a pH between 3 and 6, preferably at a pH 4. Elution may also be carried by incubating the complex with DTT. Alternatively, the whole complex of the polypeptide and the target peptide can be recovered. For instance, when the target peptide is immobilized in the target support, said immobilization can be mediated by means of a linker that comprises
25 a protease cleavage site, which is cleaved to recover to the whole complex. Said methods are well-known by an expert in the field.

In a particular embodiment, steps i) and ii) are repeated at least once, at least twice, at least three times, at least four times, at least 5 times, at least 6 times, at least 7 times at
30 least 10 times, preferably at least 2 times. In each of said repetitions, the members of the polypeptide library used in step i) correspond to the members recovered in step ii).

Thus, in another particular embodiment, steps i) and ii) of the method of the fourteenth aspect are repeated at least once, at least twice, at least three times, at least four times, at least 5 times, at least 6 times, at least 7 times at least 10 times, preferably at least 2 times, wherein the polypeptide library used in step i) in each repetition is formed by the members
5 of the library recovered in step (ii).

In a third step, the polypeptide of the library is identified. Identification of the polypeptide involves determining the sequence of the polypeptide of the library.

10 Said determination can be mediated by several methods. For instance, it can simply consist on determining the sequence of said polypeptide, by any technique well-known by a skilled person, such as mass spectrometry.

In case where the member of the library is a polypeptide of the library as a phenotype is
15 directly or indirectly linked to a nucleic acid as a genotype corresponding to said phenotype, as defined in the second aspect of the invention, the determination of the sequence of the polypeptide of the library can also consist on sequencing said nucleic acid. As indicated in the definition of the “genotype” referred in the second aspect of the invention, said nucleic acid encodes, or comprises a sequence encoding, the polypeptide
20 of the library directly or indirectly linked to it. Methods to determine the sequence of a nucleic acid directly or indirectly linked to a polypeptide are well known by an expert in the field. For instance, they might simply involve amplifying the sequence of said nucleic acid by PCR or in case the nucleic acid is an RNA molecule, by reverse transcriptase followed by PCR (RT-PCR). The amplified cDNA is then sequenced by well-known
25 techniques for a skilled person. Non-limiting examples of said sequencing techniques include the well-known Sanger method, pyrosequencing, sequencing by synthesis or sequencing by ligation. Alternatively it might comprise a first step where the nucleic acids are isolated from the polypeptide of the library to which they are directly or indirectly linked, and then, the nucleic acids are amplified by PCR or RT-PCR and sequenced, as
30 previously described. Techniques to separate a nucleic acid from a polypeptide are well-known by a skilled person. Non/limiting examples of said techniques include any technique allowing the disruption of the interaction of a polypeptide with other molecules,

or simply the degradation of a polypeptide. These techniques include an incubation with dithiothreitol (DTT), with a detergent such as sodium dodecyl sulfate (SDS), with proteinases such as proteinase K to degrade the proteins, or simply an incubation at high temperatures to denature the proteins so that the binding of any polypeptide to the nucleic acids is disrupted, preferably between 40-95°C, more preferably at 60°C. The nucleic acids are then isolated from the proteins by well-known techniques, such as phenol/chloroform, silica methods, or guanidinium thiocyanate-phenol-chloroform extraction when the nucleic acid is RNA. In case where the nucleic acid is indirectly linked to the polypeptide and is comprised within a cell from a microorganism, such as for example a bacteria or a yeast cell, the separation of the nucleic acid might comprise techniques allowing to degrade the cell membrane. Said techniques are also well-known by an expert in the field and may for instance consist on any of the techniques indicated above to disrupt the interaction of a polypeptide with another molecule. Some of the methods referred above to separate the nucleic acids from the polypeptides are described in more detail in Kelly M. Elkins, 2013, Forensic DNA Biology.

Thus, in a particular embodiment, identifying the sequence of the polypeptide that interacts with the target peptide comprises determining the sequence of said peptide. In another particular embodiment, it comprises determining the sequence of the nucleic acid directly or indirectly linked to it and encoding it.

In a particular embodiment, several polypeptides of the library are capable to specifically bind to the target peptide. In this case, the method is for identifying polypeptides that bind to a target peptide, and step iii) of the method consists on identifying the sequence of the polypeptides that interact with the target peptide. The same methods provided above for the identification of the sequence of one polypeptide are also applicable for the identification of the sequence of several polypeptides. Thus, in a particular embodiment, the identification of the sequence of the polypeptides that interact with the target peptide comprises determining the sequence to the nucleic acid directly or indirectly linked to each of them.

In another particular embodiment, after step ii), the method comprises an additional step

wherein the recovered members of the library are amplified. Said amplification might consist on transforming host organisms with the members of the polypeptide library recovered, or simply amplifying the members of the library recovered. For instance, in case the member of the library is a microorganism as escribed in the second aspect of the invention and capable to replicate upon infection of an organism, such as a phage, a bacteriophage, or a virus, the members of the library recovered in step ii) can be amplified by cultivating bacteria that have been infected by said members of the library. In case said members are microorganisms as described in the second aspect of the invention that are capable to replicate, such as bacteria or yeast, the amplification of the members of the library can simply consist on cultivating said microorganisms.

Therefore, in a particular embodiment, the method for identifying a polypeptide, or polypeptides, that bind/s to a target peptide comprises:

- i) contacting a target peptide with the polypeptide display library according to the second aspect of the invention under conditions that allow a polypeptide to interact with the target peptide,
- ii) recovering those members of the library that have interacted with the target peptide,
- iii) amplifying the members of the library that have been recovered in step ii)
- iv) identifying the sequence of the polypeptide/s that interact/s with the target peptide.

In a particular embodiment, where steps i) - iii) are repeated at least once, at least twice, at least three times, at least four times, at least 5 times, at least 6 times, at least 7 times at least 10 times, preferably at least 2 times, wherein the polypeptide library used in step i) in each repetition is formed by the members of the library recovered in step (ii) and amplified in step iii).

In a certain embodiment, all the terms and embodiments described in the previous aspects of the invention, are equally applicable to the fourteenth aspect of the invention.

XI - Use of the polypeptide of the first aspect of the invention.

As indicated in the first aspect of the invention, the polypeptides of this aspect are suitable for incorporating heterologous peptide or peptides within one or more of the loop regions, either as insertion in the loop regions or replacing partially or completely the loop regions.

- 5 These variant polypeptides can then be used to present the heterologous peptide or peptides, which can be defined as “peptides of interest”. Thus, in a fifteenth aspect, the invention relates to the use of a polypeptide according to the first aspect of the invention, for presenting a peptide, wherein said peptide is found in one of the loop regions.
- 10 The expression “for presenting”, as used herein, refers to the capacity of the polypeptide to bring the heterologous peptide into proximity of an element of interest. The term “peptide of interest”, as used herein, is not limited by any function, sequence or structural characteristic, besides the fact that it can be found in one of the loop regions of the polypeptide of the first aspect of the invention. In one embodiment, the heterologous
- 15 polypeptide is inserted within one or more of the loop regions, i.e. the loop region conserves all the amino acids found in the cognate loop domain in SEQ ID NO:62 or SEQ ID NO:63 but the heterologous polypeptide is inserted between two consecutive amino acids. In another embodiment, the heterologous polypeptide within one or more of the loop regions is found as an insertion within the loop region which replaces the partially
- 20 or completely the sequence of the loop domain. In another embodiment, wherein more than one peptide is presented in different loops of the polypeptide of the invention, one or more of the peptides may be located as insertions in the loop regions and one or more of the peptides can be found replacing part or the complete loop regions.
- 25 The length of the heterologous polypeptide or peptides to be presented is not particularly limitative. Thus, the heterologous polypeptide may comprise at least two, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95,
- 30 at least 100 or more amino acids.

In a particular embodiment, the peptide of interest is any of the agents of interest defined

in the sixth aspect of the invention, preferably, it is a therapeutic agent as defined in section IV-E.1, in particular, a cytotoxic polypeptide, an antiangiogenic polypeptide, a polypeptide encoded by a tumor suppressor gene, a pro-apoptotic polypeptide, polypeptide having anti-metastatic activity, a polypeptide encoded by a polynucleotide
5 which is capable of activating the immune response towards a tumor, an antiangiogenic molecule or a toxin.

The terms “binds” and “specifically binds” have been defined in the first aspect of the invention. Methods to determine the binding between a polypeptide and a target molecule
10 as well as the KD of a binding complex have been provided in said definition in the first aspect of the invention. In a particular embodiment, the specific binding between the peptide of interest and the at last one loop region of the polypeptide has a Kd of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M.

15 In a particular embodiment, the element of interest to which the polypeptide of the first aspect presents, or brings, the peptide of interest, is a cell. In a preferred embodiment, it is a cell in a sample. In another particular embodiment, it is a cell isolated from a tissue or organism from which it derives. In another particular embodiment, said element of
20 interest is another peptide, protein, or nucleic acid in a sample.

The term “sample” has been defined in the thirteenth aspect of the invention. The sample referred in the present aspect contains the element of interest, wherein said element is preferably as specified above.

25 In a particular embodiment, the invention relates to a non-therapeutic use of a polypeptide according to the first aspect of the invention, for presenting a peptide, wherein said peptide is found in one of the loop regions.

30 In a certain embodiment, all the terms and embodiments described in the previous aspects of the invention, are equally applicable to the fifteenth aspect of the invention.

XII - Method for determining the presence of a target peptide in a sample.

As indicated in the previous aspect, the polypeptide of the first aspect of the invention is capable of specifically binding to a target molecule or target peptide. Therefore, it might
5 be useful for determining the presence of said peptide in a sample.

Thus, in a sixteenth aspect, the invention relates to a method for determining the presence of a target peptide in a sample comprising:

- 10 (i) contacting the proteins present in the sample with a polypeptide according to the first aspect of the invention, wherein the sequence of at least one of the loop regions in the polypeptide is a sequence that is capable of specifically binding to the target peptide,
- (ii) determining if there is an interaction between the target peptide and the polypeptide,
- 15 wherein if there is an interaction between the polypeptide and the target peptide, then the target peptide is present in the sample.

The term “sample” has been defined in the thirteenth aspect of the invention. The sample of the fourteenth aspect of the invention contains any biological material suitable for
20 detecting peptides present in the sample.

The expression “target peptide”, as used herein, is not limited by any functional, sequence or structural characteristic, besides the fact that it specifically binds to the sequence of at least one of the loop region of a polypeptide of the first aspect of the invention.
25

In a particular embodiment, the target peptide is that of the thirteenth aspect of the invention, and the polypeptide is that identified in said aspect as specifically binding to said target peptide.

30 The specifically binding between said sequence of at least one loop region and the target peptide has a KD of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M

or less than 10^{-15} M. The term “specifically binding” has been defined in the first aspect of the invention. Methods to determine the binding between a polypeptide and a target peptide have also been provided in the first aspect of the invention.

- 5 Contacting the proteins present in a sample with a polypeptide of the first aspect capable to specifically bind to the target peptide, is generally a matter of simply mixing the polypeptide with the sample under conditions that allow a polypeptide to interact with a target peptide. Thus, in a particular embodiment, step i) of the method is carried under said conditions. The expression “under conditions that allow a polypeptide to interact
10 with a target peptide” has been defined in the fourteenth aspect of the invention. Said definition applies to the present aspect of the invention by substituting the term “member of the library”, or “polypeptide of the library”, by “polypeptide of the first aspect”.

- 15 Determining if there is an interaction, i.e. the formation of a polypeptide-target peptide complex, can be done in a number of ways.

- On one side, this may be done by first attaching the target peptide to a solid support by means of a specific antibody attached to said support and specific for said peptide, or of an antibody specific for a tag present in said peptide, such as a histidine tag or biotin.
20 Then, the polypeptide of the first aspect is added, and excess reagent is washed off. The presence of the polypeptide of the first aspect in the solid support is then determined. Various blocking and washing steps may be utilized as it is known in the art. The washing conditions can be any of those specified in the fourteenth aspect of the invention. As understood by a skilled person, after the washing steps, the polypeptide of the first aspect
25 detected is that bound to the target peptide. Thus, the detection of the polypeptide of the first aspect is an indirect indication of the binding of the polypeptide to the target peptide, and thus of the presence of said peptide in the sample.

- Techniques to detect the presence of a polypeptide or peptide in a sample are well-known
30 by an expert in the field. For instance, the polypeptide can be labelled and said label detected. Said label can be any radioactive, fluorescent, biological or enzymatic tags. Methods to detect said labels are well-known by an expert in the field. Patents concerning

the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Alternatively, one may find additional advantages through the use of a secondary binding ligand such as a first and second antibody and/or a biotin/avidin ligand binding arrangement, as it is known in the art. Non-limiting
5 examples of techniques based on the use of a secondary ligand to detect a polypeptide in a sample include Western blot or immunoblot, ELISA (Enzyme-Linked Immunosorbent Assay) or RIA (Radioimmunoassay).

On the other side, determining if there is an interaction can be done by first attaching the
10 polypeptide of the first aspect to a solid support, by means of an antibody attached to said solid support and specific for said polypeptide. Then, the sample suspected to comprise the target peptide is added, and excess reagent is washed off. Finally, the presence of the peptide of interest in the solid support is determined.

15 The rest of steps can be the same as those indicated above in case the target peptide is immobilized in a solid support, except that the part of the polypeptide-target peptide complex that is detected in the present case is the target peptide, by means of a label (as those indicated above) attached to the target peptide or of a secondary ligand (as those indicated above) specific for the target peptide. Thus, in this case, the detection of the
20 target peptide is an indirect indication of the binding of the polypeptide to the target peptide, and thus of the presence of said peptide in the sample.

As well-known by an expert in the field, any of the techniques specified above allowing determining the presence of a polypeptide or of a peptide in a sample or attached to a
25 solid support, also allow determining the amount of said polypeptide or peptide in said sample or attached to said solid support. Therefore, the method for determining the presence of a target peptide in a sample allows not only determining the presence of the target peptide, but also the amount of the polypeptide-target peptide complexes formed and thus the amount of target peptide in the sample.

30

Thus, in a particular embodiment, the invention also relates to a method for determining the amount of a target peptide in a sample comprising:

- 5 i) contacting the proteins present in the sample with a polypeptide according to the first aspect of the invention, wherein the sequence of at least one of the loop regions in the polypeptide is a sequence that is capable of specifically binding to the target peptide, under condition allowing a polypeptide-target peptide complex to form,
- ii) determining the amount of the polypeptide-target peptide complexes formed, wherein the amount of polypeptide-target peptide complexes formed indicates the amount of target peptide in the sample.

10 The expression “under condition allowing a polypeptide-target peptide complex to form”, as used herein, refers to the same conditions that allow a polypeptide to interact with a target peptide indicated above.

15 In a particular embodiment of the fourteenth aspect, the polypeptide of the first aspect is immobilized in a solid support. In a particular embodiment, said solid support is any of those specified in the fourteenth aspect of the invention. In a particular embodiment, the polypeptide is attached to the solid support through a linker moiety.

20 In another particular embodiment of the fourteenth aspect, the target peptide is immobilized in a solid support. In a particular embodiment, said solid support is any of those specified in the fourteenth aspect of the invention. In a particular embodiment, the polypeptide is attached to the solid support through a linker moiety.

25 In a certain embodiment, all the terms and embodiments described in the previous aspects of the invention, are equally applicable to the sixteenth aspect of the invention.

30 In another embodiment, the terms “consists” and “comprises”, “consisting” and “comprising”, used in any of the aspects and embodiment of the invention are interchangeable.

Sequence overview

SEQ ID NO	Sequence
1	Loop región AB (GSSQVP)
2	Loop región BC (MNHG)
3	Loop región CD (PETVGYSLLPLAPVGGIIGWMFAVEQDGFKNQFSITGG)
4	Loop región DE (VGHPGN)
5	Loop región EF (GIDEHGH)
6	Loop región FG (PQIPFGS)
7	Loop región HI (EPERDGASP)
8	Loop región IJ (FQECVHDDSRPALPS)
9	Loop región JK (NQEEK)
10	beta strand A (QRVNGKVKGRIFV)
11	beta strand B (IVFENTDLHSYVV)
12	beta strand C (RSYTAIS)
13	beta strand D (EFTRQAEVTF)
14	beta strand E (LVIKQRFS)
15	beta strand F (LTIDTELEGRV)
16	beta strand G (SVHIEPYTELYHYS)
17	beta strand H (VITSSSTREYTVT)
18	beta strand I (SRIYTYQWRQTIT)
19	beta strand J (TQQLSVDSVFVLY)
20	beta strand K (ILRYALSNSIG)
21	α 1 (TVGYSL)
22	α 2 (PVGGIIGWM)
23	α 3 (GFSIT)
24	CaD (TIPE)
25	T22 (RRWCYRKCYKGYCYRKCR)
26	V1 peptide (LGASWHRPDKCCLGYQKRPLP)
27	CXCL12 (KPVSLSYRCPCRFFESHVARANVKHLKI LNTPNCALQIVARLKNNNRQVCIDPKLKWIQE YLEKALN)
28	vCCL2 LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKDWVKKL MQQLPVTA)
29	Optimized EPI-X4 IVRWSKKVPCVSIVRWSKKVPCVS
30	Arg-rich sequence (RRRRRRRRR)
31	Arg-rich sequence (RRRGRGRRR)
32	Arg-rich sequence (RARGRGRRR)
33	Arg-rich sequence (RARGRGGGA)
34	A5G27 (RLVSYNGIIFFLK)
35	FNI/II/V (WQPPRARITGYIIKYEKPGSPPREVVPRPRPGVTEATITGLEPGTEYTIYVIALKNNQKS EPLIGRKKT)
36	Seq-1-7 (KYLAYPDSVHIWRKRKRK)
37	Seq-1-8 (KYLAYPDSVHIWRKRKRK)
38	Angiopep-2-7 (TFFYGGSRGKRNNFKTEEYRKR)
39	Linker region (GGSSRSS)
40	Linker region (GGGNS)

SEQ ID NO	Sequence
41	T140 (RRX ₁ CYRKX ₂ PYRX ₃ CR)
42	TN14003 (RRX ₁ CYX ₂ KX ₃ PYRX ₄ CR)
43	TC14012 (RRX ₁ CYEKX ₂ PYRX ₃ CR)
44	TE14011 (RRX ₁ CYX ₂ KX ₃ PYRX ₄ CR)
45	TZ14011 (RRX ₁ CYX ₂ KX ₃ PYRX ₄ CR)
46	GW-H1 peptide (GYNYAKKLANLAKKFANALW)
47	nucleolin-binding peptide 1 (KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK)
48	nucleolin-binding peptide 2 (KDEPQRRSARLSAKPAPPKPEPKPKKAPAKKRKRKRKRK)
49	Linker región (SGGTSGSTSGTGST)
50	Linker región (AGSSTGSSTGPGSTT)
51	Linker región (GGSGGAP)
52	Linker región (GGGVEGGG)
53	Linker región (PKPSTPPGSS)
54	Linker región (APAETKAEPMT)
55	Cleavage site (AAALE)
56	Cleavage site (DDDDK)
57	Cleavage site (IEDGR)
58	Cleavage site (LVPRGS)
59	Cleavage site (ENLYFQG)
60	Cleavage site (LEVLFQGP)
61	T22-NIDOm2-H6 RRWCYRKCYKGYCYRKRGGSSSRSSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLPLAPVGGIIGWMFAVEQDGFKNFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEHGHILTIDTELEGRVPQI PFGSSVHIE PYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKI LAYALSNSIGPVREGSPDAKHHHHHH
62	Native human nidogen-1 G2 domain SPQRVNGKVKGRIFVGSSQVPIVFENTDLHSYVVMNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN G FSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEHGHILTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY VTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPDA
63	Native human nidogen-1 G2 domain lacking SP dipeptide. QRVNGKVKGRIFVGSSQVPIVFENTDLHSYVVMNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN G FSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEHGHILTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY VTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPDA
64	Human nidogen-1 G2 domain carrying H459A., R468N, F639S and R650A mutations (NIDOm2). SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN G FSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEHGHILTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY VTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPDA
65	Human nidogen-1 G2 domain carrying H459A., R468N, F639S and R650A mutations and lacking SP dipeptide. QRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN G FSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEHGHILTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY VTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPDA
66	T22-G2-H6 RRWCYRKCYKGYCYRKRGGSSRSS

SEQ ID NO	Sequence
	SPQRVNGKVKGRIFVGSSQVPIVFENTDLHSYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NG FSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEHGHLLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY VTEPERDGA SPRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILRYALSNSIGPVREGSPDA KHHHHHH
67	BAK
68	BAK BH3 domain
69	PUMA
70	Diphtheria toxin I
71	exotoxin of <i>P. aeruginosa</i>
72	P14543-1 of the Uniprot Database (version dated July 7, 2009)
73	Hexahistidine (HHHHHH)
74	IL-6 ligand (LSLITRL)
75	IL-6 ligand (WQDPHSWNSSFYRLRFELRYRAERSKTFTTW)
76	Linker region (GGSSRSSS)
77	Positively charged peptide region (RKRKRK)
78	Positively charged peptide region (RRRRRR)
79	Positively charged peptide region (KKKKKK)
80	Positively charged peptide region (HHHHHH)
81	Positively charged peptide region (RHRHRH)
82	Positively charged peptide region (RKRKRKRK)
83	Positively charged peptide region (RKRHRK)
84	Positively charged peptide region (RKRHRH)
85	Positively charged peptide region (RHRHRH)
86	Positively charged peptide region (RKRKRKR)
87	NIDOm3 SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SPRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPD A
88	NIDOm3_V45T SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SPRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPD A
89	NIDOm3_V121Q SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SPRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPD A
90	NIDOm3_F157E SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SPRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPD A
91	NIDOm3_V215T SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SPRIYTYQWRQTITFQECTHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPD A
92	NIDOm4 SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHNHGRSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR

SEQ ID NO	Sequence
	EYTVTEPERDGA SP SRIYTYQWRQTITFQECTHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
93	NIDOm4_T215V SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
94	NIDOm5 SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
95	NIDOm3_V176T SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
96	NIDOm3_I200T SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRTYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSP DA
97	NIDOm3_V236Y SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
98	NIDOm3_L237T SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
99	NIDOm3_S65I SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNIYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN GFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY TVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPDA
100	NIDOm3_R114I SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTIQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTRE YTVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPDA
101	NIDOm3_C214S SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFK NGFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGA SP SRIYTYQWRQTITFQESVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPD A
102	NIDOm3_S65I_R114I SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNIYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN GFSITGGEFTIQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY TVTEPERDGA SP SRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPDA
103	NIDOm5_S65I_R114I SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNIYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN GFSITGGEFTIQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREY TVTEPERDGA SP SRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPDA
104	NIDOm5_S65I SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNIYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN GFSITGGEFTRQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTRE YTVTEPERDGA SP SRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALNSIGPVREGSPDA
105	NIDOm5_R114I

SEQ ID NO	Sequence
	SPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN NGFSITGGEFTIQAEVTFQGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQIPEGSSVHIEPYTELYHYSTSVITSSSTRE YTVTEPERDGASPSRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPDA
106	T22-NIDOm3-H6 RRWCYRKCYKGYCYRKCRGGSSRSS SPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKN NGFSITGGEFTIQAEVTFVGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTR EYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPVREGSPD AK HHHHHH
107	T22-NIDOm3_V45T-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFVGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQIP FGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSV LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
108	T22-NIDOm3_V121Q-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNH GNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFQGHGPN LVIKQRFSGIDEKGNLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERD GASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPV EGSPDAKHHHHHH
109	T22-NIDOm3_F157E-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNH GNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFVGHGPN LVIKQRFSGIDEKGNLTIDTELEGRVPQIPEGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERD GASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPV EGSPDAKHHHHHH
110	T22-NIDOm3_V215T-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFVGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECTHDDSRPALPSTQQLSVDSVSV LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
111	T22-NIDOm4-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFQGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQIP EGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECTHDDSRPALPSTQQLSVDSVSV LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
112	T22-NIDOm4_T215V-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNH GNSYTAISTIPETVGYSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFQGHGPN LVIKQRFSGIDEKGNLTIDTELEGRVPQIPEGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERD GASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSVLYNQEEKILAYALSNSIGPV EGSPDAKHHHHHH
113	T22-NIDOm5-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFQGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQIP EGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVSV LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
114	T22-NIDOm3_V176T-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFVGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSV LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
115	T22-NIDOm3_I200T-H6 RRWCYRKCYKGYCYRKCRGGSSRSSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLLPLAPVGGIIGWMFAVEQDGFKNNGFSITGGEFTIQAEVTFVGHGPNLVIKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVSV LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH

SEQ ID NO	Sequence
116	T22-NIDOm3_V236Y-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVS LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
117	T22-NIDOm3_L237T-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVS VTYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
118	T22-NIDOm3_S65I-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG SLLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIP FGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVS LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
119	T22-NIDOm3_R114I-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTIQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIP FGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVS LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
120	T22-NIDOm3_C214S-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG YSLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQESVHDDSRPALPSTQQLSVDSVS VLYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
121	T22-NIDOm3_S65I_R114I-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQVPIVFENTDLASYVVMNHGNSYTAISTIPETVG SLLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTIQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIP FGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVS LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
122	T22-NIDOm5_S65I_R114I-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVG SLLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTIQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIP FGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVS VLYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
123	T22-NIDOm5_S65I-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVG SLLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTIQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIP EGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVS LYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
124	T22-NIDOm5_R114I-H6 RRWCYRKCYKGYCYRKRGGSSRSPQRVNGKVKGRIFVGSSQTPIVFENTDLASYVVMNHGNSYTAISTIPETVG SLLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTIQAEVTFQGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQIP FGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIYTYQWRQTITFQESTHDDSRPALPSTQQLSVDSVS VLYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
125	Human Nidogen G2 domain shown in Fig. 13A HQQHPQVIDVDEVEETGVVFSYNTDSRQTCANNRHQCSVHAECRDYATGFCCSCVAGYTG NGRQCVAEGSPQRVNGKVKGRIFVGSSQVPIVFENTDLHSYVVMNHGRSYTAISTIPETV GYSLLPLAPVGGIIGWMFAVEQDGFKNFSGITGGEFTRQAEVTFVGHGPNLVKQRFSGI DEHGLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGAS PSRIYTYQWRQTITFQECVHDDSRPALPSTQQLSVDSVFLYNQEEKILRYALSNSIGPV REGSPDALQNPCYIGTHGCDTNAACRPGPRTQFTCECSIGFRGDGRTCYDIDECSEQPSV (human sequence in Fig. 13A)
126	Mouse Nidogen G2 domain shown in Fig. 13A PQHHHPQVIDVDEVEETGVVFSYNTGSQQTCAANNRHQCSVHAECRDYATGFCCRCVANYTGNG RQCVAEGSPQRVNGKVKGRIFVGSSQVPIVFENTDLHSYVVMNHGRSYTAISTIPETVG SLLPLAPIGGIIGWMFAVEQDGFKNFSGITGGEFTRQAEVTFVGHGPNLVKQRFSGIDE HGLTISTELEGRVPQIPYGASVHIEPYTELYHYSSSVITSSSTREYTVMEPDQDGAAPS

SEQ ID NO	Sequence
	HTHIYQWRQTITFQECADDDARPALPSTQQLSVDSVFLYNKEERILRYALSNSIGPVRD GSPDALQNPCYIGTHGCDNAACRPGPGTQFTCECSIGFRGDGQTCYDIDECSEQPSR (mouse sequence in Fig. 13A)
127	Human Nidogen (UniProt P14534) MLASSSRIRAAWTRALLPLLAGPVGCLSRQELFPFGPGQGDLLEDGDDFVSPAELLS GALRFYDRSDIDAVYVTTNGIATSEPPAKESHPLFPPTFGAVAPFLADLDTTDLGKLV YYREDLSPSITQRAAEVHRGFPEISFQPSAVVVTWESVAPYQGPSRDPDQKGRNTFQ AVLASSDSSSYAIFLYPEDGLQFHHTTFSKKNQVPAVVAFSQGSVGLWKSNGAYNIFA NDRESVENLAKSSNSGQQGVWVFEIGSPATTNGVVPADVILGTEDGAEDDEDEDYDLAT TRLGLEVDGTTTFFSYKALRRGGADTYSVPSVLSPPRAATERPLGPPTERTSRFQLAVETF HQQHPQVIDVDEVEETGVVFSYNTDSRQTCANNRHQCSVHAECRDYATGFCSCSVAGYTG NGRQCVAEGSPQVRNGKVKGRIFVGSSQVPIVFENTDLHSYVMNHGRSYTAISTIPETV GYSLLPLAPVGGIIGWMMFAVEQDGFKNFSITGGEFTRQAEVTFVGHGPNLVKQRFSGI DEHGLTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGAS PSRIITYQWRQTITFQECVHDDSRPALPSTQQLSVDSVFLYNQEEKILRYALSNSIGPV REGSPDALQNPCYIGTHGCDTNAACRPGPRTQFTCECSIGFRGDGRTCYDIDECSEQPSV CGSHTICNNHPGTFRCCEVEGYQFSDEGTCAVVDQRPINYCETGLHNCDIPQRAQCIYT GGSSYTCSCLPFGSGDQACQDQVDECQPSRCHPDAFCYNTPGSFTCQCKPGYQGDGFRVC PGEVEKTRCQHEREHILGAAGATDPQRPIPPGLFVPECDAGHYAPTQCHGSTGYCWVCD RDGREVEGTRTRPGMTPPCLSTVAPPIHQGPAVPTAVIPLPPGTHLLFAQTGKIERLPLE GNTMRKTEAKAFLHVPKVIIGLAFDCVDKMYVWTDITEPSIGRASLHGGEPTTIIRQDL GSPEGIAVDHLGRNIFWTDNSLDRIEAKLDGTQRRVLFETDLVNPRGIVTDSVRGNLYW TDWNRDNPKEIETSYMDGTNRRIILVQDDLGLPNGLTFDAFSSQLCWVDAGTNRAECLNPSQ PSRRKALEGLQYPPFAVTSYGKNLYFTDWKMNSVVALDLAISKETDAFQPHKQTRLYGITT ALSQCPQGHNYCSVNNGGCTHLCLATPGSRTCRCPDNTLGVDCIEQK (sequence in Fig. 13B)
128	T22-NIDOm2-H6 (Sequence in figure 13B) RRWCYRCKYKGYCYRCKRGSSSRSSSPQVRNGKVKGRIFVGSSQVPIVFENTDLASYVMNHGNSYTAISTIPETVG YSLPLAPVGGIIGWMMFAVEQDGFKNFSITGGEFTRQAEVTFVGHGPNLVKQRFSGIDEKGNLTIDTELEGRVPQI PFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGASPSRIITYQWRQTITFQECVHDDSRPALPSTQQLSVDSVS VLYNQEEKILAYALSNSIGPVREGSPDAKHHHHHH
129	EPIX-4-(RK)-GFP-H6 IVRWSKKVPCVSIVRWSKKVPCVSRKRKRKGGSSRSSSKGEELFTGVVPILVELDGDVNGHKF SVSGEGEGDATYGKLTILKFICTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKRHDFFKSAMPE GYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIT ADKQKNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRD HMLLEFVTAAGITHGMDELYHHHHHHH (Sequence in Fig. 20)
130	GFP SKGEELFTGVVPILVELDGDVNGHKFVS SGEGEGDATYGKLTILKFICTTGKLPVPWPTLVTTLTYGVCFSRYPDHMK RHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQ KNGIKANFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLEFVTAAGITHGM DELY
131	Optimized EPI-X4 coupled to RKRKRK positively charged peptide region IVRWSKKVPCVSIVRWSKKVPCVSRKRKRK
132	EPI-X4 ILVRYTKKVPQVSTPTL
133	EPI-X4 coupled to RKRKRK positively charged peptide region ILVRYTKKVPQVSTPTL RKRKRK

Table 6: Sequence overview

The invention will be described by way of the following examples which are to be considered as merely illustrative and not limitative of the scope of the invention.

EXAMPLES

Production and Purification of protein Nanoparticles

Plasmid vectors encoding T22-GFP-H6, T22-STM-H6 and T22-NIDOMut2-H6, with SEQ ID NO.61, modular proteins were transformed in *Escherichia coli* (*E.coli*) expression system and different proteins produced Over Night (O/N) in Luria Broth (LB) medium upon induction with isopropyl β -D-1-thiogalactopyranoside (IPTG) at early exponential growth phase. The NIDOMut2 peptide corresponds to the peptide with SEQ ID NO:61. Cells were then harvested by centrifugation (5min at 5000g), resuspended in wash buffer (20mM Tris, 500mM NaCl, 10mM Imidazole pH=8) in presence of protease inhibitors (Complete EDTA-Free, Roche) and disrupted in a French Press (2-3 rounds at 1100psi). Cell soluble fractions were then separated by centrifugation (45min at 15.000g) and soluble proteins purified by Immobilized Metal Affinity Chromatography (IMAC). For that soluble fractions were charged in immobilized Nickel containing HiTrap Chelating HP columns (GE Healthcare) and proteins eluted by a linear gradient of Elution buffer (20mM Tris, 500mM NaCl, 500mM Imidazole pH=8) in a ÄKTA pure system (GE Healthcare). Purified proteins were finally dialyzed against sodium carbonate buffer (166mM NaCO₃H pH=8) for T22-STM-H6 and T22-NIDOMut2-H6 or sodium carbonate with salt buffer (166mM NaCO₃H, 333mM NaCl, pH=8) for T22-GFP-H6.

Protein purity was determined by polyacrylamide gel electrophoresis (SDS-PAGE) and western blot immunostaining with monoclonal anti-His antibody (Santa Cruz Biotechnology). Protein integrity was verified by MALDI-TOF mass spectrometry, protein amount quantified by Bradford's assay and Nanoparticles size determined by Dynamic Light Scattering.

Production of Nanoconjugates

Protein-FdU nanoconjugates were generated by covalent binding of protein nanoparticles with oligomers containing five units of 5'-(FdU)5-hexaethyleneglycolthiol-3' (FdU) through protein lysine amines using a 6-Maleimidohexanoic acid N-hydroxysuccinimide ester (EMCS) bifunctional linker. For that thiol conjugated FdU oligomers were first reacted with the Maleimide group in the EMCS bifunctional linker in a 1:1 molar ratio at Room Temperature (RT) for 10min adding ester groups. Subsequently, active ester

groups containing FdU molecules were then reacted with external lysine-amine groups within protein nanoparticles in a 1:5 molar ratio Over Night at RT. Finally, protein-FdU nanoconjugates were dialyzed against their respective storage buffers to remove non reacted free oligo-FdU molecules. Final conjugated products were characterized by

5 MALDI-TOF mass spectrometry and Dynamic Light Scattering and conjugated FdU/protein molar ratio was calculated by UV light absorbance at 260nm (wavelength at which our FdU oligomers absorbs light with a molar extinction coefficient of $44500 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in a UV-visible light spectrophotometer. All nanoconjugates incorporated an average of 2-10 oligo FdU molecules per proteins unit and maintained their parental

10 average nanoparticle size.

T22-NIDOMut2-H6 NPs fluorescence labelling

T22-NIDOMut2-H6 molecules were covalently labelled with ATTO488 fluorescent molecules for intracellular tracking. For that, ester conjugated ATTO488 molecules (Sigma) were reacted with external lysine-amine groups in T22-NIDOMut2-H6

15 molecules at 1:2 (protein : dye molecule) for 1h at RT. Then labelled nanoparticles were dialyzed against their storage buffer (Sodium Carbonate buffer) at 4°C O/N in order to remove non-conjugated free ATTO488 molecules. Labelled nanoparticles were finally characterized by MALDI-TOF mass spectrometry and Dynamic Light Scattering.

Dynamic Light Scattering

20 Volume size distribution of all protein nanoparticles and nanoconjugates were determined by Dynamic Light Scattering (DLS) at 633nm in a Zetasizer Nano ZS (Malvern Instruments) in triplicate. All nanoparticles and nanoconjugates were within nanometric range and showed average nanoparticles sizes between 10 – 50 nm.

Nanoparticles CXCR4-dependent Internalization

25 CXCR4 receptor specific internalization was studied with labelled T22-NIDOMut2-H6-ATTO488 nanoparticles over CXCR4+ Human Cervix HeLa cells. For that, HeLa cells acquired at ATCC (CCL-2) were cultured on 24well plates in MEM alpha medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere at 37°C and 5% CO₂ until reaching a 70% confluence. Then labelled T22-NIDOMut2-

H6-ATTO488 nanoparticles were incubated with HeLa cells in serum-free OptiPRO medium (Gibco) supplemented with L-glutamine for different times and concentrations. Cells fluorescence was then analyzed in a FACS-Canto flow cytometer (Becton Dickinson) using an argon ion laser at 488nm (for ATTO488 molecule excitation) and a
5 D detector (530/30nm filter) after a “harsh” trypsin digestion designed to complete remove protein externally attached to cells.

For competition assays, cells were pre-incubated with the CXCR4 specific antagonist AMD3100 (octahydrochloride hydrate-Sigma) for 1h before the addition of labelled T22-NIDOmut2-H6-ATTO488 nanoparticles.

10 **Confocal Laser Microscopy:**

Labelled T22-NIDOmut2-H6-ATTO488 nanoparticles’ intracellular localization was also studied in HeLa cells by Confocal Laser Microscopy. For that, HeLa cells were cultured in MatTek culture dishes (MatTek Corp.) in MEM alpha medium supplemented with 10% Fetal bovine serum until reaching 70% of confluence. Then labelled T22-
15 NIDOmut2-H6-ATTO488 nanoparticles were incubated with HeLa cells in serum-free OptiPRO medium (Gibco) supplemented with L-glutamine for different times and concentrations. Cells membranes and nuclei were then stained with CellMask™ DeepRed and Hoechst (Molecular Probes) respectively for 10min before being washed with PBS. Living cells were finally recorded in a Leica TCS-SP5 confocal laser scanning
20 microscope (Leica Microsystems) using a Apo63x/1.4 (oil HC x PL APO lambda blue) objective and a blue diode (405nm), Argon laser (488nm) and HeNe laser (633nm) to visualize cell nuclei, cells membranes and labelled nanoparticles respectively.

MTT

In-Vitro cell cytotoxicity of FdU containing nanoconjugates (T22-GFP-H6-FdU, T22-
25 STM-H6-FdU, T22-NIDOmut2-H6-FdU) were evaluated over CXCR4+ HeLa cells with luminescent cells viability assays. For that, HeLa cells were cultured on 96-well plates in 90ul of MEM alpha medium supplemented with 10% Fetal bovine serum for 24h. Then 10ul of different concentrations of FdU containing protein nanoconjugates were added and incubated for additional 48h. Finally, cell viability was determined by CellTiter-Glo
30 Luminescent Cell Viability Assay (Promega) following supplier’s instructions.

Generation of the CXCR4+ colorectal cancer model.

All in vivo procedures were approved by the Hospital de Sant Pau Animal Ethics Committee and performed according to European Council directives. Five-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice weighing between 18 and 20 g (Charles River, L-Abreslle, France) and maintained in specific-pathogen-free (SPF) conditions, were used to generate the CXCR4 overexpressing (CXCR4+) subcutaneous (SC) colorectal (CRC) xenograft mouse model to study the antitumor effect of the nanoconjugates. To that purpose, we used the patient-derived M5 colorectal tumor tissue, perpetuated as a tumor line in donor animals. Thus, 10 mg of M5 SC CRC tumor tissue obtained from these animals were and implanted in the subcutis of NSG mice.

Antitumor effect of the nanoconjugates in the M5 CXCR4+ SC CRC model

Once the tumors reached a volume within the 120-200 mm³ range, mice were randomly allocated in four groups, one Control (K, n=4) and three experimental: T22-STM-FdU (n=4); T22-NIDOm2-FdU (n=4) and T22-GFP-FdU (n=4). All experimental groups received intravenous injections of the corresponding nanoconjugate, in a repeated dose schedule following a dosage regime of 20µg, every 3 days, per 5 doses. The control group received 200µl of 166 mM NaHCO₃ pH8.0, following the same schedule. The antitumor effect was evaluated as inhibition of tumor growth, measuring the evolution of tumor volume along time, by registering every three days the longer (D) and shorter (d) tumor diameters with a caliper. Tumor volume was calculated using the ellipsoid formula, Volume = 1/2(D×d²). Fourteen days after the initiation of the nanoconjugate administration, mice were euthanized, the subcutaneous tumors taken for the evaluation of apoptotic induction, whereas the liver and kidney tissues were processed for histological analysis. Mouse body weight was registered over the experimental period 2 times a week.

Evaluation of apoptotic induction in tumor and histology in normal tissues

Tumor, liver and kidney tissues were collected and fixed with 4% formaldehyde in phosphate-buffered solution for 24 h and then embedded in paraffin for histological analysis. Apoptotic induction analyses were performed in 4 µm thick sections of tumors at the end of the experiment (two days after the last nanconjugate dose) that were

processed for hematoxylin and eosin (H&E) staining. Apoptotic induction was evaluated by counting the number of cell death bodies in H&E stained tumor slices per 10 high-power fields (magnification 400×), in blinded samples evaluated by two independent researchers. Liver and kidney were also taken for histopathologically analysis.

5 Representative pictures were taken using CellAB software (Olympus Soft Imaging v 3.3, Nagano, Japan).

Determination of intrinsic fluorescence

Fluorescence spectra were recorded in a Cary Eclipse spectrofluorimeter (Agilent Technologies, Mulgrave, Australia). A quartz cell with 10 mm path length and a

10 thermostated holder was used. The excitation and emission slits were set at 5 nm. Excitation wavelength (λ_{ex}) was set at 295 nm. Emission spectra were acquired within a range from 310 to 450 nm. The protein concentration was 0.2 mg/mL in carbonate buffer (166 mM NaCO_3H , pH 8). In order to evaluate the conformation stability against heating we obtained the fluorescence spectra at each temperature at a 1°C/min scan rate and we

15 calculated the Centre of Spectral Mass (CSM) for each spectrum. CSM is a weighted average of the fluorescence spectrum peak. Also it is related with the relative exposure of the Trp to the protein environment. The maximum red-shift in the CSM of the Trp, is compatible with a large solvent accessibility (Li, T. M.; et al., *Biochemistry* 1976, 15, 5571-80; Ruan, K and Weber, G, *Biochemistry* 1989, 28 (5), 2144-53 and Mohana-

20 Borges, R. et al., *Proceedings of the National Academy of Sciences of the United States of America* 1999, 96 (14), 7888-93) and the protein unfolding.

The Centre of Spectral Mass (CSM) was calculated for each of the fluorescence emission according to Eq.1 (Lakowicz, J. R.; et al., 1991, *Biophys Chem* 39(1), 79-84), where I_i is the fluorescence intensity measure at the wavelength λ_i .

$$\lambda = \frac{\sum \lambda_i \cdot I_i}{\sum I_i} \quad (i)$$

25

Unfolding temperature determination

Midpoint unfolding temperature (T_m) was determined as the temperature value that correspond to the inflexion point of the CSM vs T° curve. We also determined the T_{onset} as the temperature where the CSM value begins to increase.

Statistical analysis

- 5 Mann–Whitney U tests were used for pairwise comparisons of the differences in tumor volume or the number of apoptotic bodies between groups. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using SPSS version 11.0 package (IBM, NY, USA), and values were expressed as mean $\pm$ standard error of the mean (SEM).

10

Obtention and characterization of new protein clones: T22-NIDOMut3-H6 and all its further derivatives T22-NIDOMut3_V45T-H6, T22-NIDOMut3_V121Q-H6, T22-NIDOMut3_F157E-H6, T22-NIDOMut3_V215T-H6, T22-NIDOMut4_T215V-H6, T22-NIDOMut4-H6, and T22-NIDOMut5-H6

- 15 Genes encoding for the new T22-NIDOMut3-H6 and all its further derivatives T22-NIDOMut3_V45T-H6, T22-NIDOMut3_V121Q-H6, T22-NIDOMut3_F157E-H6, T22-NIDOMut3_V215T-H6, T22-NIDOMut4_T215V-H6, T22-NIDOMut4-H6, and T22-NIDOMut5-H6 were provided by Genart (Thermo Fisher) and subcloned into pET26b plasmids (Novagen). Protein-encoding plasmids were transformed into Escherichia coli
- 20 BL21 DE3 (Novagen) strains and the protein was produced overnight (O/N) at 20 °C in Luria Broth (LB) medium upon induction with 0.1 mM isopropil- β -D-1-thiogalactopyranoside (IPTG). Cells were then harvested by centrifugation (15 min at 5000 g) and resuspended in wash buffer (20 mM Tris, 500 mM NaCl, 10 mM Imidazole, pH 8) in presence of protease inhibitors (cOmplete™ EDTA-Free, Roche). Cells were then
- 25 disrupted in an EmulsiFlex-C5 system (Avestin) by 3 rounds at 8000 psi. The soluble fraction of the cell lysate, containing the proteins, was separated by centrifugation (45 min at 15000 g) and then charged into a HisTrap HP column (GE Healthcare) for purification by immobilized metal affinity chromatography (IMAC) in an ÄKTA pure system (GE Healthcare). Protein elution was achieved applying a lineal gradient of
- 30 elution buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazole, pH 8). Purified protein

fractions were then dialyzed against sodium carbonate (166 mM NaCO₃H, pH 8). Protein purity was determined by SDS-PAGE gel electrophoresis and subsequent western-blot immunodetection using anti-His monoclonal antibody (Santa Cruz Biotechnology). Protein integrity was also determined by MALDI-TOF mass spectrometry. Final protein concentration was determined by Bradford's assay and Nanodrop.

For the stability study in other buffers, purified protein fractions were dialyzed against chosen FDA-approved buffer and carbonate as control. Protein concentration was initially set at 2.0 mg/ml in carbonate buffer and dialysed into 0.5 ml of each new buffer. Samples were centrifuged for 15 min 15000g to remove precipitate and the remaining soluble protein concentration was determined by Bradford's assay and Nanodrop.

Morphometric characterization of proteins T22-NIDOMut3-H6 and all its further derivatives

Volume size distribution of protein nanoparticles was determined in a Zetasizer Nano ZS (Malvern Instruments) by Dynamic Light Scattering (DLS) at 633nm in triplicates.

Oligo-FdU conjugation for proteins used in experiments addressed to the analysis of proteins T22-NIDOMut3-H6 and all its further derivative

T22-NIDOMut3-H6 and T22-NIDOMut2-H6 were covalently linked to oligo 5-Fluoro-2'-deoxyuridine (oligoFdU) molecules through the amine groups of exposed lysines in a two-step reaction using a bi-functional linker. To that end, TCEP was first added to cleave the S-S bond protecting the thiol group of the oligoFdU molecules and the product was cleaned of impurities using NAP-10 sephadex gravity columns (GE Healthcare). Then, thiol-oligoFdU molecules were reacted to a 6-Maleimidohehexanoic acid N-hydroxysuccinimide ester bifunctional linker (EMCS) by thiol-maleimide reaction in a 1:1 molar ratio for 30 min at room temperature. Resulting hydroxysuccinimide-functionalized oligoFdU molecules were finally reacted to amino groups of external lysines, in a 1:5 molar ratio 5 h at R.T. and subsequently purified using Zeba™ Spin Desalting Columns (Thermo Scientific) and dialyzed against carbonate buffer in order to remove non-covalently bonded oligoFdU molecules. Reaction efficiency was finally checked by MALDI-TOF mass spectrometry and conjugated oligoFdU molecules determined by FdU absorbance at 260 nm in a UV/visible light spectrophotometer using

molar extinction coefficient (ϵ : 43500 M⁻¹ cm⁻¹). Absorbance values were corrected by subtracting the baseline 260 nm absorbance of the non-conjugated protein at an equivalent concentration.

In vitro cell viability assay performed for the analysis of new proteins T22-NIDOMut3-H6 and all its further derivative:

HeLa cells (ATCC, CCL-2) were incubated in opaque 96-well plates in 90 μ l of MEM alpha medium (Gibco) containing 10 % of Fetal bovine serum (Gibco) in humidified atmosphere and 5 % CO₂ at 37 °C. Then, 10 μ l of T22-NIDOMut3-H6-FdU and T22-NIDOMut2-H6-FdU nanoconjugates were added along with the control T22-NIDOMut3-H6 at 25 nM final concentration and incubated for additional 48 h. Cell viability was finally tested by CellTiter-Glo® Luminescent Cell Viability Assay (Promega) in a Victor 3 luminescent plate reader (Perkin Elmer). All samples were analyzed in triplicate and data expressed as mean % of viability (related to control cells) +/- standard error.

Protein design, production, and purification of EPIX4-(RK)-GFP-H6, T22-GFP-H6 and T22-BFP-H6 proteins.

Synthetic genes encoding the modular proteins were designed in-house. The EPIX-4 sequence used was the optimized dimeric version with higher receptor affinity. In the case of EPIX4-(RK)-GFP-H6, a six cationic amino acid sequence (RKRRKK) was added after the EPIX-4 ligand to favor protein self-assembling. Also, between EPIX-4 and the protein scaffold GFP a flexible linker (GGSSRSS) was added conferring the ligand accessibility to bind CXCR4 receptor. The gene codon usage was optimized for *E. coli* to be inserted into the plasmid pET22b (Novagen) and the construct was provided by Geneart (ThermoFisher). The recombinant versions of the vector were transformed in *E. coli* BL21 (DE3) (F⁻ ompT hsdSB (rB⁻, mB⁻) gal dcm DE3) (Novagen). The encoded proteins were produced in Luria-Bertani (LB) media in 500 mL cell Erlenmeyer flasks at 20 °C overnight (O/N) upon addition of 0.1 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside). When the OD₅₅₀ of the cell culture reached around 0.5, bacterial cells were harvested and centrifuged at 7000 rpm, for 15 min at 4 °C. The cell pellet was resuspended in Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 40 mM imidazole pH=8) in presence of protease inhibitor cocktail Complete EDTA-Free (Roche). Bacterial cells

were disrupted with French Press for three rounds at 1200 PSI, centrifuged (45 min, 15000 g, 4 °C) and soluble fraction purified by affinity chromatography with a HisTrap Chelating HP column in an AKTA purifier FPLC, (GE Healthcare). After the samples were filtered (0.22 µm) and injected into the column, the fractions to be collected were
5 eluted with elution buffer approximately (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazol pH 8). Purified protein fractions were dialyzed against carbonate buffer (166 mM NaCO₃H, pH 8). In addition, the CXCR4-targeted protein T22-GFP-H6 and T22-BFP-H6 were produced and purified for the formation of biparatopic nanoparticles as previously described (U. Unzueta et al., Nanotechnology 2017, 28, 505102). BFP
10 corresponds to the protein provided in the GenBank database under accession number EF064258.1.

Protein characterization of EPIX4-(RK)-GFP-H6, T22-GFP-H6 and/or T22-BFP-H6 proteins

The integrity of the recombinant proteins was checked by mass spectrometry (MALDI-
15 TOF), TGX (Tris-Glycine eXtended) Stain-Free acrylamide gels electrophoresis (BioRad) and Western Blot analysis using anti-His monoclonal antibody (1:1000; Santa Cruz, ref. 57598). Protein concentration was determined by Bradford (Biorad) assay with an Albumin (Roche) standard curve. GFP fluorescence emission (510 nm) was determined on purified proteins with a Cary Eclipse fluorescence spectrophotometer
20 (Agilent Technologies) using an excitation wavelength of 450 nm. The volume and size distribution of nanoparticles were measured by dynamic light scattering (DLS) at 633 nm through a Zetasizer Nano ZS (Malvern Instruments) using quartz cuvettes.

Ultrastructural characterization of the nanoparticles produced with EPIX4-(RK)-GFP-H6, T22-GFP-H6 and/or T22-BFP-H6 proteins

25 Size and shape of protein nanoparticles at nearly state were evaluated with a field emission scanning electron microscope (FESEM) Merlin (Zeiss). Protein samples were directly deposited over silicon wafers, excess of liquid blotted with Whatman filter paper, air dried and observed without coating in a FESEM Zeiss Merlin operating and 1 kV and equipped with a high resolution *in-lens* secondary electron detector. Representative

images of nanoparticles were taken at a range of high magnifications (from 80.000 x to 300.000 x).

Cell culture, flow cytometry and cytotoxicity assay with EPIX4-(RK)-GFP-H6, T22-GFP-H6 and/or T22-BFP-H6 proteins

- 5 Experiments were performed in CXCR4⁺ cervical and colorectal cell lines (HeLa and SW1417, respectively). HeLa cells were cultured in Eagle's Minimum Essential Medium (Gibco) and SW1417 in Dulbecco's Modified Eagle's Medium (Gibco). Both cell lines were supplemented with 10% fetal bovine serum (Gibco) and incubated in a humidified atmosphere at 37 °C and 5 % (HeLa) or 10 % (SW1417) of CO₂.
- 10 For testing protein internalization, cells were seeded in 24-well plates (Nunc) (30000 cells · wells⁻¹) for 24 h. Briefly, the medium was removed, and cells were washed with PBS. Then protein was incubated at 1 and 2 μM, diluted in OptiPro medium supplemented with L-Glutamine and incubated at different times, at suitable cell line conditions. Then, harsh trypsin digestion (1 mg ml⁻¹ for 15 min) (Gibco) was carried out to remove protein
- 15 particles bound to the outer size of the cell membranes. Intracellular green fluorescence was analyzed by flow cytometry on an FACS-Calibur system (Becton Dickinson) using a 15 mW air-cooled argon ion laser at 488 nm excitation. Fluorescence emission was measured with a D detector (530/30 nm band pass filter), and manually corrected by the specific fluorescence of purified protein, to get data representative of the amount of
- 20 internalized protein for comparative purposes. For competition assays, a specific CXCR4 antagonist AMD3100 (octahydrochloride hydrate, Sigma-Aldrich) was added 1 h before nanoparticles addition in a 1:10 (protein: AMD3100) molar ratio. All experiments were done by triplicate.

Production and characterization of biparatopic nanoparticles

25

T22-GFP-H6, T22-BFP-H6 and EPIX4-(RK)-GFP-H6 protein nanoparticles (at 1.5 mg ml⁻¹) were disassembled by different methods. In T22-GFP-H6 and T22-BFP-H6 samples, we added NaCl (500 mM Na⁺ final concentration) and imidazole (300 mM final

concentration) and in EPIX4-(RK)-GFP-H6 we added 0.2 % SDS, all of them into carbonate buffer (166 mM NaCO₃H, pH 8) for 2 h. at RT.

T22-GFP-H6/ EPIX4-(RK)-GFP-H6 and T22-BFP-H6/ EPIX4-(RK)-GFP-H6 biparatopic nanoparticles were generated by mixing the building blocks respectively in a 1:1 molar ratio, and subsequently dialyzing them against carbonate buffer (166 mM NaCO₃H, pH 8). We performed an exhaustive dialysis (4 changes every 30 min at RT, 1 change O/N at 4 °C and finally, 4 changes every 30 min). T22-BFP-H6/ EPIX4-(RK)-GFP-H6 biparatopic nanoparticles were used for FRET and confocal microscopy experiments and T22-GFP-H6/ EPIX4-(RK)-GFP-H6 for FESEM, cell culture and *in vivo* experiments.

To determine if EPIX4-(RK)-GFP-H6 is capable to form heterogeneous nanoparticles, carrying at the same time EPIX4-(RK)-GFP-H6 and T22-BFP-H6 protein, FRET analysis was performed. Fluorescence emission of protein nanoparticles was measured in a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) upon excitation at 387 nm. The emission was collected from 400-600 nm.

Confocal assay for experiments that involve the use of EPIX4-(RK)-GFP-H6, T22-GFP-H6 and/or T22-BFP-H6 proteins

HeLa cells were grown on Mat-Tek plates (25,000 cells·wells⁻¹) in Eagle's Minimum Essential Medium (Gibco) supplemented with 10% foetal bovine serum (Gibco) at 37 °C and 5 % for 24 h. Then, 2 µM of protein nanoparticles were added in OptiPro medium supplemented with L-Glutamine and incubated for 24 h at suitable cells conditions. Upon protein exposure cell nuclei were labelled with 5 µg ml⁻¹ Hoechst 33342 (Thermo) and the plasma membrane with 2.5 µg ml⁻¹ CellMask™ Deep Red (Thermo) for 10 min at room temperature. Confocal images were collected on an inverted TCS SP5 Leica Spectral confocal microscope (Leica) using 63× (1.4 NA) oil immersion objective lenses. Excitation was reached via a 405 nm blue diode laser (nucleic acids), 488 nm line of an argon ion laser (nanoparticles) and 633 nm line of a HeNe laser (Cell membrane). The confocal pinhole was set to 1 Airy unit and z-stacks acquisition intervals were selected to satisfy Nyquist sampling criteria. Confocal images were processed using the Surpass Module in Imaris X64 v.7.2.1. software (Bitplane).

Statistical analyses for experiments that involve the use of EPIX4-(RK)-GFP-H6, T22-GFP-H6 and/or T22-BFP-H6 proteins

Pairwise comparisons of cell internalization, competition assay and number of apoptotic and mitotic foci in the affected organs were made with Tukey's tests. All statistical tests were performed using GraphPad Prism version 8.0. All quantitative values both *in vitro* and *in vivo* experiments were expressed as mean $\pm$ standard error of mean ($\bar{x} \pm \text{SEM}$). Differences among groups were considered significant at $p < 0.05$.

EXAMPLE 1: Characterization of the T22-NIDOm2-H6 protein nanoparticles.

Human Nidogen (P14543) is a 136.4kDa structural protein from the basement membranes that naturally binds collagen IV, perlecan and laminin with high affinity. This protein consists of 3 globular domains that play a key role in the control of the extracellular matrix formation during development by probably linking collagen IV and laminin networks. G2 domain contains an 11-stranded β -barrel with a central α -helix (Figure 12A, B) with the same folding as green fluorescent protein from *Aequorea victoria* (Figure 12C, D).

In a first step, in order to generate a GFP-like human protein scaffold, a superposition of the mouse Nidogen G2 crystal structure (1GL4), and GFP β -barrel (1Q4A) was performed to select the exact G2 domain sequence which structure perfectly superposes, discarding non-matching domain fragments. A mouse Nidogen sequence as it is currently the only available resolved structure and is highly homologous in sequence to human Nidogen G2 (Figure 13A). In this sense, the designed human Nidogen protein was decided to start at Ser430 as it is the first superposed residue on both structures (backbone rmsd = 1.67) and previous Gly429, being a very flexible amino acid, could produce unwanted folding patterns of preceding N-terminal ligands. Therefore, the designed protein encompasses Ser430-Ala667 amino acids of Human Nidogen (with accession number P14543 in Uniprot Database version dated July 7, 2009) and has SEQ ID NO.62.

In a second step, in order to produce a biologically neutral protein scaffold, we decided to selectively mutate different residues implicated in the Nidogen G2 domain interaction with described natural ligands such as perlecan. Amino acids His456, Arg650, R468 and

F639 were mutated. As shown in 13B, selected residues were completely buried by perlecan when interacting with G2 β -barrel according to this model, confirming their implication in the interaction hotspot (Figure 13B).

- 5 Finally, from supramolecular organization point-of-view, residues proposed to be implicated in protein-protein contacts in GFP-based nanoparticle model were found to be highly similar (same group) in its relative Nidogen β -barrel structure suggesting that they could also be a homologous interaction point upon Nidogen-derived building blocks oligomerization. In this sense, selected amino acids, candidates to be mutated, although
10 being slightly overlapping with proposed protein interaction area (specifically F639), introduced mutation (F639S) favors this protein-protein contact point. Therefore, any possible effect of Nidogen G2 mutations over its future oligomerization capacity was discarded. Considering all these analysis, the following mutations were incorporated in Ser430-Ala667 sequence of human Nidogen with accession number P14543 in Uniprot
15 Database version dated July 7, 2009 (SEQ ID NO.72): H459A, R468N, F639S and R650A and was named NIDOMut2 (Figure 13B) and is shown as SEQ ID NO:64.

- Genetic engineering allows rational design and recombinant production of modular self-assembling proteins. In this example, three different modular proteins containing 1) a N-
20 terminal cationic CXCR4-specific ligand (T22), 2) a structurally stable and biologically neutral protein scaffold namely mutated human Nidogen-1 G2 domain (NIDOMut2, with SEQ ID NO.64), Stefin A Triple Mutant protein (STM) or Green Fluorescent Protein (GFP) and 3) a C-terminal poly-Histidine have been designed and produced in a recombinant way (T22-NIDOMut2-H6 with SEQ ID NO.61, T22-STM-H6 and T22-
25 GFP-H6) in *E.coli*. Produced proteins were successfully purified by IMAC affinity chromatography were full-length and pure proteins were obtained as determined by MALDI-TOF mass spectrometry and western-blot immunodetection. All of them self-assemble in regular size nanoparticles in a range of 25 nm for T22-STM-H6, 12nm for T22-NIDOMut2-H6 and 11nm for T22-GFP-H6 as determined by Dynamic Light
30 Scattering (Fig. 2 and Fig.3). T22-NIDOMut2-H6 protein, which shows a molecular mass of 30,3kDa, was successfully labelled with an ATTO488 fluorescent dye molecule for intracellular tracking purposes as determined by MALDI-TOF mass spectrometry where

additional peaks (with molecular mass additions around 600Da) are detected, corresponding each peak to the acquisition of an additional ATTO488 molecule (Fig. 4).

EXAMPLE 2: T22-NIDOMut2-H6 with SEQ ID NO.61 CXCR4-dependent internalization.

- 5 Labelled T22-NIDOMut2-H6-ATTO488 nanoparticles can be internalized into HeLa cells in a CXCR4-dependent process as determined by intracellular fluorescence accumulation measured in a flow cytometer (Fig. 5). HeLa are tumor cells that highly overexpress CXCR4 receptor. CXCR4-mediated nanoparticles uptake is demonstrated by competition assays where the pre-incubation of HeLa cells with a CXCR4-specific
- 10 antagonist AMD3100 efficiently prevents T22-NIDOMut2-H6-ATTO488 internalization. Confocal laser microscopy image shows punctuated intracellular perinuclear accumulation of T22-NIDOMut2-H6-ATTO488 nanoparticles within CXCR4+ HeLa cells.

EXAMPLE 3: Characterization of T22-NIDOMut2-H6-FdU nanoconjugates.

- 15 Characterization of T22-NIDOMut2-H6-FdU nanoconjugates was performed by MALDI-TOF mass spectrometry where additional peaks over 30,3kDa (with mass increments around 2kDa) corresponding to additional oligo-FdU molecules incorporation are detected. In figure-6B, peaks corresponding up to 3-oligoFdU molecules incorporation can be detected. T22-NIDOMut2-H6-FU nanoconjugates maintain their
- 20 nanometric nanoparticle size range as determined by Dynamic Light Scattering. T22-NIDOMut2-H6-FdU show similar cytotoxic activity than T22-STM-H6-FdU or T22-GFP-H6-FdU nanoconjugates over CXCR4+ HeLa cells in-vitro as determined by MTT cells viability assay (Fig.7). Incubation of HeLa cells in presence of different concentrations (25nM and 100nM) of the three different nanoconjugates resulted in a very
- 25 efficient cell viability inhibition with IC50s in nanomolar range (IC50< 25nM) that in all cases were significantly more efficient than equimolar concentration of free oligo-FdU (Fig.7).

EXAMPLE 4: T22-NIDOMut2-H6-FdU nanoconjugate shows higher antitumor effect in the CXCR4+ M5 SC CRC model than T22-STM-H6-FdU or T22-GFP-H6-FdU.

The CXCR4⁺ M5 CRC model was used to assess the relative potency of the three compared nanoconjugates (T22-STM-H6-FdU, T22-NIDOMut2-H6-FdU and T22-GFP-H6-FdU), because it displays high CXCR4-overexpression. This feature is highly relevant to discriminate the potency of the three nanoconjugates that are capable of targeting
5 CXCR4⁺ cancer cells and, therefore, capable of selectively eliminating CXCR4⁺ CRC stem cells.

After repeated intravenous administration of the nanoconjugates at a 20 µg q3dx5 dosage regime, all three nanoconjugates induced a significant reduction in tumor growth, as
10 measured by tumor volume along time, in comparison to vehicle-treated animals. Reduction in tumor size was more pronounced after T22-NIDOMut2-H6-FdU nanoconjugate treatment than after T22-STM-H6-FdU or T22-GFP-H6-FdU treatment. Moreover, at the end of the experiment the reduction in tumor volume (mm³) was significantly higher ($p=0.018$) for T22-NIDOMut2-H6-FdU (456.5 ± 134.9) as compared
15 to the tumor volume in the control group (1285.2 ± 149.4) than the difference registered for T22-STM-H6-FdU (699.2 ± 144.6 , $p=0.027$) or that registered for T22-GFP-H6-FdU (661.2 ± 169.2 , $p=0.050$) (Figure 8).

EXAMPLE 5: T22-NIDOMut2-H6-FdU nanoconjugate shows higher potency as inductor
20 of apoptosis than T22-STM-H6-FdU or T22-GFP-H6-FdU in the CXCR4⁺ M5 SC CRC model.

The evaluation of apoptotic induction in 10 high-power fields of H&E stained tumor tissue sections, at mouse sacrifice two days after the last nanoconjugate dose, yielded a
25 significantly higher number of apoptotic figures for T22-NIDOMut2-H6-FdU (22.7 ± 2.9 , $p<0.001$), as compared to those registered in the control vehicle-treated group (7.4 ± 0.8), or those counted in T22-STM-H6-FdU-treated (12.0 ± 1.7 , $p=0.018$) or T22-GFP-H6-FdU-treated (12.3 ± 1.5 , $p=0.018$) tumors (Figure 9 A-B). In contrast, no significant differences in apoptotic induction were observed between T22-STM-H6-FdU or T22-
30 GFP-H6-FdU-treated tumor and control tumors

In summary, T22-NIDOMut2-H6-FdU nanoconjugate induces a significantly more potent antitumor effect than T22-STM-H6-FdU or T22-GFP-H6-FdU nanoconjugates. This finding and the fact that it also induces a significantly higher number of apoptosis figures in treated tumors than the other two nanoconjugates indicates that the nanoconjugate incorporating the mutated G2 domain of human Nidogen protein, described above, is the selected protein to be used for the development and clinical translation of this nanoconjugate. Importantly, the potent antitumor activity displayed by the T22-NIDOMut2-H6-FdU nanoconjugate is observed in the absence of toxicity in normal organs, including the lack of histological alterations in liver or kidney at the end of treatment (Figure 10), which determines its significantly higher therapeutic index, as compared to the other two nanoconjugates.

EXAMPLE 6: The isolated native G2 domain of Nidogen with SEQ ID NO. 62 as well as the NIDOMut2 variant containing H459A, R468N F639S and R650A mutations with SEQ ID NO.64 are thermostable polypeptides

The midpoint unfolding temperature (T_m) from NidoWTH6 and STMH6 samples could not be determined as the maximal unfolding state was not reached in the temperature range studied. This behavior has also been described for T22-GFP-H6 (Sánchez, JM et al., Biomacromolecules, 2018, 19:3788-3797; doi:10.1021/acs.biomac.8b00924), a highly stable protein. On the other hand a $T_m=55^\circ\text{C}$ from Nidomut2H6 was acquired but in this case two thermal transitions were observed. Once again the higher T_m could not be obtained (Fig. 11). Alternatively we suggest a T_m value (Table 7, numbers in italic format) assuming that the maximal CSM values represent the unfolding state of the protein.

Proteins	T_m	$T_{\text{onset},1}$	$T_{\text{onset},2}$	Source
STMH6	$\cong 76^\circ\text{C}^*$	70°C		
NidoWTH6	$\cong 65^\circ\text{C}^*$	60°C		
Nidomut2H6	55°C	50°C	70°C	
T22-GFP-H6		75°C		Sánchez et al, 2018 (ibid.)
Human Collagen Type I	40.3°C	38.2°C		Leikina et al, (PNAS, 2002, 31314–1318)

HSA	63°C			Picó, G (Int. J. Biol. Macromol. 1997, 20: 63-7)
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Table 7. Protein unfolding parameters (* T_m value assuming that the maximal CSM values represent the entirely unfolding state of the protein)

All the proteins analyzed could be described as thermostable ($T_m > 65^\circ\text{C}$) and mesostable ($T_m < 65^\circ\text{C}$) proteins⁴ all of them are more stable than the human collagen type I (Leikina et al, (PNAS, 2002, 31314–1318) and with comparable T_m values to human serum albumin (HAS) (Picó, G (Int. J. Biol. Macromol. 1997, 20: 63-7).

EXAMPLE 7. Expression test of proteins T22-NIDOMut3-H6 and all its further derivatives

10 An expression test was carried out as a first approach to evaluate the viability and quality of each new mutant protein T22-NIDOMut3-H6, T22-NIDOMut3_V45T-H6, T22-NIDOMut3_V121Q-H6, T22-NIDOMut3_F157E-H6, T22-NIDOMut3_V215T-H6, T22-NIDOMut4_T215V-H6, T22-NIDOMut4-H6, and T22-NIDOMut5-H6 with the exception of T22-NIDOMut5-H6, which was produced and purified directly. All cultures
15 were grown in 20 mL of LB culture media and reached ODs of 3.3–4.5 after O/N induction. Each protein was successfully detected via western blot of the cell lysates (Fig. 14). Molecular weight was within the expected marker bands and it was noted that proteins containing incorporated the mutation F157E (T22NIDOMut3_F157E-H6, T22-NIDOMut4_V215T-H6 and T22-NIDOMut4) exhibited a slightly higher band. Study of
20 T22-NIDOMut3_V121Q-H6 and T22-NIDOMut3_F157E-H6 was halted at this point because the main fraction of these proteins was produced in an insoluble form.

EXAMPLE 8. Protein production scaled-up of T22-NIDOMut3-H6 and all its further derivatives

Following this test, protein production was scaled to 500mL of LB media in 2L
25 Erlenmeyer flasks and purification was performed via metal affinity chromatography. Yield is provided in Table 8. Proteins were purified efficiently (>95% purity) and their integrity was corroborated again via SDS-PAGE/western blot and MALDI-TOF (Fig. 15). All proteins were stable after dialysis in 133mM NaCO₃H buffer.

Mutant	Culture growth (OD)	Protein yield (mg/L)
T22-NIDOMut3-H6	4.10-5.32	14-24
T22-NIDOMut3_45-H6	3.52	9
T22-NIDOMut3_121-H6	4.07	Not purified
T22-NIDOMut3_157-H6	4.13	Not purified
T22-NIDOMut3_215-H6	4.52	15
T22-NIDOMut4_215-H6	3.28	26
T22-NIDOMut4-H6	2.60-3.78	12-24
T22-NIDOMut5-H6	3.17-3.54	20-28

Table 8. Culture growth (OD) and protein yield of the protein mutants derived from T22-NIDOMut2-H6.

EXAMPLE 9. Size distribution of resulting nanoparticles

5 T22-NIDOMut3-H6, T22-NIDOMut4_T215V-H6 and T22-NIDOMut5-H6 were selected to proceed with the characterization due to their best production yields (24, 26 and 28 mg/L, respectively). At this point, volume-size distribution of each candidate was studied via DLS (Fig. 16). All three candidates were equivalent to the original T22-NIDOMut2-H6 in size before and after assembly into nanoparticles with ZnCl₂ (0.04 mM). Only T22-
10 NIDOMut5-H6 exhibited nanoparticles of higher size, but well within the desired nanoscale (10–100 nm range). Precisely because Zn is used to mediate this assembly, it was deemed appropriate to perform a precipitation assay against increasing concentrations of ZnCl₂, up to 0.16 mM, (Fig. 17A) to test whether the new proteins were resistant to Zn-induced precipitation or not. It was demonstrated that all new proteins
15 derived from T22-NIDOMut3-H6 inherited the ability to remain soluble when in contact with Zn, as opposed to the control T22-NIDOMut2-H6, which exhibited losses up to 66% of the initial soluble protein. Furthermore, nanoparticle size evaluation at conducted at most of the previous Zn concentrations (Fig. 17B) revealed that T22-NIDOMut3-H6 nanoparticles are the ones assembled with a lowest dispersion at a stable size of 17 nm.

EXAMPLE 10. Stability study in buffers

The stability of resulting proteins was studied in three FDA-approved buffers, and carbonate as control buffer. To this end, T22-NIDOMut2-H6 (control), T22-NIDOMut3-

H6 and T22-NIDOMut5-H6 were further assessed to study whether the overall stability and solubility could be improved after dialysis in more complex buffers. The composition of the selected three FDA-approved buffers are provided in Table 9.

Code	Composition
A9	Polysorbate 80 (0.4 mg/ml), sucrose (80 mg/ml), sodium citrate 2-hydrate (2.7 mg/ml), citric acid anhydrous (0.146 mg/ml), pH 6.5.
B6	Sucrose (70 mg/ml), glacial acetic acid (0.12 mg/ml), sodium acetate 3-hydrate (2.45mg/ml), pH 5.3.
D1	Polysorbate 80 (0.05 mg/ml), sucrose (50 mg/ml), sodium phosphate monobasic 1-hydrate (0.22 mg/ml), sodium phosphate dibasic anhydrous (0.49 mg/ml), pH 7.2.

Table 9. Dialysis buffer composition.

- 5 After dialysis with the aforementioned buffers, precipitation was evident in most of the conditions, including the sodium bicarbonate control (Table 10). However, T22-NIDOMut2-H6 and T22-NIDOMut3-H6 remained highly soluble in buffer B6 while buffer A9 proved to be the best suited for T22-NIDOMut5-H6.

Sample	Buffer	% Precipitation (Bradford)	% Precipitation (Nanodrop)
T22-NIDOMut2-H6	Carbonate	17.6	23.9
	A9	12.7	29.0
	B6	None	None
	D1	84.1	86.5
T22-NIDOMut3-H6	Carbonate	17.6	24.5
	A9	10.25	15.4
	B6	5.4	None
	D1	83.6	84.3
T22-NIDOMut5-H6	Carbonate	60.4	24.2
	A9	None	None
	B6	15.4	26.6
	D1	50.1	62.4

Table 10. Soluble protein loss due to precipitation evaluating the FDA-approved buffers.

- 10 Intrinsic fluorescence of each protein was used to assess unfolding parameters linked to temperature. CSM profiles (Fig. 18 A-C) suggested that all three FDA-approved buffers contributed to a better stability of the protein than the regular carbonate buffer (133 mM NaCO₃H, pH 8). This trend is evident in graphs A-C, where the continuous line belonging to carbonate buffer (C) is maintained at higher CSM values than the rest of buffers
- 15 throughout most temperature points. This trend indicates that buffers A9, B6 and D1

retain the proteins in a more compact state. To assess which buffers are actually providing better stability to each of the proteins, key indicators were extracted from each CSM graph and represented in figure 18D. Ideal candidates should exhibit high values of all three indexes T_m , T_{onset} and ΔT . Comparing the 3 protein candidates, T22-NIDOMut3-H6 shows the highest values, firstly for buffer A9 and secondly for B6., Considering together precipitation and stability data the best choice for this protein is buffer B6, with good stability indices and showing almost no precipitation in dialysis. Second protein with better stability indexes is T22-NIDOMut5-H6, delivering buffer D1 with the best profile in terms of stability and precipitation.

10 **EXAMPLE 11- Drug conjugates**

T22-NIDOMut3-H6 and T22-NIDOMut2-H6 were conjugated to oligoFdU using a similar procedure and identical molar ratios. OligoFdU and protein quantification of the clean conjugated samples indicated payloads of 1.79 and 0.96 FdU/protein, respectively for T22-NIDOMut3-H6 and T22-NIDOMut2-H6. Payload estimation and conjugation efficacy were confirmed via MALDI-TOF (Fig. 19A-B). Both nanoconjugates had similar cytotoxic efficacy in vitro (CXCR4+ HeLa cell line, at 25 nM concentration of nanoconjugate drug). Remarkably, the same protein concentration of non-conjugated protein T22-Nidomut3-H6 did not cause any cytotoxicity (Fig. 19C). 22-NIDOMut3-H6-FdU assembly into nanoparticles is attained in carbonate buffer with 2 mM $ZnCl_2$; nanoparticle size remains at 17 nm as seen with the non-conjugated version of the protein (Fig. 19D).

EXAMPLE 12. Nanoparticle EPIX4-RK-GFP-H6

The capacity of the human ligand EPI-X4 was tested as a tumour-homing peptide in protein-based self-assembling nanoparticles, and also, its potential, together with T22, to form biparatopic nanoparticles to target and internalize into CXCR4-overexpressing tumour cells.

To address this purpose, we have performed a rational protein design to display the EPI-X4 peptidic sequence (Figure 20A), with the aim to generate self-assembling protein vehicles with suitable size for cancer therapy. An optimized EPI-X4 tandem version with higher receptor affinity and serum stability was placed at the N-terminal of H6-tagged

GFP. The combination of a cationic peptide at amino terminus plus the polyhistidine (H6) carboxy terminal favors the controlled protein assembling in nanoparticles of around 10-80 nm in size, via divalent cation coordination; ideal size for improving enhanced permeability and retention (EPR) effect and cell uptake, but also to minimize renal
5 filtration (kidney cut-off around 6-8 nm). The EPI-X4 polypeptidic sequence has only 25 % of cationic residues. Therefore, additional cationic amino acids (RKRKRK)[21] were incorporated into EPI-X4 to reach the 50 % in an alternative presentation of EPI-X4 (Figure 20A).

Two versions of the protein (namely EPIX4-GFP-H6 and EPIX4-(RK)-GFP-H6) were
10 efficiently produced in *Escherichia coli* and purified as pure full-length polypeptides with expected molecular masses (Figure 20B). While the parental version (EPIX4-GFP-H6) reached an unstable oligomerization in form of nanoparticulate entities of different sizes (from monomeric or dimeric forms of 4.8- 8 nm to nanoparticles of 10 and 50 nm), the protein version carrying the extra cationic sequence (EPIX4-(RK)-GFP-H6)
15 spontaneously self-assembled as regular nanoparticles of around 40 nm ($Pdi=0.343$) (Figure 20C and D). In agreement, and fully supporting these results, FESEM examinations showed toroid (ring-shaped) materials with ultrastructural morphometry (Figure 20E), that confirmed the measurements obtained by Dynamic light scattering (DLS) and Size-exclusion chromatography (SEC).

20 When exposed to cultured CXCR4+ HeLa cells, only EPIX4-(RK)-GFP-H6 efficiently penetrated target cells and accumulated intracellularly by a specific receptor entry, that was confirmed through its inhibition by the chemical CXCR4 antagonist AMD3100 (Figure 20F). In addition, confocal images fully supported these data, showing the intracellular location of EPIX4-(RK)-GFP-H6 without protein attached to the cell
25 membrane (Figure 20G). EPIX-4 ligand has been previously described as receptor internalization antagonist by its interaction with the CXCR4 second extracellular loop. The rational addition of a cationic sequence after EPIX-4 promotes protein self-assembling, and it also might mimic the mechanism of action of the natural ligand (CXCL12). The interaction between a set of arginine residues and the CXCR4 N-terminal
30 facilitates the rapid binding and efficient anchoring on the receptor, that favors cellular internalization.

EXAMPLE 13. Biparatopic nanoparticles

The combination of different cell-ligands in the same construct is an appealing approach in cancer therapy, which might dramatically increase cell specificity and avoid the development of drug resistance.. EPIX4-(RK)-GFP-H6 protein nanoparticles could be
5 disassembled to building blocks of 8.7 nm by using a mild detergent and reassembled by dialysis to materials of the same size of the parental nanoparticle (Figure 21B). In addition, biparatopic nanoparticles were successfully generated by mixing disassembled entities of EPIX4-(RK)-GFP-H6 with T22-BFP-H6, both CXCR4-targeted proteins (Figure 21A). The resulting biparatopic nanoparticles presented a monodisperse
10 population (PDI=0.179) of about 18 nm (Figure 21B) morphologically indistinguishable (Figure 21C) from the original EPIX4-(RK)-GFP-H6 (Figure 1E) or T22-BFP-H6 (U. Unzueta et al., Nanotechnology 2017, 28, 505102). Also, the presence of both proteins in the same entity was corroborated by Förster resonance energy transfer (FRET) (from blue to green fluorescence), as determined by comparing fluorescence emission scans of
15 different protein monomers against biparatopic nanoparticles upon excitation at 387nm. In this last case, when BFP is excited at 387 nm, the fluorescence emission energy of BFP is transferred to GFP chromophore only observing GFP fluorescence emission at 510 nm (Figure 21D).

To evaluate the biological properties of biparatopic nanoparticles, CXCR4+ cells lines
20 (the cervix cancer HeLa and the human colorectal SW1417 cell lines) were exposed at different times to the nanoparticles produced. Biparatopic nanoparticles keep the cell targeting properties (Figure 21E) without losing receptor specificity (Figure 21F). Regarding cell uptake, T22-GFP-H6 has high uptake at short times, while EPIX4-(RK)-GFP-H6 at longer times; biparatopic nanoparticles combine the uptake behavior of both,
25 being the cell uptake even better at short times (Figure 21E). Confocal microscopy confirms the cell internalization and green and blue proteins co-localization supports the occurrence of biparatopic nanoparticle formation.

At this stage, to assess in detail the potential of the targeted nanoparticles, in vivo biodistribution analyses were performed in CXCR4+mouse model of patient-derived M5
30 colorectal cancer. Upon systemic administration, mice were treated with a single dose of 200 µg at different times (0.5 1, 2, 5 and 24 h). Biparatopic nanoparticles elicited a much

faster tumour accumulation than EPIX4-(RK)-GFP-H6 nanoparticles reaching higher levels of intracellular material at shorter times (from 0.5 to 2 h) as predicted by in vitro analysis (Figure 21F). Meanwhile, EPIX4-(RK)-GFP-H6 showed a progressive accumulation, that peaked at 5 h, and remained relatively stable at least for 24 h (Figure 22A). When analyzing normal tissues, fluorescence was undetectable in non-tumoral tissues for all nanoparticle versions, indicating the exquisite in vivo specificity of the CXCR4-targeting. Interestingly, the absence of fluorescence in kidney at longer times is an indicator that this heteromeric oligomeric platform retains the nanoparticle stability in vivo as the parental version does. (Table 11). In addition, histopathological analyses corroborate the lack of systemic toxicity neither in CXCR4⁻ tissues (kidney and liver) or CXCR4⁺ (spleen) (Figure 22D).

	30 min		1 h		2 h		5 h		24 h	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
CONTROL	4.59 ±0.9	3.24 ±0.1	4.35 ±0.0	3.62 ±0.7	4.57 ±0.2	3.73 ±0.3	4.78 ±0.3	4.3 ±0.4	4.48 ±1.3	4.48 ±1.8
EPIX4-(RK)-GFP-H6	7.14 ±0.6	7.14 ±0.7	4.93 ±0.3	4.93 ±0.4	4.46 ±0.1	4.46 ±0.4	4.53 ±0.2	4.53 ±2.2	5.36 ±0.2	5.36 ±2.2
BIPARATOPIC NPs	7.02 ±0.5	7.02 ±0.8	4.73 ±0.2	4.73 ±1.1	4.64 ±0.4	4.64 ±0.5	3.93 ±0.3	3.93 ±1.9	4.38 ±2.0	4.38 ±2.2

Table 11. Biodistribution of CXCR4-targeted protein nanoparticles upon systemic administration. Fluorescence emitted by normal organs after the administration at the analyzed times of 300 µg of each nanoparticle, expressed as mean ± SE of radiant efficiency ($\times 10^6$;[p s⁻¹ cm⁻² sr⁻¹]/µW cm⁻²)

In addition, throughout time, it was observed a dramatic increase of apoptotic events, with a drop in the number of mitotic cells in the tumour samples, being both significant at 5 h (Figure 22C). The rapid uptake of biparatopic nanoparticles provoked higher levels of receptor internalization than the other material versions. This fact elicits a substantially higher number of apoptotic bodies at 24 h (Figure 22B). Longer administration of these nanoparticles could trigger cell death by sustained caspase-3 activation (M. V. Céspedes et al., Sci Rep 2016, 6, 35765.) which could also be enhanced by either chemotherapy conjugation (M. V. Céspedes, et al., EMBO Mol Med 2018, 10.) or by toxic protein fusion. (R. Díaz et al., Small 2018, 14, e1800665; L. Sánchez-García et al., J Control Release 2018, 274, 81.).

Taken together, all these observations strongly support the high potential of EPI-X4 as a human tumour-homing peptide. The proper protein engineering resulted into regular and stable protein nanoparticles with a potent CXCR4-targeting in vivo. In addition, the plasticity of this protein material permitted to combine with another CXCR4 targeted
5 protein in a structurally robust biparatopic nanoparticle type, with high penetrability at short times and maintaining the parental specificity and biodistribution pattern.

Regarding the antitumor activity of the biparatopic nanoparticles, the results suggest that the multivalent display of the ligands EPI-X4 and T22, which are both CXCR4 antagonists, and most likely interact with different CXCR4 domains, are probably
10 responsible for the faster rate of internalization and the enhanced induction of apoptosis in the biparatopic setting. Thus, the peak, reached 24 h post-treatment, of around 30 apoptotic figures per 400 x magnification tumor field, are of a similar magnitude for cell death induction as shown in previous results in colorectal tumor models after treatment with nanoparticles based on T22 that incorporate potent cytotoxic agents, such as
15 Floxuridine or the catalytic domain of bacterial toxins (L. Sánchez-García et al., J Control Release 2018, 274, 81; M. V. Céspedes, et al., J Control Release 2020, 320, 96).

CLAIMS

1. A polypeptide comprising:

5 (i) eleven beta strand domains designated A, B, C, D, E, F, G, H, I, J and K and

(ii) ten loop regions designated as AB, BC, CD, DE, EF, FG, GH, HI, IJ and JK loops wherein each loop region connects each two consecutive beta strand domains;

10 wherein at least one of the loop regions is a variant of the cognate loop region in SEQ ID NO:62, wherein the cognate loop regions in SEQ ID NO:62 are as defined in SEQ ID NO. 1 (loop region AB), SEQ ID NO:2 (loop region BC), SEQ ID NO:3 (loop region CD), SEQ ID NO:4 (loop region DE), SEQ ID NO:5 (loop region EF), SEQ ID NO:6 (loop region FG), amino acids 149 to 150 in SEQ ID NO:62 (loop region GH), SEQ ID NO:7 (loop region HI), SEQ ID NO:8 (loop region IJ) and SEQ ID NO:9 (loop region JK),

15 and

wherein at least one beta strand domain is a variant of the cognate beta strand in SEQ ID NO:62 and has at least 50% sequence identity with said cognate beta strand domain, wherein the cognate beta strand domains in SEQ ID NO:62 are as defined in SEQ ID NO:9 (beta strand domain A), SEQ ID NO:11 (beta strand domain B), SEQ ID NO:12 (beta strand domain C), SEQ ID NO:13 (beta strand domain D), SEQ ID NO:14 (beta strand domain E), SEQ ID NO:15 (beta strand domain F), SEQ ID NO:16 (beta strand domain G), SEQ ID NO:17 (beta strand domain H), SEQ ID NO:18 (beta strand domain I), SEQ ID NO:19 (beta strand domain J) and SEQ ID NO:20 (beta strand domain K).

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2. The polypeptide according to claim 1 wherein the variant of the at least one of the loop regions results from the mutation by deletion, substitution or addition of at least one amino acid in the sequence of the cognate loop region.

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3. The polypeptide according to aspects 1 or 2 comprising a mutation in one or more beta strands, wherein the mutation is located at position 9 in beta strand B as defined in SEQ ID NO:11, at position 1 in beta strand C as defined in SEQ ID NO:12, at

position 10 in beta strand J as defined in SEQ ID NO:19 or at position 3 in beta strand K as defined in SEQ ID NO:20.

4. The polypeptide according to claims 1-3, comprising one or more mutations, wherein
5 the mutation is located at position 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 580, 604, 638, 640, 641, 469 and/or 518 wherein the numbers are as defined in the protein sequence with accession number P14543-1 in the Uniprot Database version dated July 7, 2009.
- 10 5. The polypeptide according to claim 4 wherein the one or more mutations at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 580, 604, 638, 641, 469 and/or 518 in SEQ ID NO.62 are a H459A, a R468N, a F639S, a R650A, a H543K, a H545N, a V449T, a V525Q, a F561E, a V619T, a V619T, a C618S, a V580T, a I604T, a V640Y, a L641T, a S469I and a R518I.
- 15 6. The polypeptide according to any of claims 4 or 5 wherein the one or more mutations are as defined in Table 1.
7. A polypeptide display library comprising a plurality of polypeptides according to any
20 of claims 1-6, wherein the plurality of polypeptides is formed by polypeptides that differ in the sequence of one or more loop regions.
8. The polypeptide display library according to claim 7, wherein in the library, each polypeptide as a phenotype is linked directly or indirectly to a nucleic acid as a
25 genotype corresponding to said phenotype.
9. A polynucleotide encoding the polypeptide according to any of claims 1 to 6 or a collection of polynucleotides encoding a plurality of polypeptides of the display library according to claim 7 or 8.
- 30 10. A vector comprising the polynucleotide according to claim 9.

11. A host cell comprising the polynucleotide according to claims 9 or the vector according to claim 10.
12. A conjugate comprising
- 5 (i) a first polypeptide region comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- (ii) an agent of interest.
13. The conjugate according to claim 12 wherein the polypeptide is a functionally
- 10 equivalent variant of the G2 domain of nidogen-1 as defined in any of claims 1 to 6.
14. The conjugate according to claims 12 or 13 wherein the polypeptide region comprises amino acids 430 to 667 with respect to the sequence of human nidogen-1 defined in the UniProt database with accession number P14543-1 in the version dated July 7,
- 15 2009.
15. The conjugate according to any of claim 12-14 wherein the polypeptide is a functionally equivalent variant of the domain G2 of nidogen-1 comprising one or more mutations, wherein the mutation is located at position 459, 468, 639, 650, 543,
- 20 545, 449 , 525, 561, 618, 619, 580, 604, 638, 640, 641, 469 and/or 518 wherein the numbers are as defined in the protein sequence with accession number P14543-1 in the Uniprot Database version dated July 7, 2009.
16. The conjugate according to claim 15 wherein the one or more mutations at positions
- 25 459, 468, 639, 650, 543, 545, 449 , 525, 561, 618, 619, 580, 604, 638, 641, 469 and/or 518 in SEQ ID NO.62 are a H459A, a R468N, a F639S, a R650A, a H543K, a H545N, a V449T, a V525Q, a F561E, a V619T, a V619T, a C618S, a V580T, a I604T, a V640Y, a L641T, a S469I and a R518I.
- 30 17. The conjugate according to claims 15 or 16 wherein the one or more mutations are as defined in Table 1.

18. The conjugate according to any of claims 12 to 17 wherein the polypeptide further comprises a second polypeptide region which is capable of specifically binding to a target of interest.
- 5 19. The conjugate according to claim 18 wherein the second polypeptide region which is capable of specifically binding to a target of interest is a polycationic peptide.
20. The conjugate according to claim 19 wherein the polycationic peptide is selected from the group consisting of
- 10 (i) a sequence which is capable of specifically binding to a receptor on a cell surface and promoting internalization of the conjugate on said cell,
- (ii) an arginine-rich sequence,
- (iii) the GWH1 peptide,
- (iv) a CD44 ligand,
- 15 (v) a peptide capable of crossing the blood brain barrier,
- (vi) a cell penetrating peptide and
- (vii) a nucleolin-binding peptide.
21. The conjugate according to claim 20 wherein the polycationic peptide comprises a
- 20 sequence which is capable of specifically interacting with a receptor on a cell surface and promoting internalization of the conjugate on said cell, said sequence being a CXCR4 ligand.
22. The conjugate according to claim 21 wherein the CXCR4 ligand is a peptide selected
- 25 from the group consisting of a peptide comprising the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 (SEQ ID NO:27) peptide, the vCCL2 (SEQ ID NO:28), the EPI-X4 sequence (SEQ ID NO:29) or a functionally equivalent variant thereof.
- 30 23. The conjugate according to claim 22 which comprises a positively charged peptide sequence, preferably RKRKRK (SEQ ID NO. 77), located at the N- or C-terminal end of the CXCR4 ligand, preferably at the C-terminal end of the CXCR4 ligand.

24. The conjugate according to claim 23 wherein the CXCR4 ligand is the EPI-X4 sequence (SEQ ID NO:29) or a functionally equivalent variant thereof.
- 5 25. The conjugate according to claim 20 wherein the polycationic peptide is an arginine-rich sequence comprising a sequence selected from the group consisting of RRRRRRRRRR (SEQ ID NO:30), RRRGRGRRR (SEQ ID NO:31), RARGRGRRR (SEQ ID NO:32), and RARGRGGGA (SEQ ID NO:33).
- 10 26. The conjugate according to claim 20 wherein the polycationic peptide is the CD44 ligand A5G27 (SEQ ID NO:34) or FNI/II/V (SEQ ID NO:35).
27. The conjugate according to claim 20 wherein the polycationic peptide is the peptide capable of crossing the blood brain barrier selected from the group consisting of Seq-1-7 (SEQ ID NO:36), Seq-1-8 (SEQ ID NO:37), Angiopep-2-7 (SEQ ID NO:38).
- 15 28. The conjugate according to any of claims 12 to 27 wherein the polypeptide further comprises a third polypeptide region which is a positively charged amino acid-rich region.
- 20 29. The conjugate according to claim 28 wherein the positively charged amino acid-rich region is a polyhistidine region.
30. The conjugate according to claim 29 wherein the polyhistidine region comprises between 2 and 10 contiguous histidine residues.
- 25 31. The conjugate according to any of claims 12 to 30 wherein the polycationic peptide is located at the N-terminus and the positively charged amino acid-rich region is located at the C-terminus of the polypeptide or wherein the positively charged amino acid-rich region is located at the N-terminus and the polycationic peptide is located at the C-terminus of the polypeptide.
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32. The conjugate according to any of claims 12 to 31 wherein the agent of interest is a therapeutic agent or an imaging agent.
33. The conjugate according to claim 32 wherein the therapeutic agent is selected from the group consisting of
- (i) a chemotherapy agent,
 - (ii) a cytotoxic polypeptide,
 - (iii) an antiangiogenic polypeptide,
 - (iv) a polypeptide encoded by a tumor suppressor gene,
 - (v) a pro-apoptotic polypeptide,
 - (vi) a polypeptide having anti-metastatic activity,
 - (vii) a polypeptide encoded by a polynucleotide which is capable of activating the immune response towards a tumor and
 - (viii) an antiangiogenic molecule.
 - (ix) a toxin
34. The conjugate according to any of claims 32 or 33 wherein the polypeptide is conjugated to a plurality of therapeutic agents, wherein said plurality of therapeutic agents are the same or different.
35. The conjugate according to claims 33 or 34 wherein the therapeutic agent is a chemotherapy agent.
36. The conjugate according to claim 35 wherein the chemotherapy agent is an antimetabolite.
37. The conjugate according to claims 36 wherein the antimetabolite is a pyrimidine analogue or an oligomeric form thereof.
38. The conjugate according to claims 37 wherein the pyrimidine analogue is floxuridine or a pentameric form thereof.

39. The conjugate according to any of claims 28 to 38 wherein the second polypeptide region is connected to the first polypeptide region via a first peptide linker and/or wherein the first polypeptide region is connected to the third polypeptide region via a second peptide linker.

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40. A method for preparing a conjugate according to any of claims 12 to 39 comprising

- (i) providing the polypeptide of the conjugate according to any of claims 12-39 comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- (ii) contacting said polypeptide with an activated form of the agent of interest of the conjugate according to any of claims 12-39 which is capable of reacting with at least one group in the polypeptide and wherein the contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the agent of interest and the group in polypeptide.

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41. The method according to claim 40 wherein the activated form of the agent of interest agent contains a group which reacts with at least one of the side chains in the polypeptide.

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42. The method according to 41 wherein the group which reacts with at least one of the side chains in the polypeptide region is a thiol group, an amino group or a carboxylic acid group.

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43. A method for preparing a conjugate according to any of claims 12 to 39 comprising

- (i) providing the polypeptide of the conjugate according to any of claims 12-39 comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof wherein the polypeptide is provided in an activated form and
- (ii) contacting said polypeptide with the agent of interest which is capable of reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a

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bond between the reactive group in the polypeptide and the group in the agent of interest.

44. A polypeptide comprising

- 5 (i) a first region comprising the G2 domain of nidogen-1 or a functionally equivalent variant thereof and
- (ii) a second region which comprises an antagonistic CXCR4 ligand.

45. The polypeptide according to claim 44 wherein the first region is a functionally
10 equivalent variant of the G2 domain of nidogen-1 as defined in any of claims 1 to 6.

46. The polypeptide according to claims 44 or 45 wherein the first region comprises amino acids 430 to 667 with respect to the sequence of human nidogen-1 defined in the UniProt database with accession number P14543-1 in the version dated July 7, 2009.

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47. The polypeptide according to claims 44 or 45 wherein the polypeptide is a functionally equivalent variant of the domain G2 of nidogen-1 comprising one or more mutations, wherein the mutation is located at position 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 580, 604, 638, 640, 641, 469 and/or 518 wherein the
20 numbers are as defined in the protein sequence with accession number P14543-1 in the Uniprot Database version dated July 7, 2009.

48. The polypeptide according to claim 47 wherein the one or more mutations at positions 459, 468, 639, 650, 543, 545, 449, 525, 561, 618, 619, 580, 604, 638, 641, 469 and/or
25 518 in SEQ ID NO.62 are a H459A, a R468N, a F639S, a R650A, a H543K, a H545N, a V449T, a V525Q, a F561E, a V619T, a V619T, a C618S, a V580T, a I604T, a V640Y, a L641T, a S469I and a R518I.

49. The polypeptide according to claims 47 or 48 wherein the one or more mutations are
30 as defined in Table 1.

50. The polypeptide according to any of claims 44 to 49 wherein the second region comprises the EPI-X4 sequence (SEQ ID NO:29) or a functionally equivalent variant thereof.
- 5 51. The polypeptide according to claim 50 wherein the second region further comprises a positively-charged amino acid region.
52. The polypeptide according to claim 51 wherein at least 50% of the amino acids of the second region are positively charged amino acids.
- 10 53. The polypeptide according to claims 51 or 52 wherein the positively charged amino acid region comprises the RKRKRK sequence.
54. The polypeptide according to any of claims 44 to 53 wherein the first region is located at the N-terminus and the positively charged amino acid-rich region is located at the C-terminus of the polypeptide.
- 15 55. The polypeptide according to any of claims 44 to 54 wherein the polypeptide further comprises a third polypeptide region which is a positively charged amino acid-rich region.
- 20 56. The polypeptide according to claim 55 wherein the positively charged amino acid-rich region is a polyhistidine region.
- 25 57. The polypeptide according to claim 56 wherein the polyhistidine region comprises between 2 and 10 contiguous histidine residues.
58. The polypeptide according to any of claims 55 to 57 wherein the first region is located at the N-terminus and the third region is located at the C-terminus of the polypeptide.
- 30 59. A method for preparing a nanoparticle comprising multiple copies of the polypeptide according to any of claims 44 to 58, said method comprising placing a preparation of

said polypeptide under conditions adequate for the assembly of a plurality of copies of the polypeptide into a nanoparticle.

60. A method for preparing a nanoparticle comprising multiple copies of the conjugate
5 according to any of claims 28 to 39 or multiple copies of the polypeptide according to any of claims 55-58 selected from

(i) a method which comprises placing a preparation of said conjugate or said polypeptide under conditions adequate for the assembly of a plurality of copies of the conjugate or of the polypeptide into a nanoparticle or
10 (ii) a method which comprises

i. placing a plurality of polypeptides each comprising
1. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,
2. a second polypeptide region which is capable of
15 specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide and
3. a third polypeptide region which is a positively charged amino acid-rich region,

wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptide
20 and wherein the polypeptide is provided in an activated form, wherein said activated form of the polypeptide contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of the polypeptide and
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ii. contacting the nanoparticle obtained in step i with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in the polypeptide, wherein said contacting is carried out under conditions adequate
30 for the formation of a bond between the reactive group in the polypeptide and the group in the agent of interest.

61. The method according to claim 60 wherein the first, second and third polypeptide regions are as defined in the conjugates of any of claims 13 to 31 or 45-58.

5 62. A method for preparing a biparatopic nanoparticle comprising multiple copies of a first type of conjugate and multiple copies of a second type of conjugate, wherein the first and second types of conjugates are as defined in any of claims 28 to 39 and wherein the first and the second type of conjugates differ in the sequence of the polycationic peptide, said method being selected from

10 (i) A method which comprises contacting a preparation of said first type of conjugate with a preparation of said second type of conjugate under conditions adequate for the assembly of a plurality of copies of the two types of conjugates into a nanoparticle or

(ii) A method which comprises
15 i. contacting a preparation of a first polypeptide with a preparation of a second polypeptide wherein the first and second types of polypeptides comprise

a. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,
20 b. a second polypeptide region which is capable of specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide, and/or comprises an additional positively charged peptide sequence located at its N- or C-terminal end and the sequence of the polycationic peptide of one polypeptide is
25 different from the sequence of the polycationic peptide of the other polypeptide,

c. a third polypeptide region which is a positively charged amino acid-rich region,
30 d. optionally, a positively charged peptide sequence located at the N- or C-terminal end of the polycationic peptide,

- wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptides,
- wherein the first and second polypeptides differ in their polycationic peptide,
- wherein the first and/or second polypeptides are as the conjugates defined in claims 28-39,
- wherein the first and/or the second polypeptides are provided in an activated form, wherein said activated form of the polypeptides contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of polypeptides,
- ii. contacting the nanoparticle obtained in step i with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in each polypeptide, wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptides and the group in the agent of interest.
63. The method according to claim 62 wherein the first, second and third polypeptide regions are as defined in the conjugates of any of claims 13 to 31.
64. A method for preparing a biparatopic nanoparticle comprising multiple copies at least one conjugate according to claims 28 to 39 and multiple copies of at least one polypeptide according to any of claims 55 to 58 wherein the polycationic peptide of the first type of conjugate is different from the sequence of the second region of the at least one polypeptide, said method being selected from
- (i) A method which comprises placing a preparation of multiple copies of said at least one conjugate and multiple copies of said at least one polypeptide under conditions adequate for the assembly of a plurality of copies of the two conjugates into a nanoparticle or
- (ii) A method which comprises

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- i. contacting a preparation of a first polypeptide with a preparation of a second polypeptide wherein the first and second type of polypeptide comprise
 - a. a first polypeptide region which is the G2 domain of nidogen-1 or a functionally equivalent variant thereof,
 - b. a second polypeptide region which is capable of specifically binding to a target of interest wherein said second polypeptide is a polycationic peptide, and/or comprises an additional positively charged peptide sequence located at its N- or C-terminal end and the sequence of the polycationic peptide of one polypeptide is different from the sequence of the polycationic peptide of the other polypeptide,
 - c. a third polypeptide region which is a positively charged amino acid-rich region,wherein the polycationic peptide and the positively charged amino acid-rich region are located at the ends of the polypeptides,
wherein the first polypeptide is as the conjugate defined in any of claims 28-39 and the second polypeptide is as defined in any of claims 55 to 58,
wherein the polycationic peptide of the first polypeptide is different from the polycationic peptide of the second polypeptide,
 - 25 wherein the first and/or the second polypeptides are provided in an activated form, wherein said activated form of the polypeptides contains a reactive group, wherein said placing is carried out under conditions adequate for the formation of a nanoparticle containing a plurality of copies of polypeptides,
 - 30 ii. contacting the nanoparticle obtained in step i with an activated form of the agent of interest which contains a group which is capable of reacting with the reactive group in each polypeptide,

wherein said contacting is carried out under conditions adequate for the formation of a bond between the reactive group in the polypeptides and the group in the agent of interest.

- 5 65. The method according to claim 64 wherein the first, second and third polypeptide regions of the conjugate and polypeptide of alternative (i) or of the first and second polypeptides in alternative (ii) are as defined in the conjugates of any of claims 13 to 31 or 44-58.
- 10 66. The method according to claims 64 or 65 wherein the polycationic peptide of the conjugate in alternative (i) or of the first polypeptide in alternative (ii) is selected from the group consisting of the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 (SEQ ID NO:27) peptide, the vCCL2 (SEQ ID NO:28) and functionally equivalent variant thereof.
- 15 67. The method according to claim 66, wherein the polycationic peptide of the conjugate in alternative (i) or of the first polypeptide in alternative (ii) is selected from the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26), the CXCL12 (SEQ ID NO:27) peptide, the vCCL2 (SEQ ID NO:28) and
- 20 a functionally equivalent variant thereof, is preferably RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), and the polycationic peptide of the polypeptide in alternative (i) or of the second polypeptide in alternative (ii) is the EPI-X4 sequence (SEQ ID NO:29) or a functionally equivalent variant thereof.
- 25 68. The method according to claim 67 wherein the EPI-X4 sequence (SEQ ID NO:29) is joined to a RKRKRK (SEQ ID NO:77) sequence.
69. The method according to any of claims 59 to 68 wherein the conditions adequate for the assembly of a plurality of copies of the polypeptide into a nanoparticle comprise
- 30 an incubation in a low salt buffer.

70. The method according to claim 69 wherein the low salt buffer is selected from the group consisting of a carbonate buffer, a citrate buffer, an acetate buffer, a Tris buffer and a phosphate buffer.
- 5 71. The method according to claim 70 wherein the pH of the buffer is between pH 4 and pH 8, preferably between pH 5 and pH 7.5, more preferably is of about PH 5.3, PH 6.5 or PH 7.2.
72. The method according to claims 70 or 71 wherein the citrate buffer, the acetate buffer
10 and/or the phosphate buffer further comprise Polysorbate 80 and/or sucrose.
73. The method according to claim 72 wherein the sucrose is found at a concentration between 20 mg/ml and 100 mg/ml.
- 15 74. The method according to claim 73 wherein the sucrose is found at a concentration between 50 mg/ml and 90 mg/ml, preferably of 70 mg/ml.
75. The method according to any of claims 70 to 74 wherein the citrate buffer comprises Polysorbate 80 (0.4 mg/ml), sucrose (80 mg/ml), sodium citrate 2-hydrate (2.7
20 mg/ml) and citric acid anhydrous (0.146 mg/ml) and has a pH of about 6,5.
76. The method according to claims 70 to 74 wherein the acetate buffer comprises sucrose (70 mg/ml), glacial acetic acid (0.12 mg/ml), sodium acetate 3-hydrate (2.45mg/ml) and has a pH of about 5.3.
- 25 77. The method according to claims 70 to 74 wherein the phosphate buffer comprises Polysorbate 80 (0.05 mg/ml), sucrose (50 mg/ml), sodium phosphate monobasic 1-hydrate (0.22 mg/ml), sodium phosphate dibasic anhydrous (0.49 mg/ml) and has a pH of about 7.2.
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78. A nanoparticle comprising multiple copies of the conjugate according to any of claims 28 to 39, multiple copies of a polypeptide according to any of claims 55 to 58 or which has been obtained by a method according to any of claims 59 to 77.
- 5 79. A biparatopic nanoparticle that comprises multiple copies of a first and a second type of conjugates, wherein both said first and second types of conjugates are as defined in any claims 28-39 or as the polypeptide defined in claims 55-58 and wherein the first and second types of conjugates differ in the polycationic peptide or a biparatopic nanoparticle that has been obtained by a method according to claims 62-77.
- 10 80. The biparatopic nanoparticle according to claim 79 wherein the polycationic peptide of the first type of conjugate and of the second type of conjugate is a CXCR4 ligand, preferably selected from the group consisting of the peptide that comprises the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ
- 15 ID NO:26) the CXCL12 peptide (SEQ ID NO:27), the vCCL2 peptide (SEQ ID NO:28), the EPI-X4 sequence (SEQ ID NO:29) and a functionally equivalent variant thereof.
- 20 81. The biparatopic nanoparticle according to claim 80 wherein the polycationic peptide of the first type of conjugate consists of the peptide that comprises the sequence RRWCYRKCYKGYCYRKCR and wherein the polycationic peptide of the second type of conjugate is the EPI-X4 sequence (SEQ ID NO:29).
- 25 82. A biparatopic nanoparticle that comprises multiple copies of a conjugate according to any of claims 28-39 and multiple copies of a polypeptide according to any of claims 55-58, wherein the polycationic region of the conjugate is different from the first region of the polypeptide, or a biparatopic nanoparticle that has been obtained by a method according to any of claims 62-77.
- 30 83. The biparatopic nanoparticle according to claim 82 wherein the polycationic peptide of the conjugate is a CXCR4 ligand, preferably selected from the group consisting of the peptide that comprises the sequence RRWCYRKCYKGYCYRKCR (SEQ ID

NO:25), the V1 peptide (SEQ ID NO:26) the CXCL12 peptide (SEQ ID NO:27) and the vCCL2 peptide (SEQ ID NO:28).

84. The biparatopic nanoparticle according to claim 83 wherein the antagonistic CXCR4
5 ligand is the EPI-X4 sequence (SEQ ID NO:29).

85. The nanoparticle according to claim 78 to 84 having a diameter of between 1 and 100 nm.

10 86. A conjugate according to any of claims 12 to 39, a polypeptide according to any of claims 44 to 58 or a nanoparticle according to claims 78 to 85 for use in medicine.

87. A conjugate according to any of claims 12 to 39, a polypeptide according to any of claims 44 to 58 or a nanoparticle according to claims 78 to 85 wherein the conjugate/s
15 or polypeptide/s comprise/s a sequence which is capable of specifically interacting with a receptor on a cell surface and promoting internalization of the conjugate or of the nanoparticle on said cell, wherein said cell is a tumor cell present in a cancer for use in the treatment of cancer.

20 88. The conjugate, polypeptide or nanoparticle for use according to claim 87 wherein the polycationic peptide of the conjugates or polypeptides forming the nanoparticle is a CXCR4 ligand and wherein the cancer is characterized in that it comprises cancer cells that express or overexpress CXCR4.

25 89. The conjugate or nanoparticle for use according to claim 88 wherein the CXCR4 ligand is selected from the group consisting of the peptide that comprises the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25), the V1 peptide (SEQ ID NO:26) the CXCL12 peptide (SEQ ID NO:27), the vCCL2 peptide (SEQ ID NO:28), the EPI-X4 sequence (SEQ ID NO:29).

90. The conjugate or nanoparticle for use according to claim 89 wherein the CXCR4 ligand is a peptide having the sequence RRWCYRKCYKGYCYRKCR (SEQ ID NO:25).
- 5 91. The conjugate or nanoparticle for use according to claim 90 wherein the CXCR4 ligand is the EPI-X4 peptide (SEQ ID NO.29).
92. The nanoparticle for use according to any of claims 86 to 91 wherein the nanoparticle is a biparatopic nanoparticle as defined in any of claims 79-85.
- 10 93. The conjugate, polypeptide or nanoparticle for use according to any of claims 88 to 92 wherein the cancer cells that express or overexpress CXCR4 are metastatic stem cells.
- 15 94. The conjugate, polypeptide or nanoparticle for use according to claims 87 to 93 wherein the cancer is pancreatic or colorectal cancer.
95. The conjugate, polypeptide or nanoparticle for use according to any of claims 87 to 94 wherein the cancer is a primary tumor or a metastasis.
- 20 96. A method for the imaging of a target cell which comprises specific binding sites for one or more components of the conjugate according to any of claims 12 to 39, of the polypeptide according to any of claims 44 to 58 or of the nanoparticle according to claims 78 to 85, the method comprising
- 25 (i) contacting a sample containing said cell with a conjugate according to any of claims 12 to 39, with a polypeptide according to any of claims 44-58, or with a nanoparticle according to claims 78 to 85 under conditions adequate for the binding of the conjugate, of the polypeptide or of the nanoparticle to the cell and wherein the agent of interest is an imaging agent and
- 30 (ii) imaging the cell by detecting the signal provided by the imaging agent.

97. The method according to claim 96 wherein the cell expresses or overexpresses CXCR4 and wherein the one or more components of the conjugate or of the nanoparticle is a polycationic peptide and said polycationic peptide is a CXCR4 ligand.

5

98. A method for identifying a polypeptide that binds to a target peptide, said method comprising:

- i) contacting a target peptide with the polypeptide display library according to claims 7 or 8 under conditions that allow a polypeptide to interact with the target peptide,
- ii) recovering those members of the library that have specifically interacted with the target peptide, and
- iii) identifying the sequence of the polypeptide that interacts with the target peptide.

15

99. The method according to claim 98, wherein steps i) –ii) are repeated at least once wherein the polypeptide library used in step i) in each repetition is formed by the members of the library recovered in step (ii).

100. The method according to claims 98 or 99 wherein the target peptide is immobilized in a solid support.

101. Use of a polypeptide according to any of claims 1 to 6 for presenting a peptide, wherein said peptide is found in one of the loop regions.

25

102. A method for determining the presence of a target peptide in a sample comprising:

- i) contacting the proteins present in the sample with a polypeptide according to any of claims 1 to 6, wherein the sequence of at least one of the loop regions in the polypeptide is a sequence that is capable of specifically binding to the target peptide,
- ii) determining if there is an interaction between the target peptide and the polypeptide,

30

wherein if there is an interaction between the polypeptide and the target peptide, then the target peptide is present in the sample.

103. The method according to claim 102 wherein the polypeptide is immobilized in a
5 solid support.

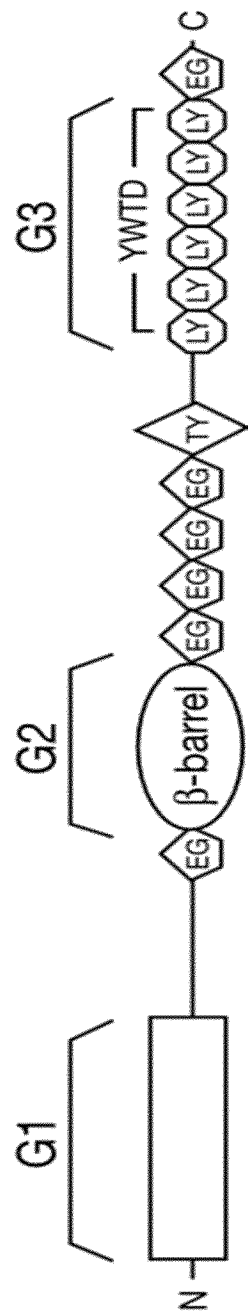


Fig. 1

A

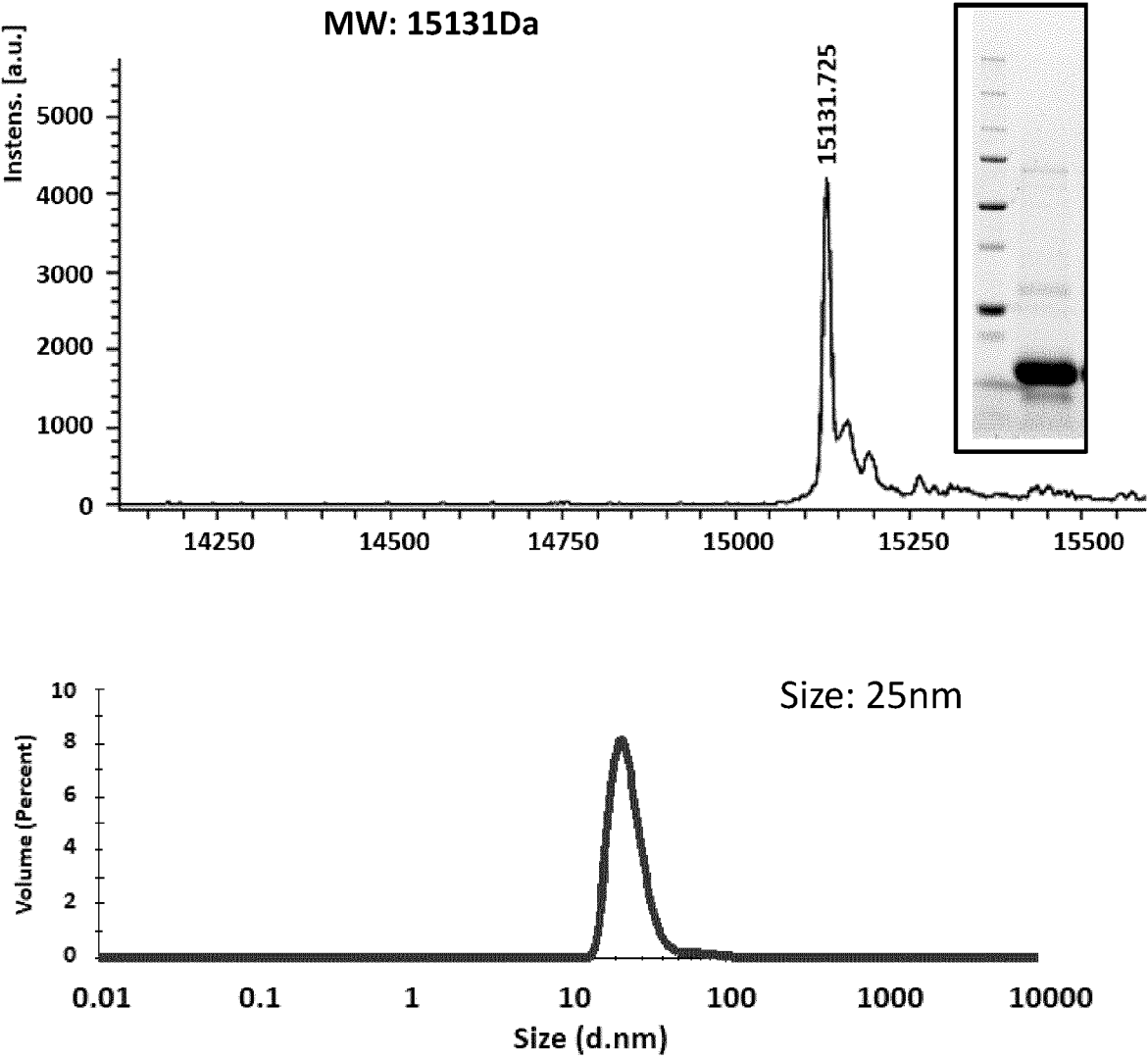


Fig. 2

B

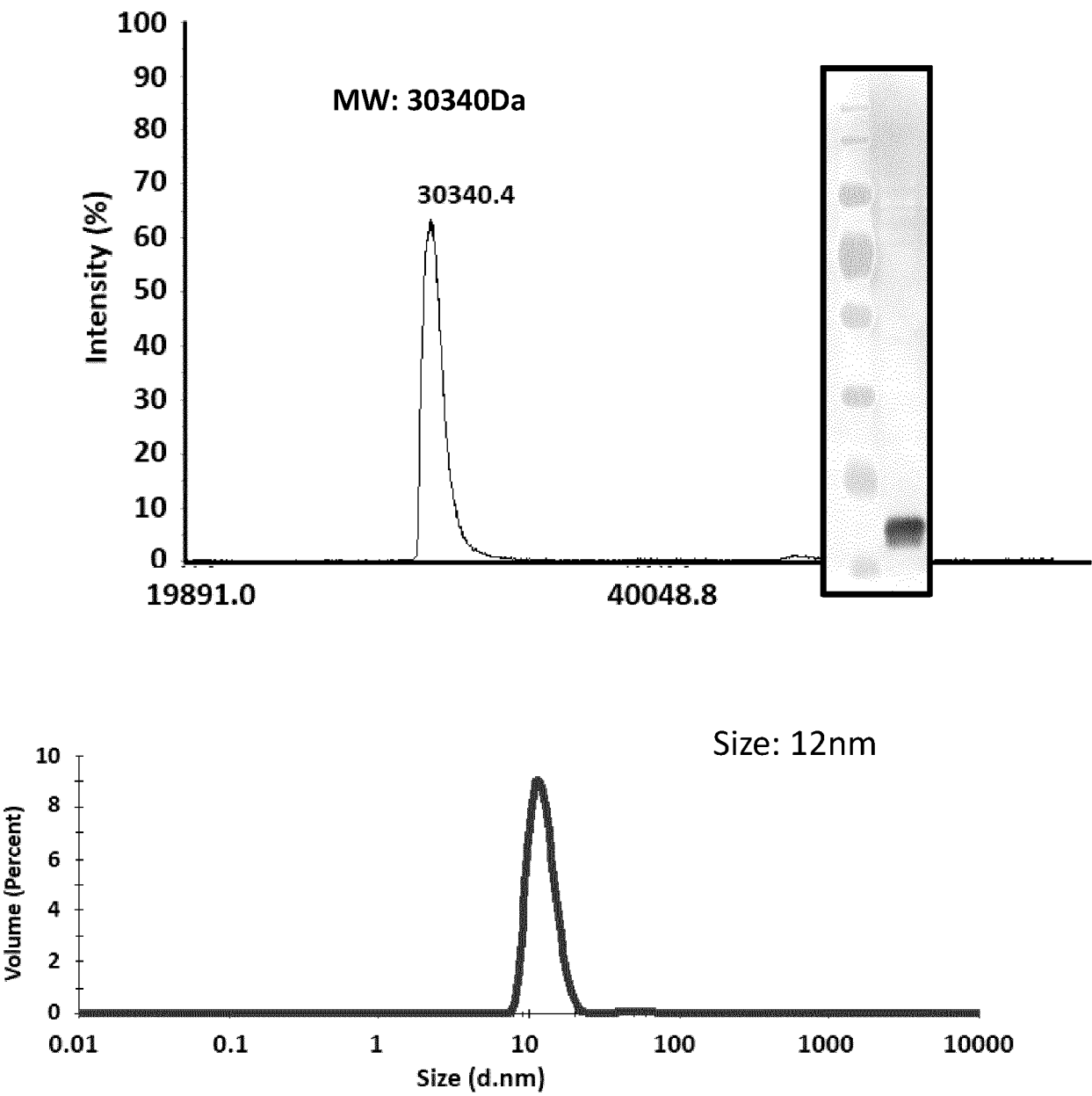


Fig. 2 (cont.)

C

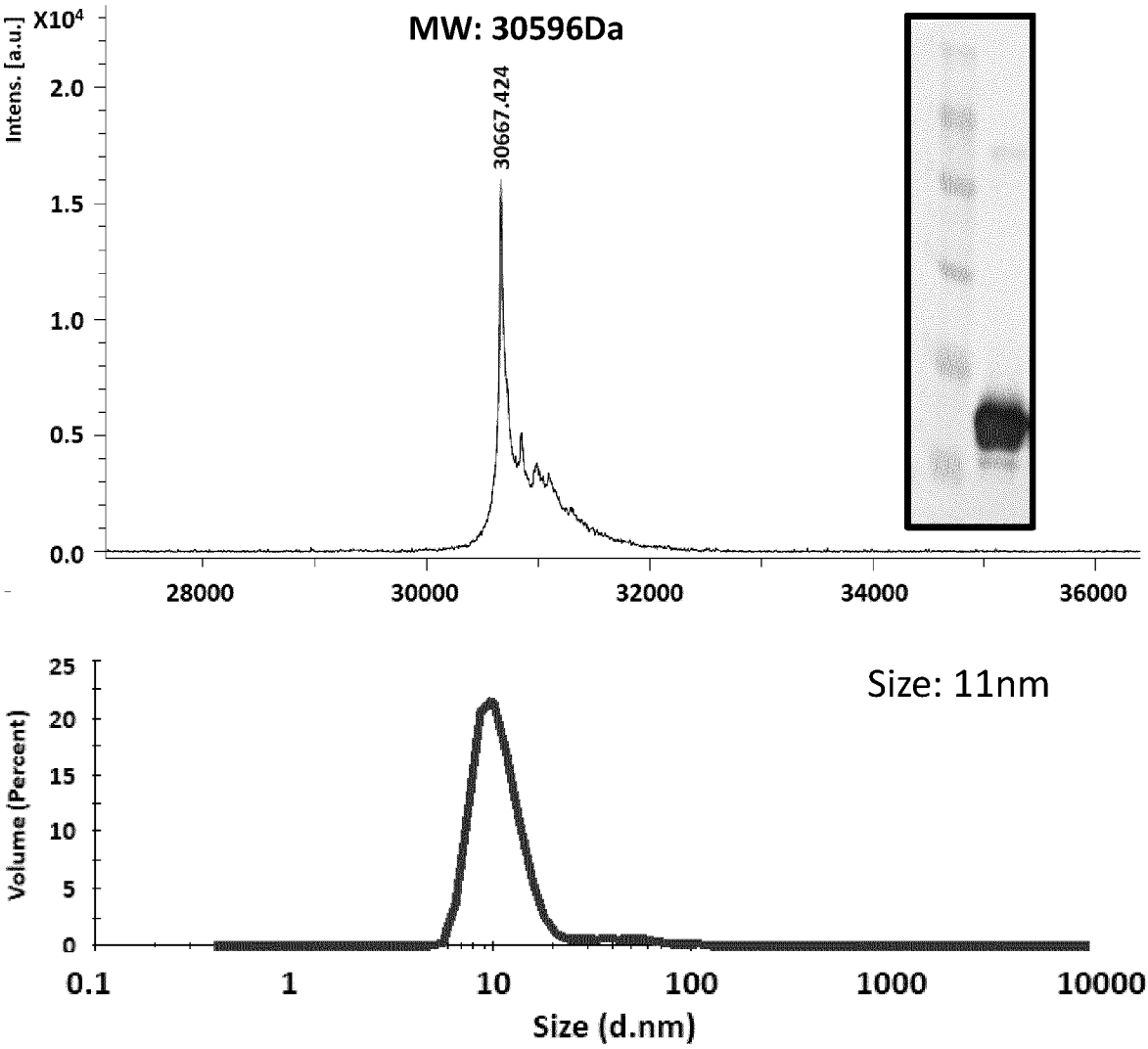


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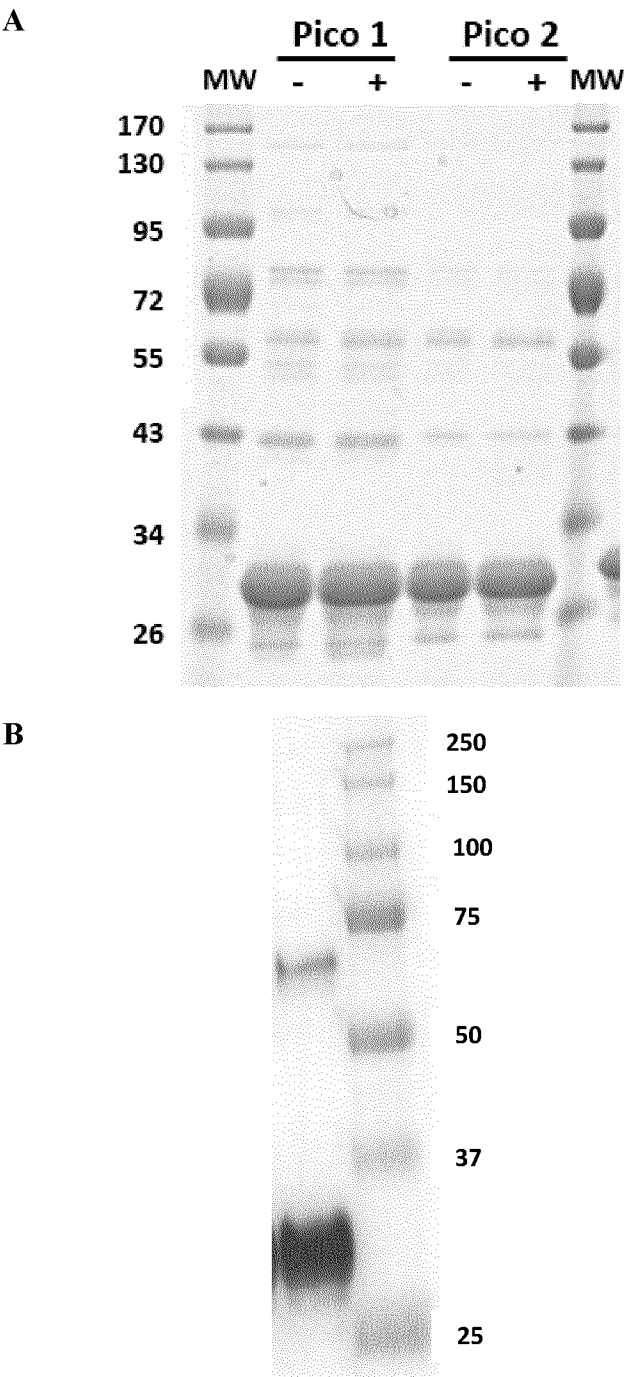
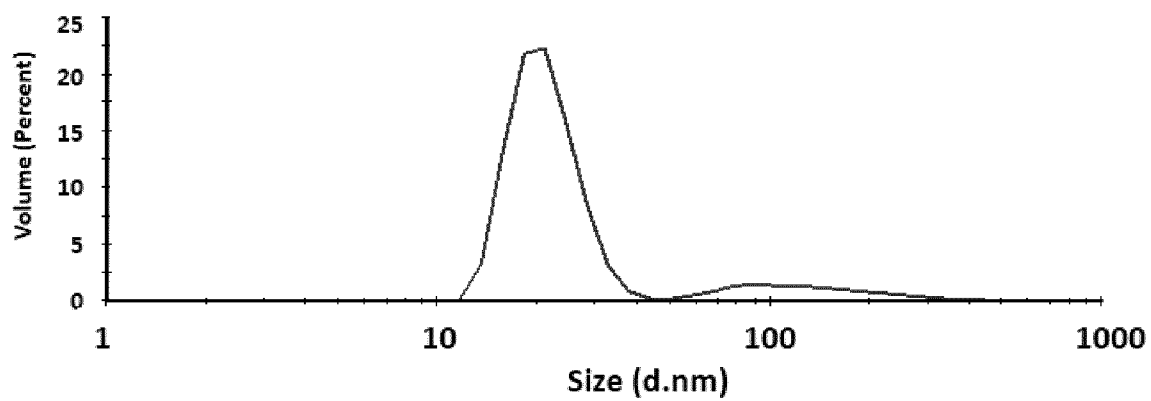


Fig. 3

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C



D

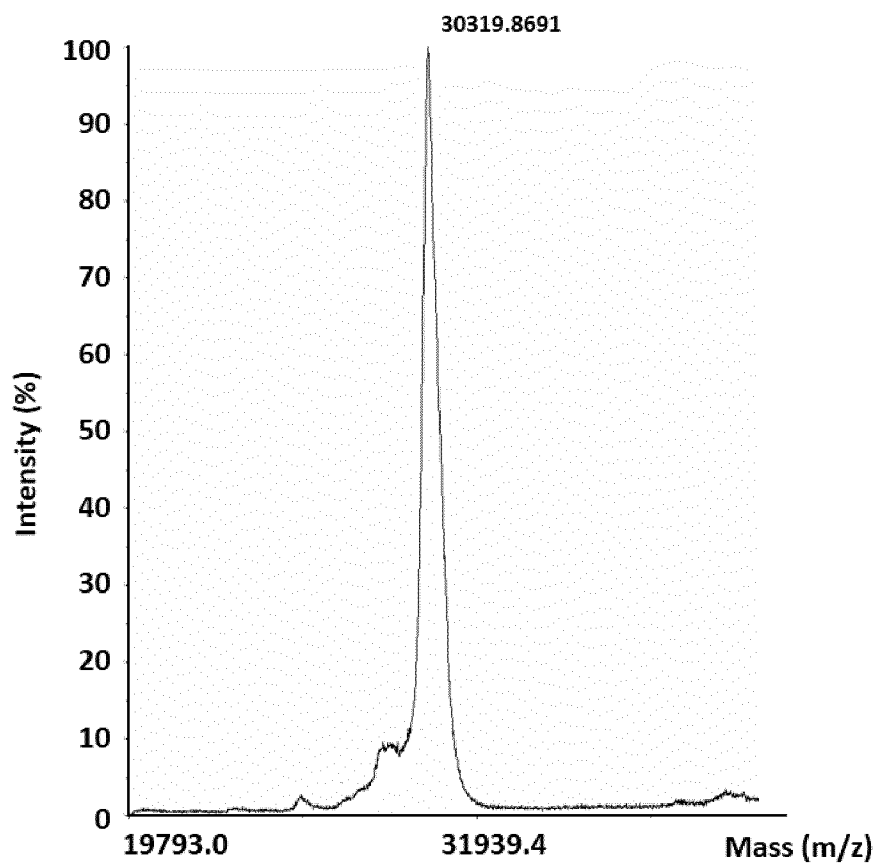
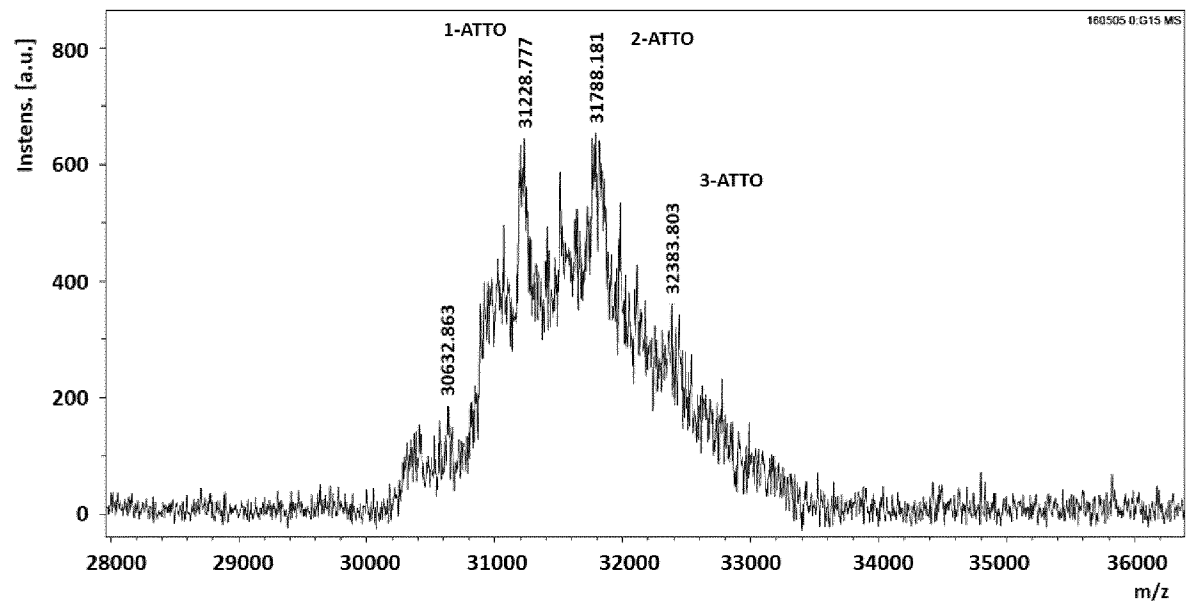


Fig. 3 (cont.)

A



B

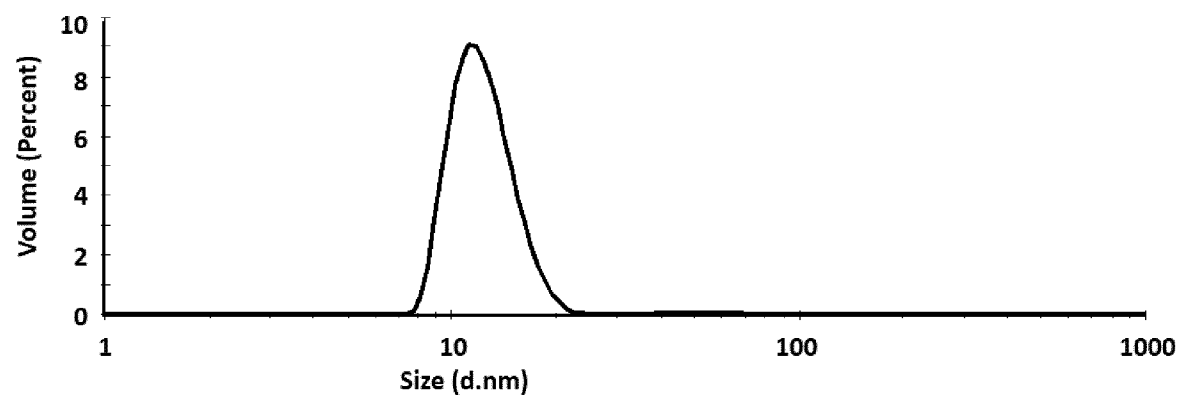
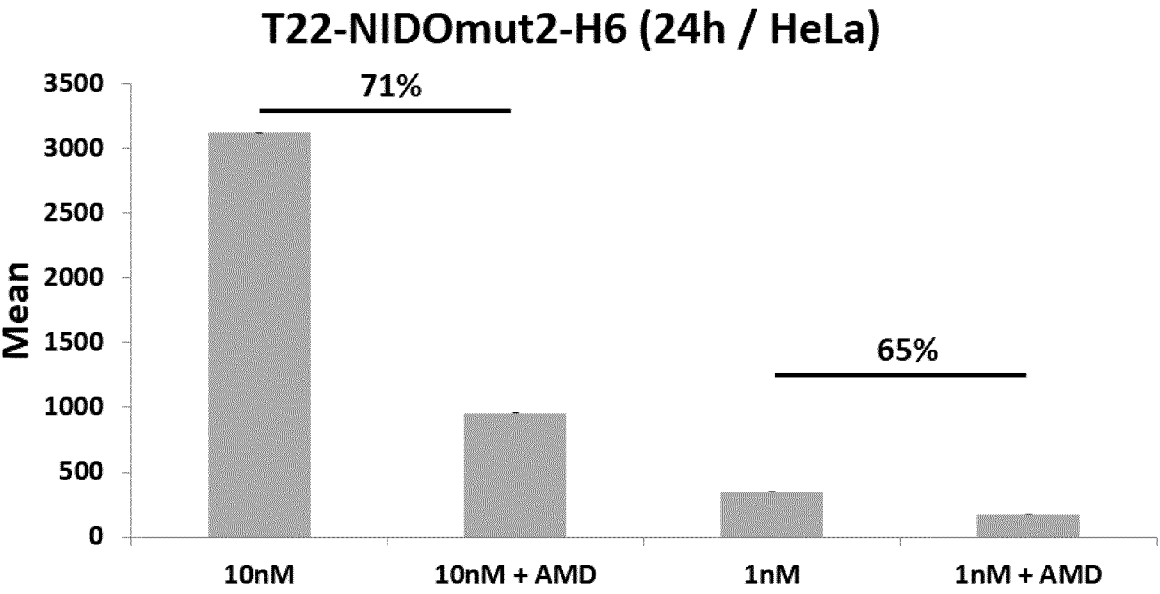


Fig. 4

A



B

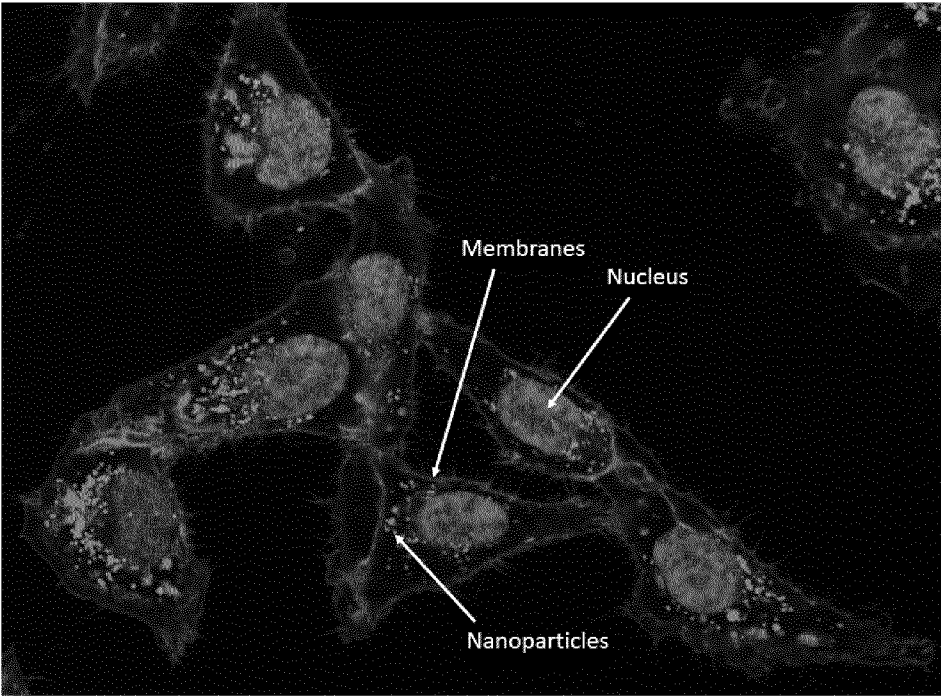


Fig. 5

A

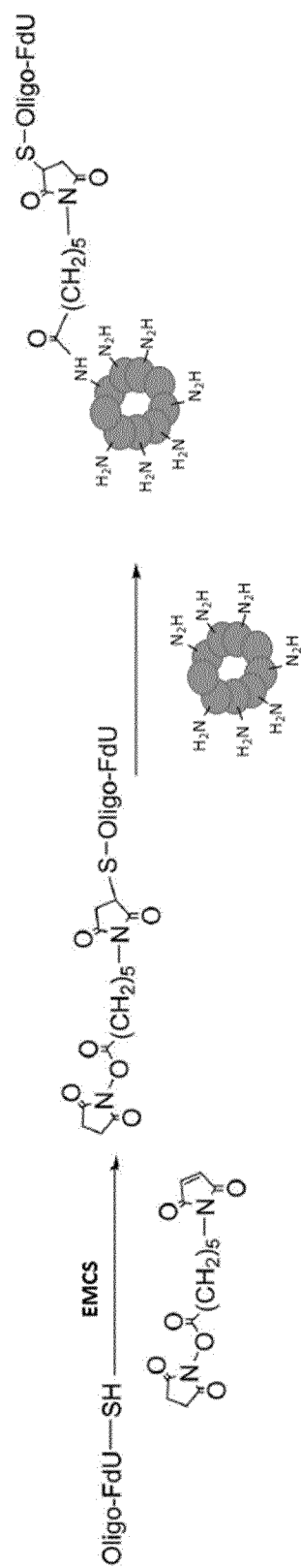


Fig. 6

B

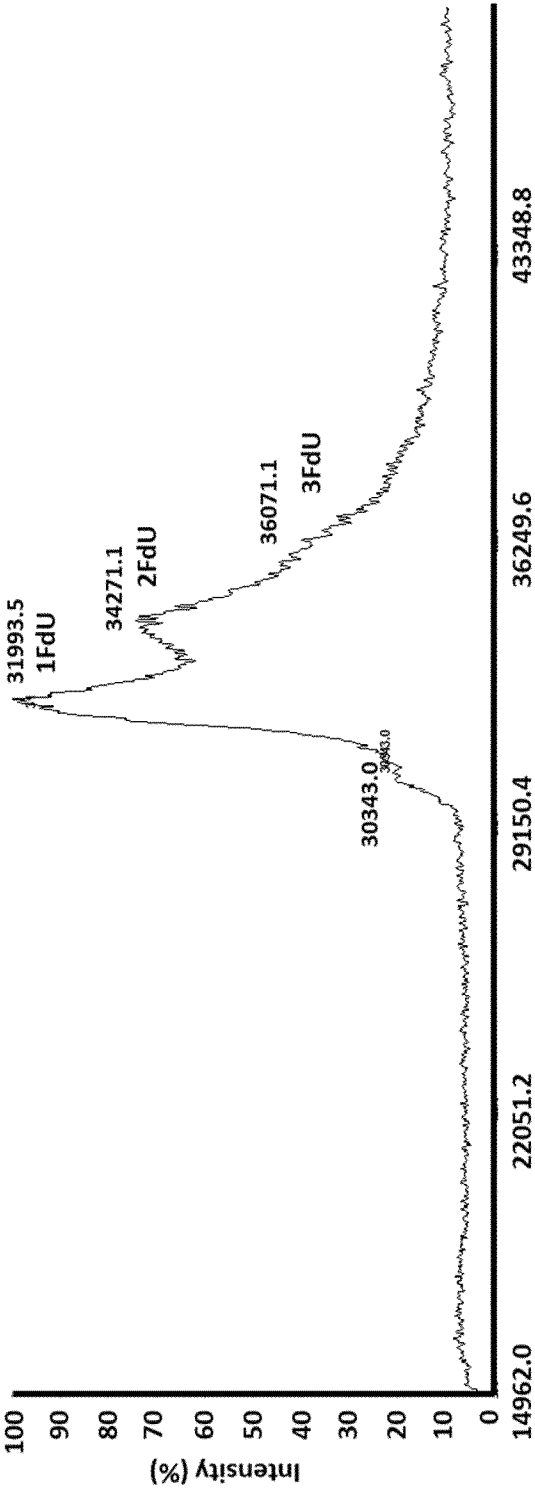


Fig. 6 (cont.)

C

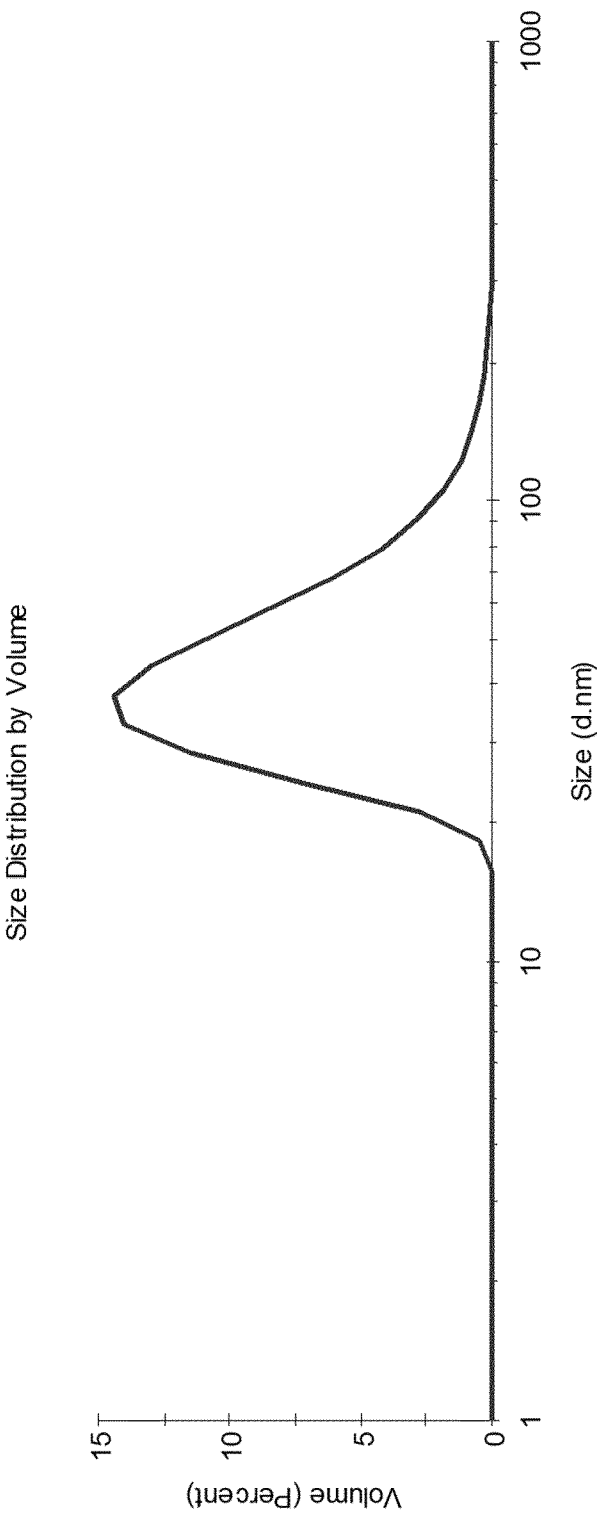


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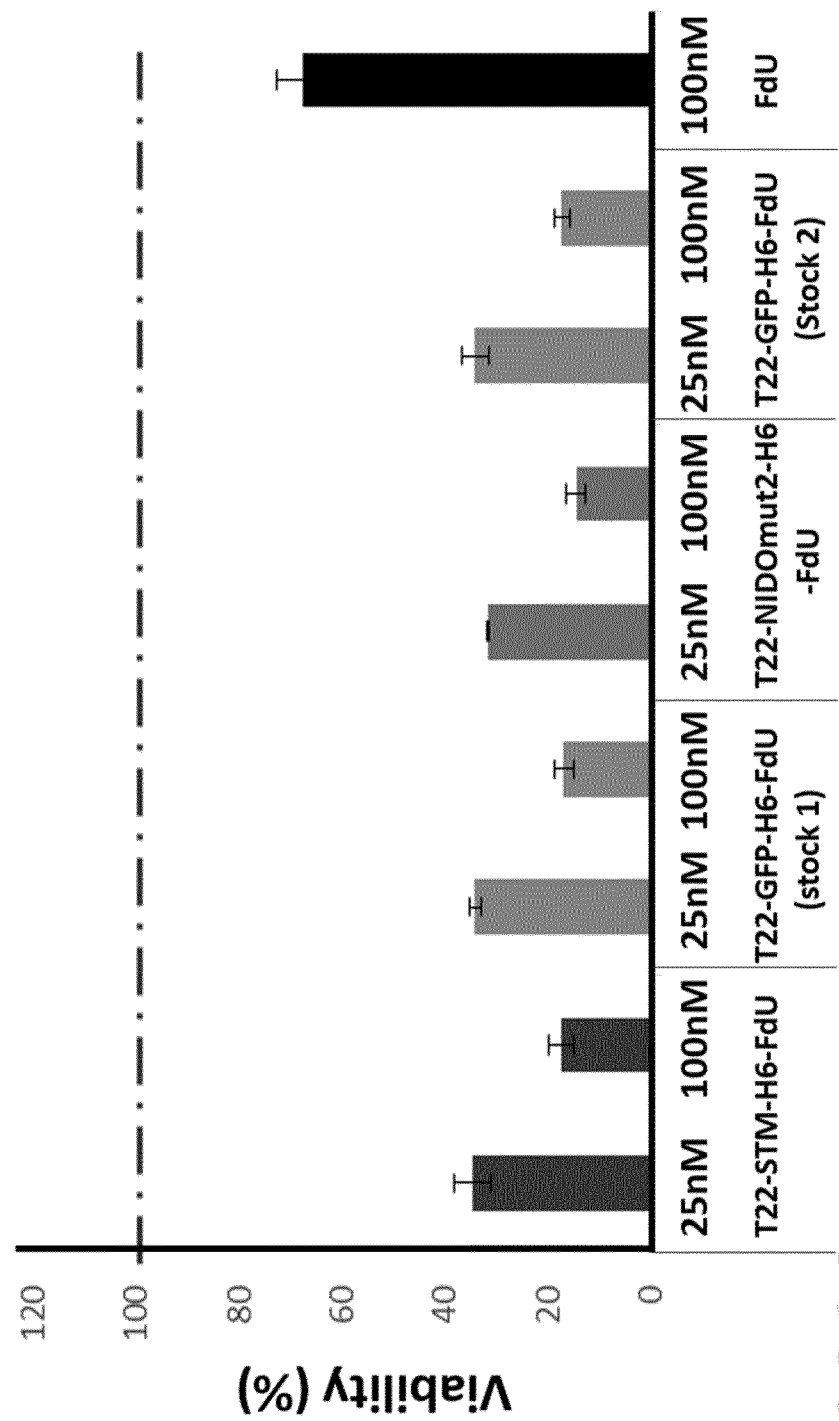


Fig. 7

Antitumoral effect in a SC M5 NSG model in a repeated dose regime
(20 µg q3d x5 doses)

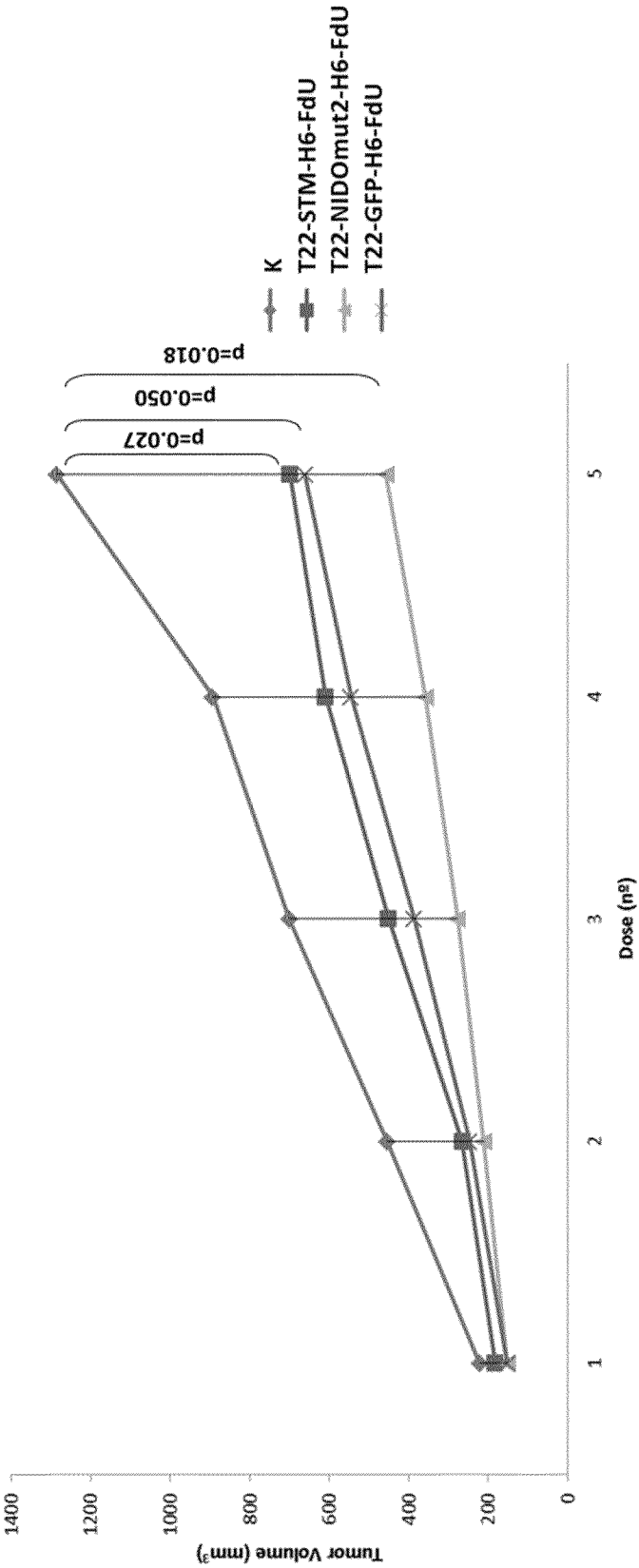


Fig. 8

A

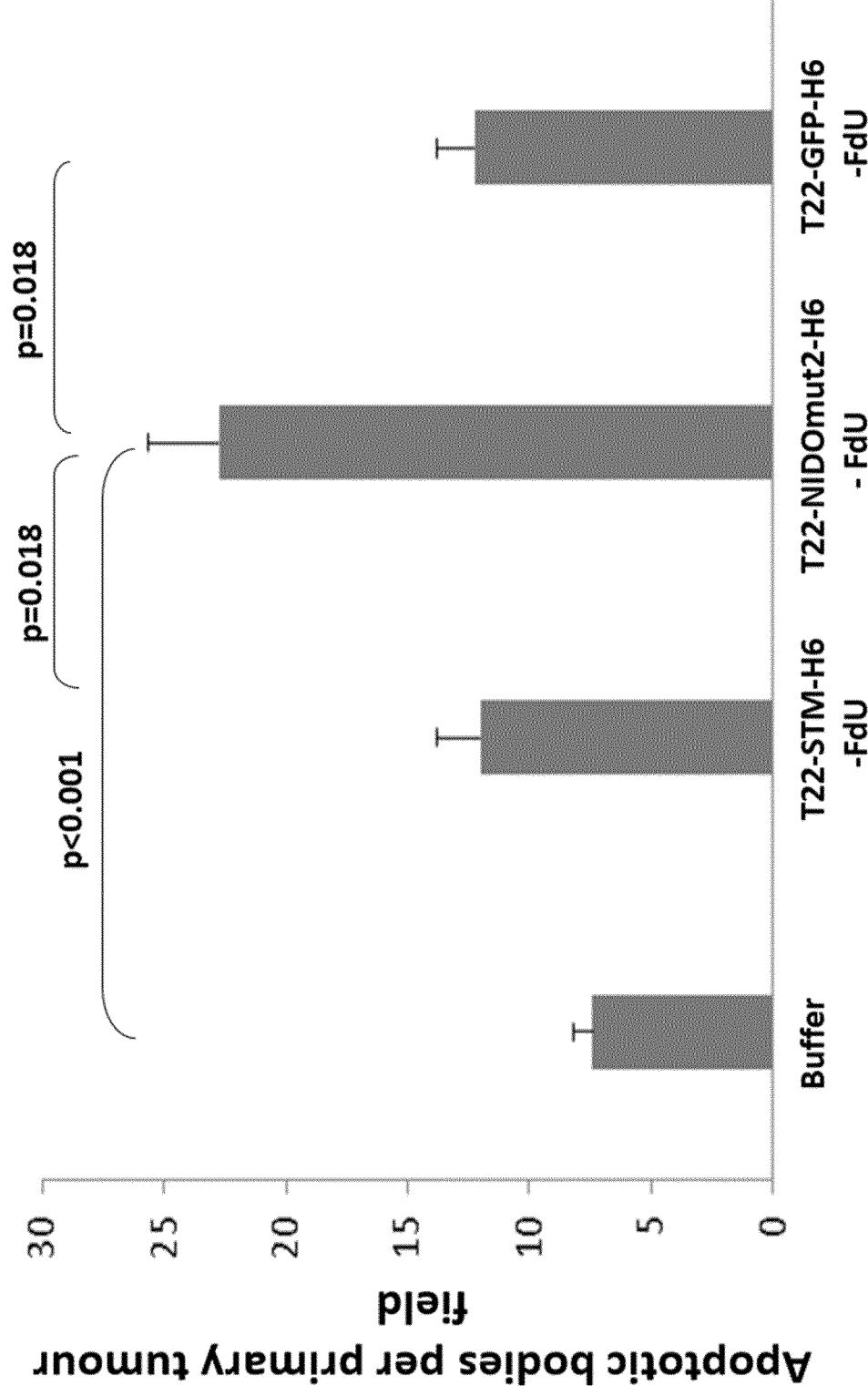


Fig. 9

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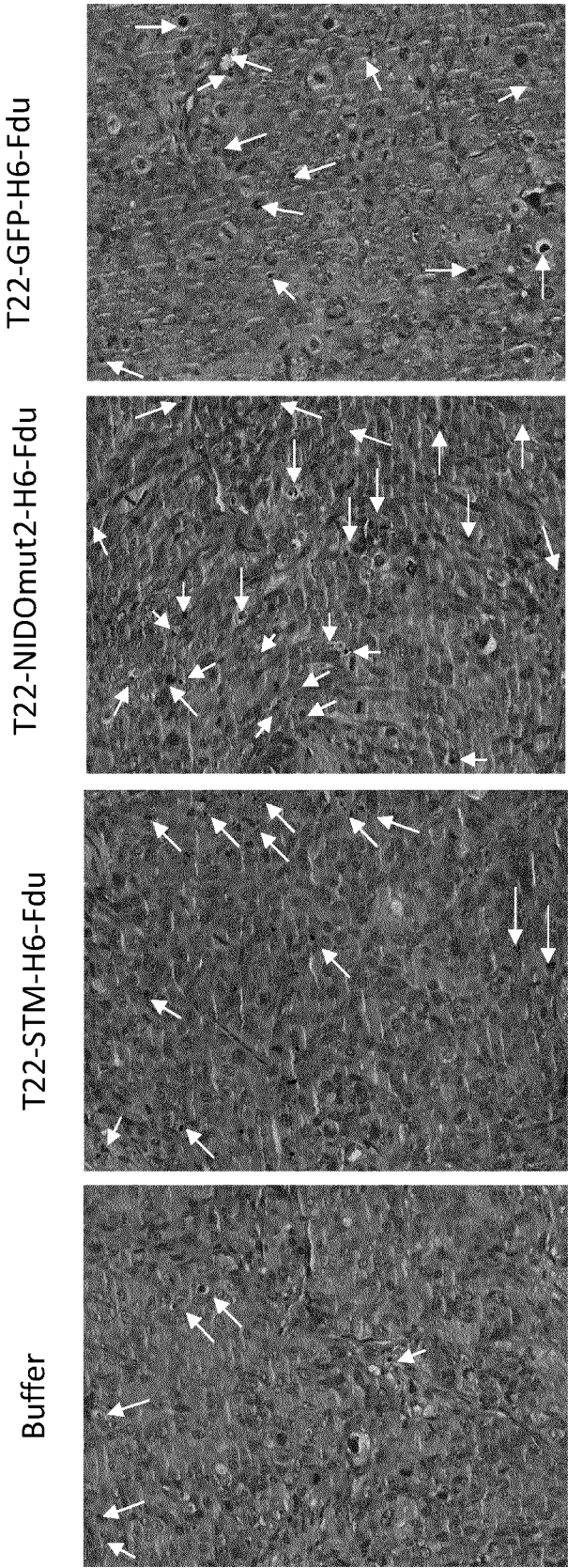
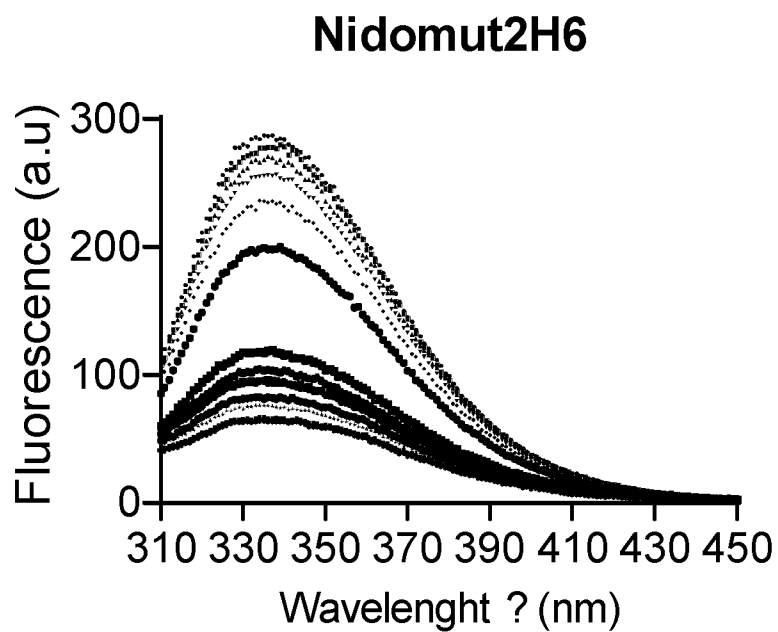


Fig. 9 (cont.)

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A



B

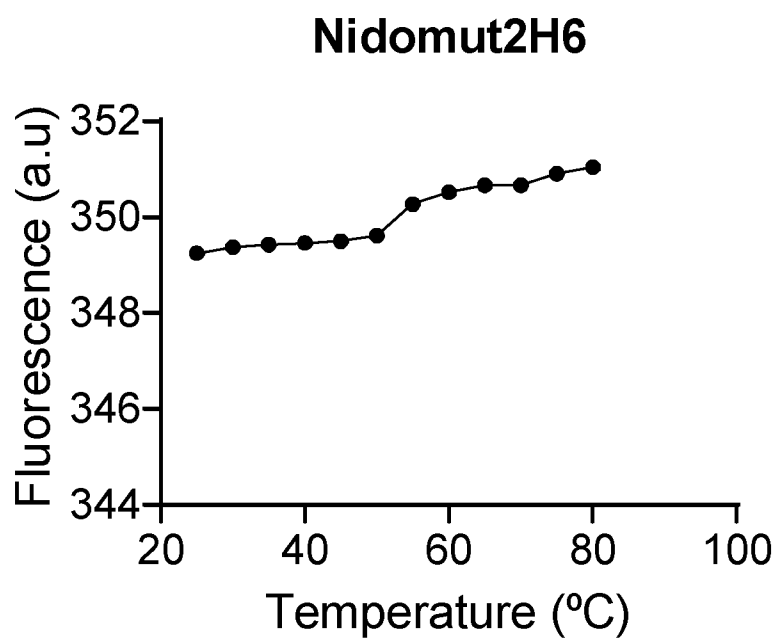
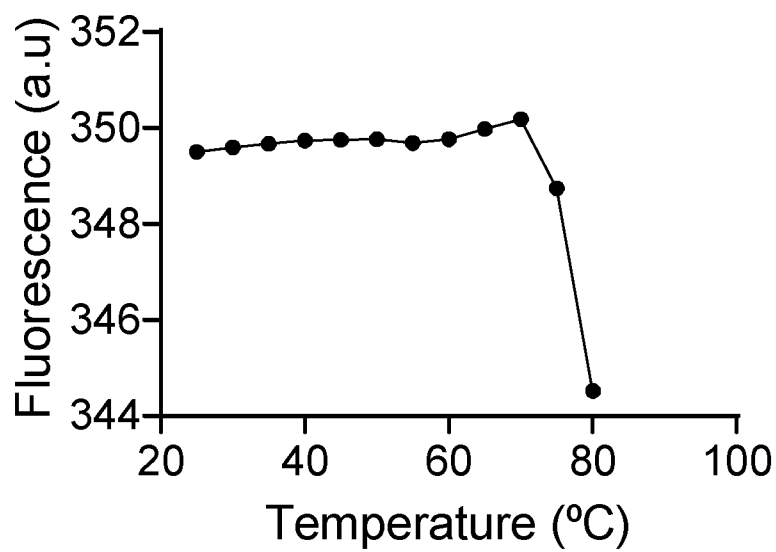


Fig. 11

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C

NidoWTH6

D

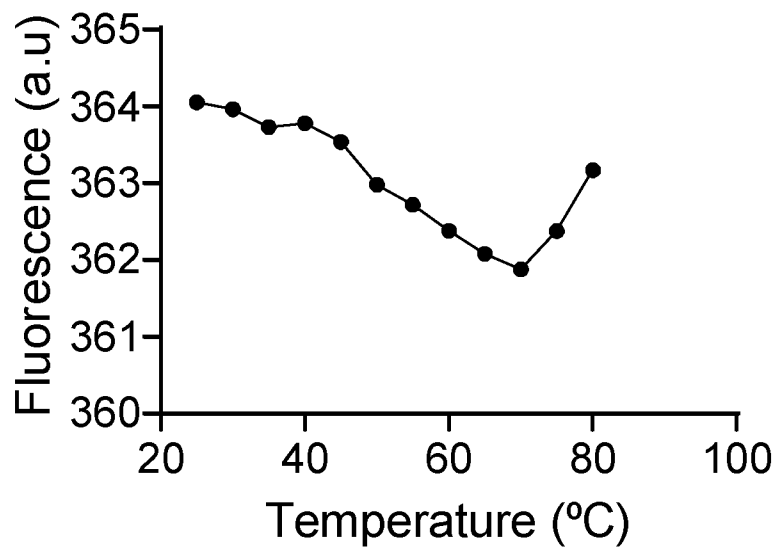
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Fig. 11 (cont.)

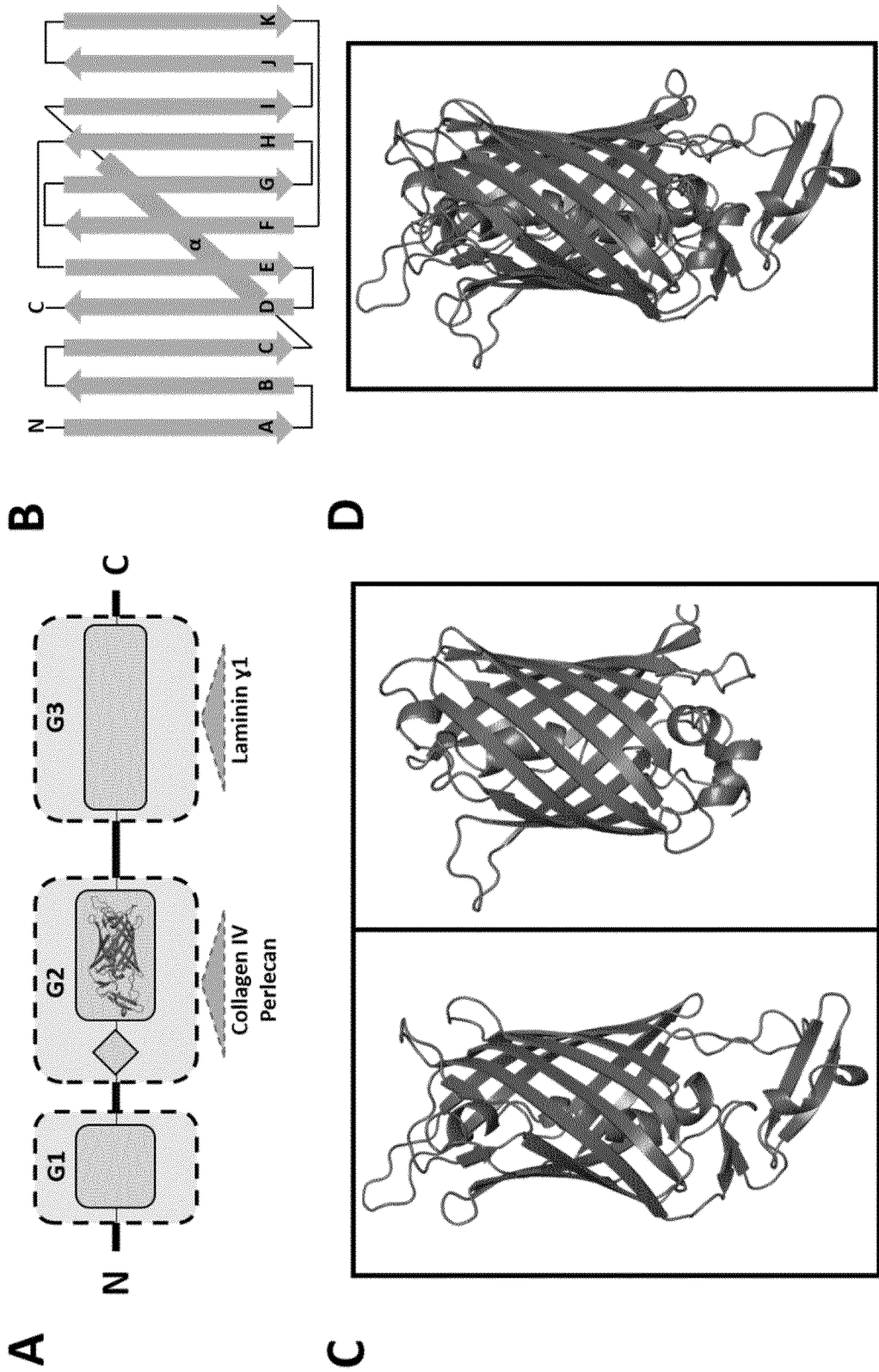


Fig. 12

[illegible]

Fig. 13A

Human Nidogen (P14543):

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 NGGCTHLCLATPGSRTCRCPDNTLGVDCIEQK

NIDOMut2:

SPQRVNGKVKGRIFFVGSSQVPIVFENTDL**A**SYVVMNHG**N**SYTAISTIPETVGYSLPLAPVGGIIGWMFAVEQDGFKNFSGSITGGEFTRQAEVT
 FVGHGPNLVKQRFSGIDEHGHILTIDTELEGRVPQIPFGSSVHIEPYTELYHYSTSVITSSSTREYTVTEPERDGAASPRIITYQWRQTITFQECVH
 DDSRPALPSTQQLSVDSV**SV**LYNQEEKIL**A**YALSNSIGPVREGSPDA

T22-NIDOMut2-H6:

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Fig. 13B

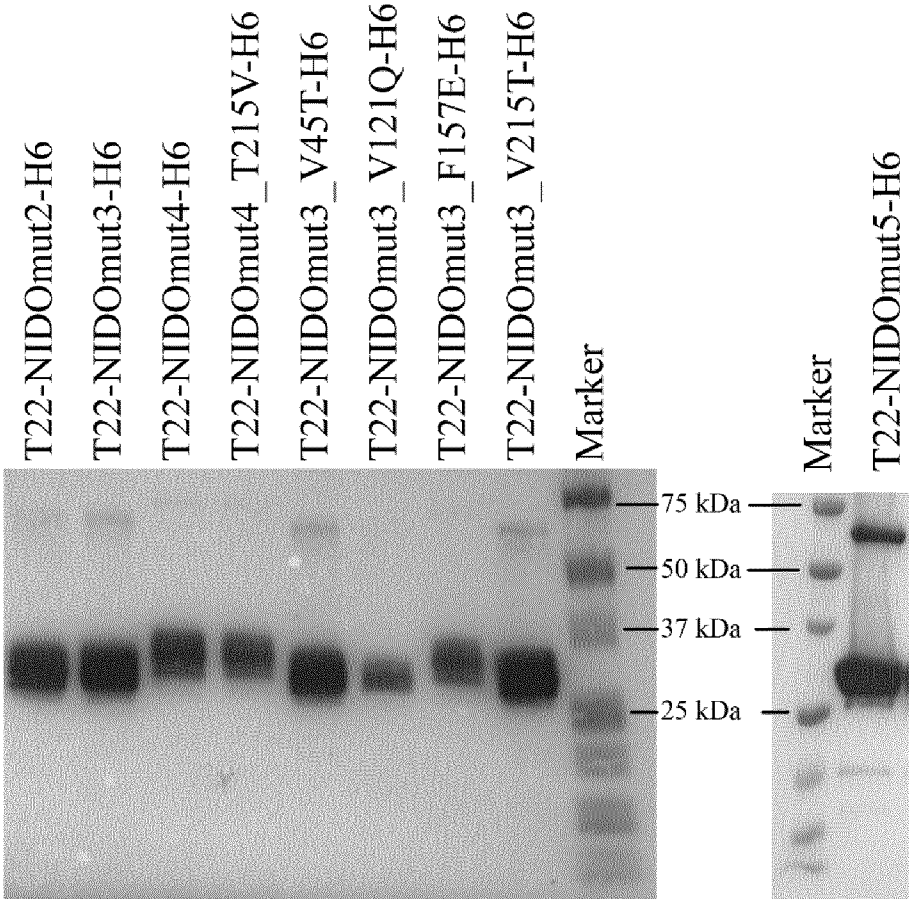


Fig. 14

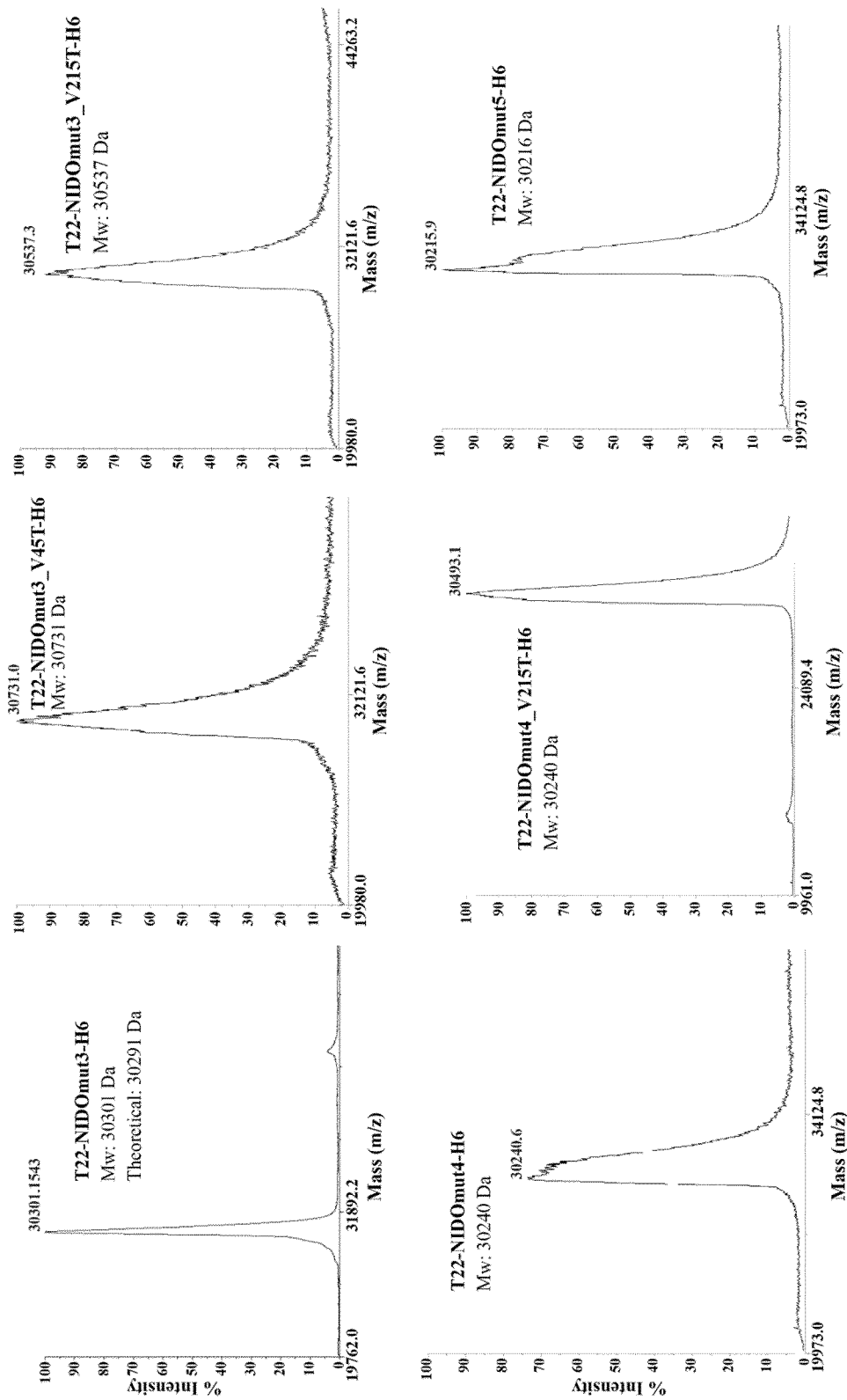


Fig. 15

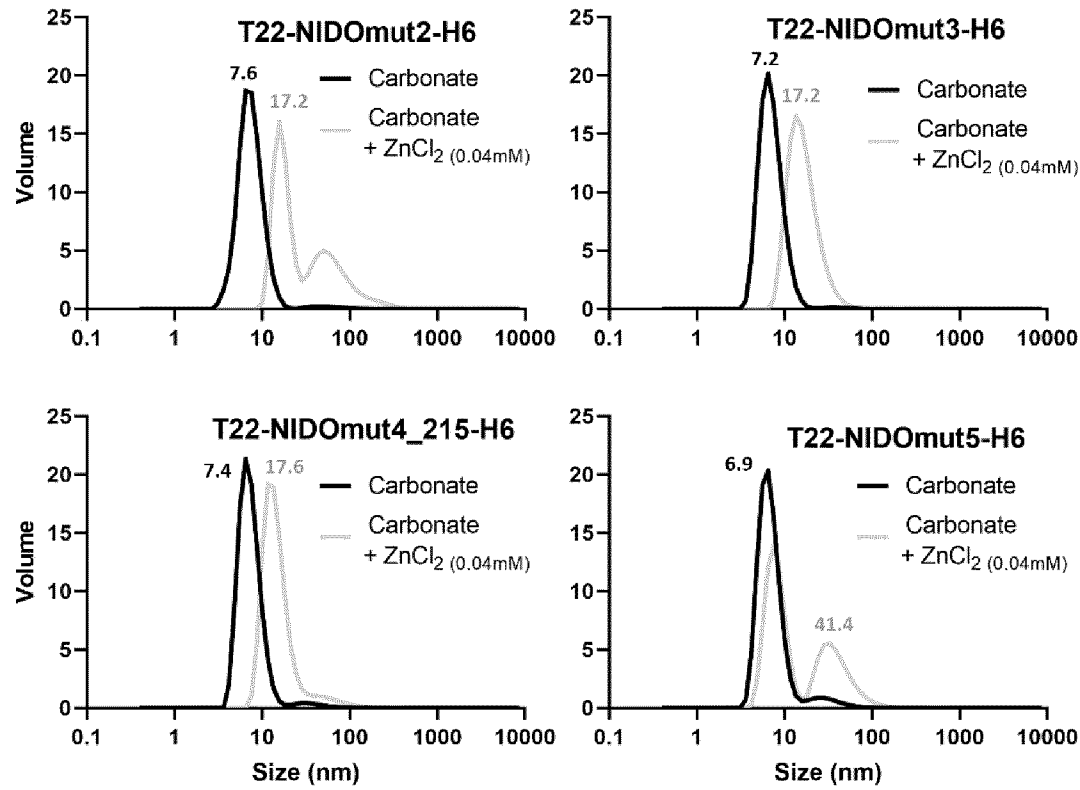


Fig. 16.

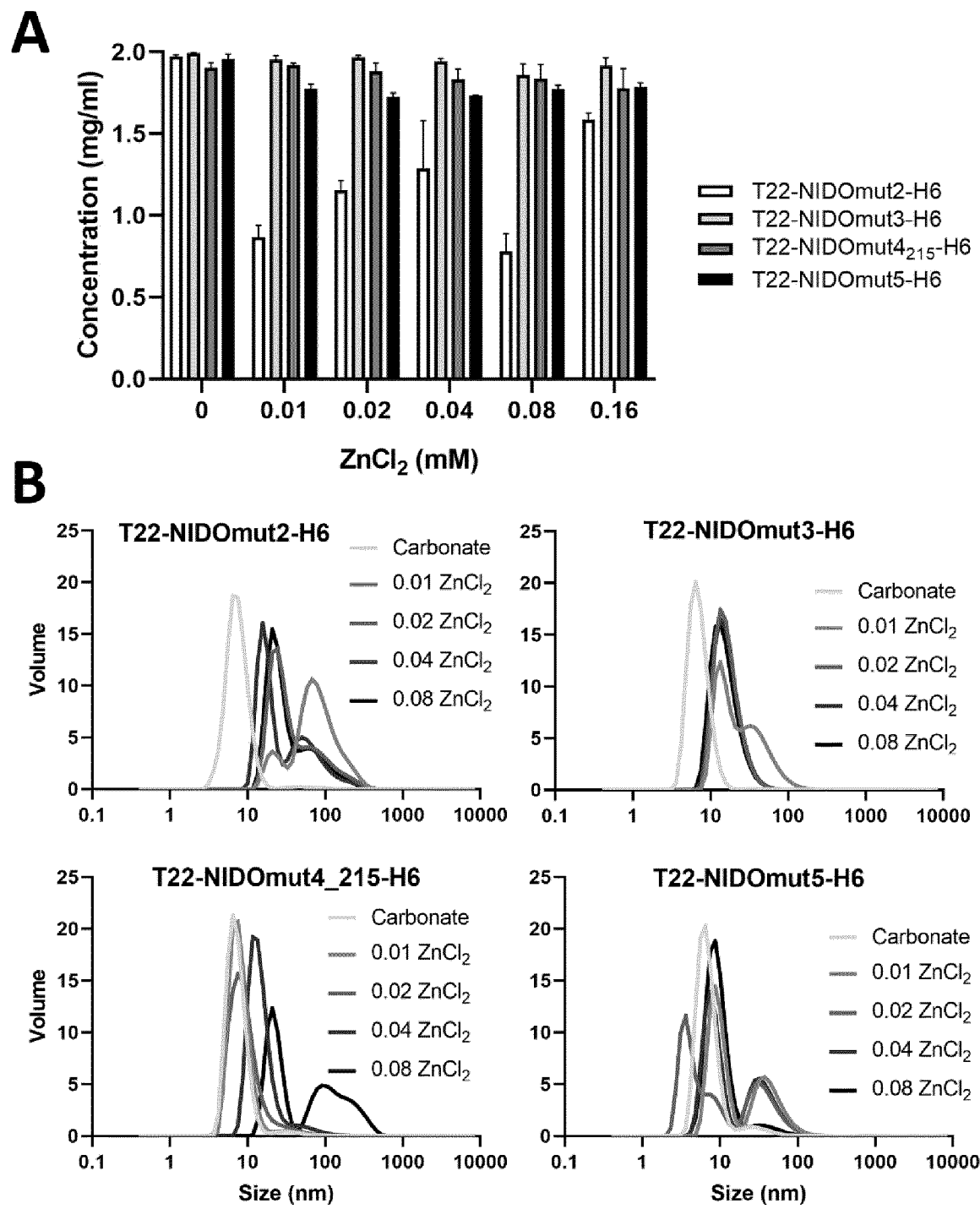


Fig. 17

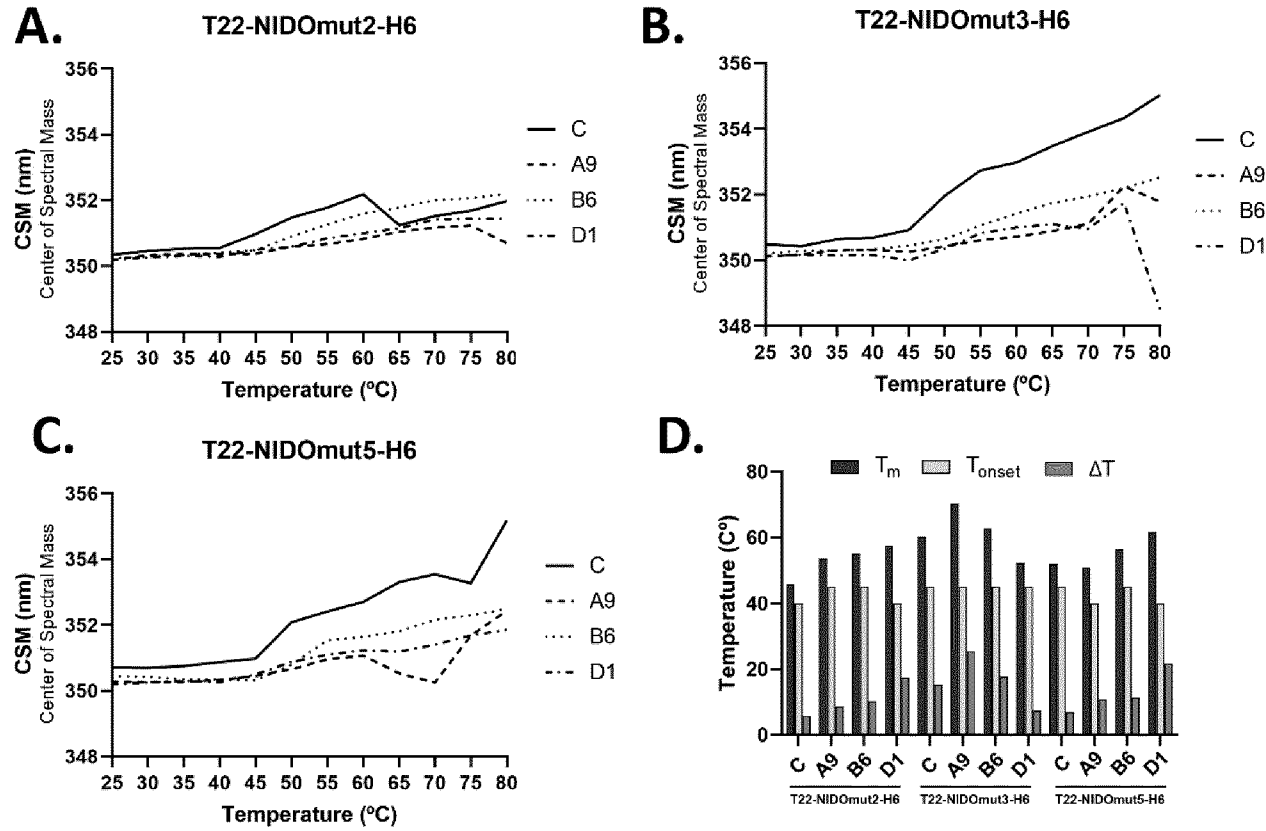


Fig. 18

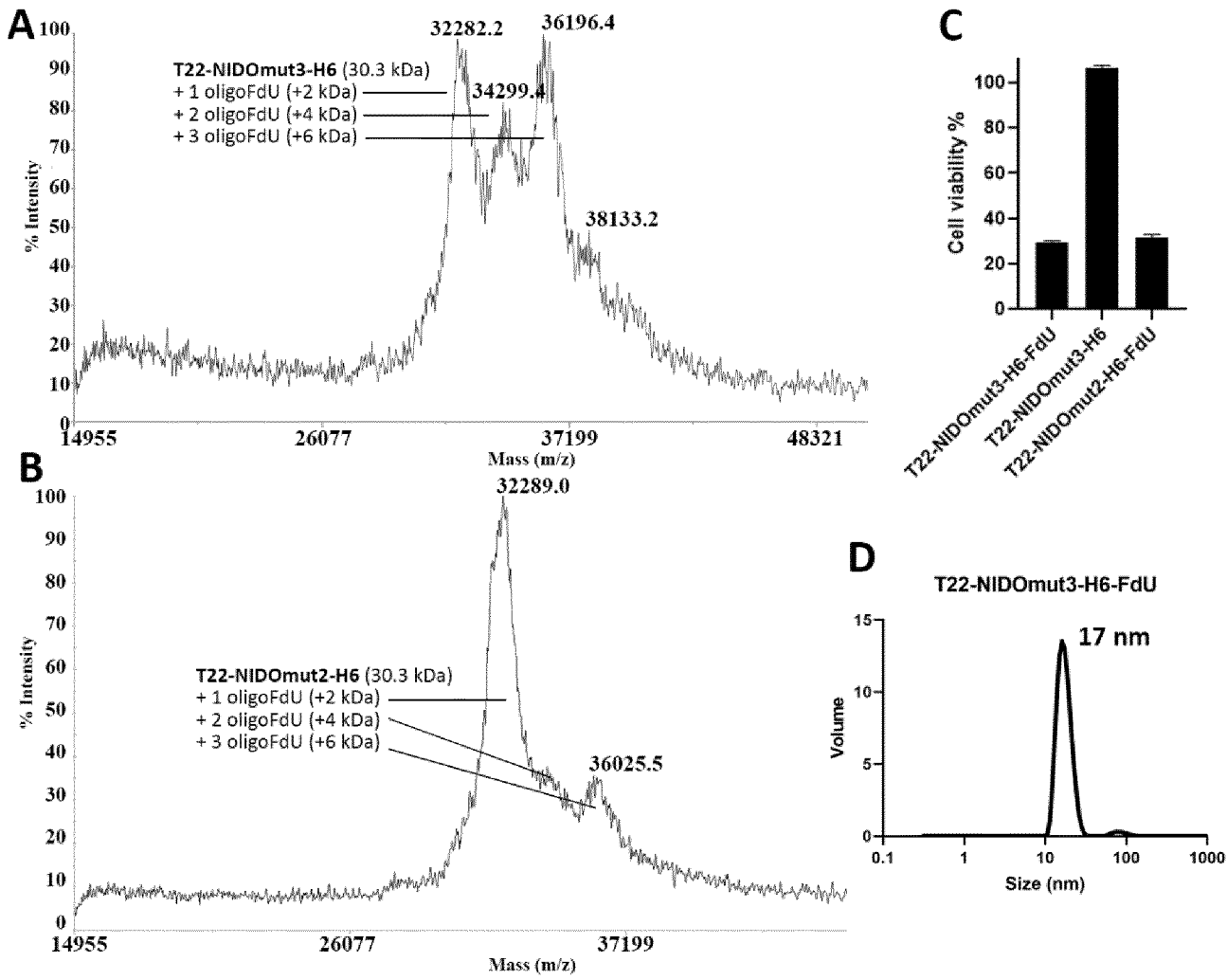


Fig. 19.

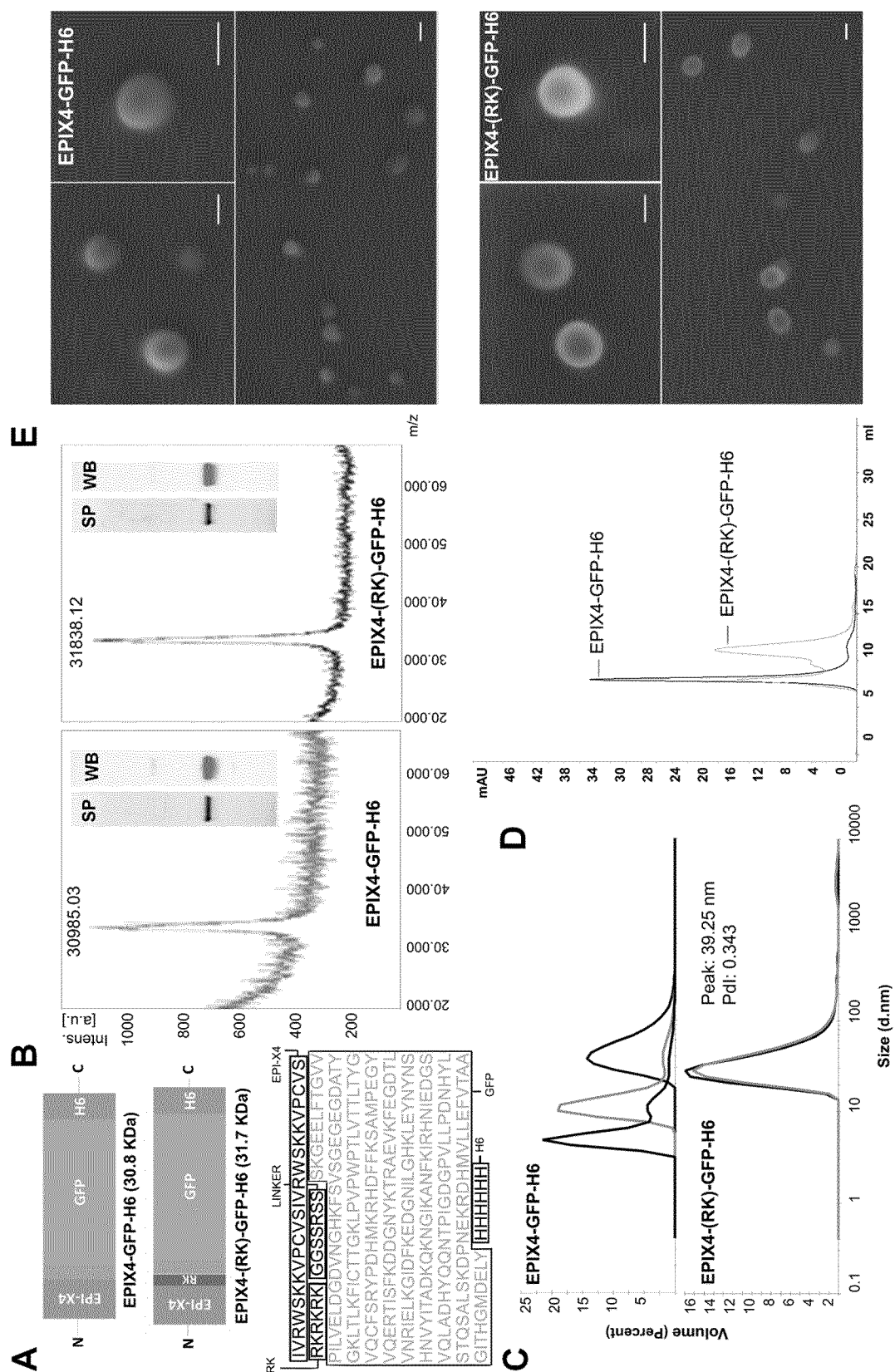


Fig. 20.

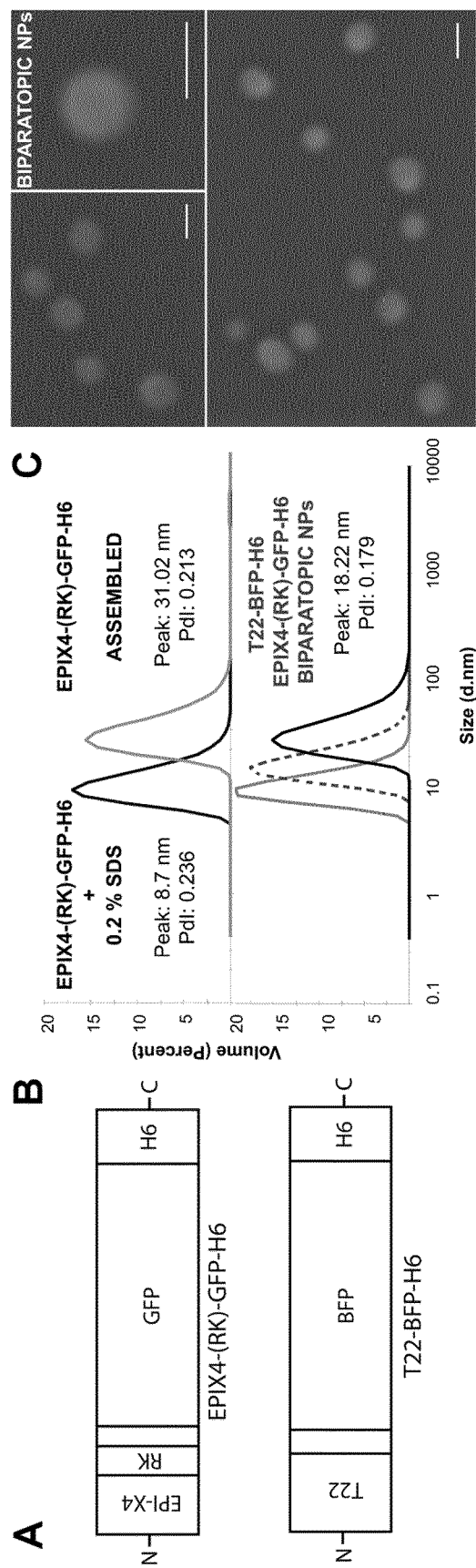


Fig. 21

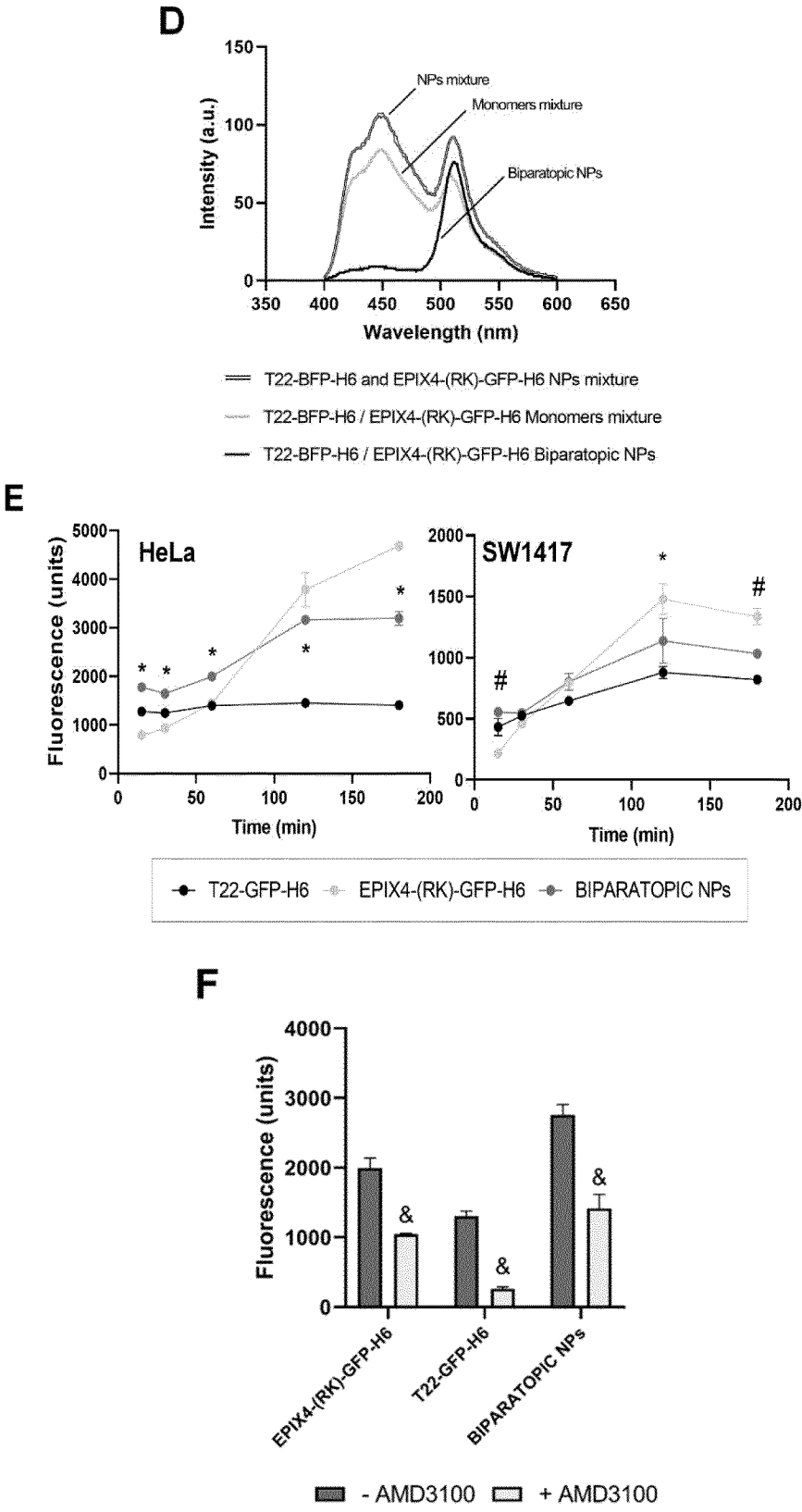


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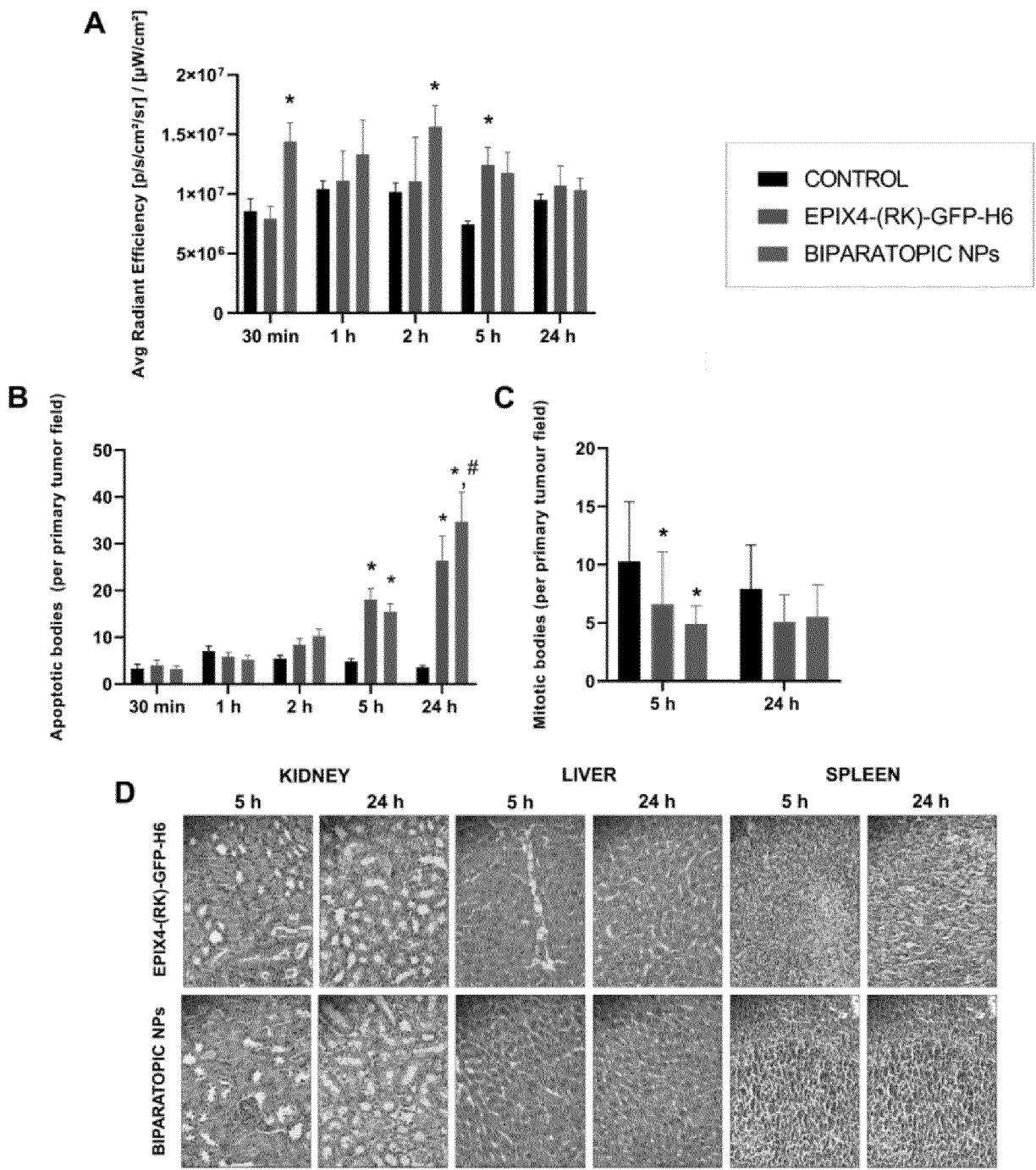


Fig. 22

SEQUENCE LISTING

<110> UNIVERSITAT AUTNOMA DE BARCELONA
FUNDACI INSTITUT DE RECERCA DE L' HOSPITAL DE LA SANTA
CREU I SANT PAU
CONSORCIO CENTRO DE INVESTIGACIN BIOMDICA EN RED, M.P.
NANOLIGENT S.L.

<120> SCAFFOLD PROTEINS AND THERAPEUTIC NANOCONJUGATES BASED ON NIDOGEN

<130> P18306PC00

<150> EP19383201

<151> 2020-01-14

<160> 133

<170> PatentIn version 3.5

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Thr Phe Phe Tyr Gly Gly Ser Arg Gly Lys Arg Asn Asn Phe Lys Thr
1 5 10 15

Glu Glu Tyr Arg Lys Arg
20

<210> 39

<211> 7

<212> PRT

<213> Artificial

<220>

<223> Linker

<400> 39

Gly Gly Ser Ser Arg Ser Ser
1 5

<210> 40

<211> 5

<212> PRT

<213> Artificial

<220>

<223> Linker

<400> 40

Gly Gly Gly Asn Ser
1 5

<210> 41

<211> 14

<212> PRT

<213> Artificial

<220>

<223> T140 peptide

<220>

<221> Xaa is L-3-(2-naphthyl)alanine

<222> (3)..(3)

<220>

<221> Xaa is D-Lys

<222> (8)..(8)

<220>

<221> Xaa is L-Citrulline

<222> (12)..(12)

<400> 41

Arg Arg Xaa Cys Tyr Arg Lys Xaa Pro Tyr Arg Xaa Cys Arg
1 5 10

<210> 42

<211> 14

<212> PRT

<213> Artificial

<220>

<223> TN14003 peptide

<220>

<221> Xaa is L-3-(2-naphthyl)alanine

<222> (3)..(3)

<220>

<221> Xaa is L-Citrulline

<222> (6)..(6)

<220>

<221> Xaa is D-Lys

<222> (8)..(8)

<220>

<221> Xaa is L-Citrulline

<222> (12)..(12)

<400> 42

Arg Arg Xaa Cys Tyr Xaa Lys Xaa Pro Tyr Arg Xaa Cys Arg
1 5 10

<210> 43

<211> 14

<212> PRT

<213> Artificial

<220>

<223> TC14012 peptide

<220>

<221> Xaa is L-3-(2-naphthyl)alanine

<222> (3)..(3)

<220>

<221> Xaa is D-Citrulline

<222> (8)..(8)

<220>

<221> Xaa is L-Citrulline

<222> (12)..(12)

<400> 43

Arg Arg Xaa Cys Tyr Glu Lys Xaa Pro Tyr Arg Xaa Cys Arg
1 5 10

<210> 44

<211> 14

<212> PRT

<213> Artificial

<220>

<223> TE14011 peptide

<220>

<221> Xaa is L-3-(2-naphthyl)alanine

<222> (3)..(3)

<220>

<221> Xaa is L-Citrulline
<222> (6)..(6)

<220>
<221> Xaa is D-Glutamic acid
<222> (8)..(8)

<220>
<221> Xaa is L-Citrulline
<222> (12)..(12)

<400> 44

Arg	Arg	Xaa	Cys	Tyr	Xaa	Lys	Xaa	Pro	Tyr	Arg	Xaa	Cys	Arg
1				5					10				

<210> 45
<211> 14
<212> PRT
<213> Artificial

<220>
<223> TZ14011 peptide

<220>
<221> Xaa is L-3-(2)-naphthyl)alanine
<222> (3)..(3)

<220>
<221> Xaa is L-Citrulline
<222> (6)..(6)

<220>
<221> Xaa is D-Lysine
<222> (8)..(8)

<220>
<221> Xaa is L-Citrulline
<222> (12)..(12)

<400> 45

Arg	Arg	Xaa	Cys	Tyr	Xaa	Lys	Xaa	Pro	Tyr	Arg	Xaa	Cys	Arg
1				5					10				

<210> 46
<211> 20
<212> PRT
<213> Artificial

<220>
<223> GW-H1 peptide

<400> 46

Gly	Tyr	Asn	Tyr	Ala	Lys	Lys	Leu	Ala	Asn	Leu	Ala	Lys	Lys	Phe	Ala
1				5					10					15	

Asn	Ala	Leu	Trp
		20	

<210> 47
<211> 31
<212> PRT

<213> Artificial

<220>

<223> Nucleolin binding peptide

<400> 47

Lys Asp Glu Pro Gln Arg Arg Ser Ala Arg Leu Ser Ala Lys Pro Ala
1 5 10 15

Pro Pro Lys Pro Glu Pro Lys Pro Lys Lys Ala Pro Ala Lys Lys
20 25 30

<210> 48

<211> 39

<212> PRT

<213> Artificial

<220>

<223> Nucleolin-binding peptide

<400> 48

Lys Asp Glu Pro Gln Arg Arg Ser Ala Arg Leu Ser Ala Lys Pro Ala
1 5 10 15

Pro Pro Lys Pro Glu Pro Lys Pro Lys Lys Ala Pro Ala Lys Lys Arg
20 25 30

Lys Arg Lys Arg Lys Arg Lys
35

<210> 49

<211> 14

<212> PRT

<213> Artificial

<220>

<223> Linker region

<400> 49

Ser Gly Gly Thr Ser Gly Ser Thr Ser Gly Thr Gly Ser Thr
1 5 10

<210> 50

<211> 15

<212> PRT

<213> Artificial

<220>

<223> Linker region

<400> 50

Ala Gly Ser Ser Thr Gly Ser Ser Thr Gly Pro Gly Ser Thr Thr
1 5 10 15

<210> 51

<211> 7

<212> PRT

<213> Artificial

<220>
<223> Linker region

<400> 51

Gly Gly Ser Gly Gly Ala Pro
1 5

<210> 52
<211> 8
<212> PRT
<213> Artificial

<220>
<223> Linker region

<400> 52

Gly Gly Gly Val Glu Gly Gly Gly
1 5

<210> 53
<211> 10
<212> PRT
<213> Artificial

<220>
<223> Linker region

<400> 53

Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser
1 5 10

<210> 54
<211> 11
<212> PRT
<213> Artificial

<220>
<223> Linker region

<400> 54

Ala Pro Ala Glu Thr Lys Ala Glu Pro Met Thr
1 5 10

<210> 55
<211> 5
<212> PRT
<213> Artificial

<220>
<223> Cleavage site

<400> 55

Ala Ala Ala Leu Glu
1 5

<210> 56
<211> 5
<212> PRT

<213> Artificial

<220>

<223> Cleavage site

<400> 56

Asp Asp Asp Asp Lys
1 5

<210> 57

<211> 5

<212> PRT

<213> Artificial

<220>

<223> Cleavage site

<400> 57

Ile Glu Asp Gly Arg
1 5

<210> 58

<211> 6

<212> PRT

<213> Artificial

<220>

<223> Cleavage site

<400> 58

Leu Val Pro Arg Gly Ser
1 5

<210> 59

<211> 7

<212> PRT

<213> Artificial

<220>

<223> Cleavage site

<400> 59

Glu Asn Leu Tyr Phe Gln Gly
1 5

<210> 60

<211> 8

<212> PRT

<213> Artificial

<220>

<223> Cleavage site

<400> 60

Leu Glu Val Leu Phe Gln Gly Pro
1 5

<210> 61

<211> 270

<212> PRT
<213> Artificial

<220>
<223> T22-NidMut-H6

<400> 61

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu His Gly His Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val

245

250

255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
 260 265 270

<210> 62

<211> 238

<212> PRT

<213> Artificial

<220>

<223> Nidogen G2 domain

<400> 62

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
 1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu His Ser Tyr
 20 25 30

Val Val Met Asn His Gly Arg Ser Tyr Thr Ala Ile Ser Thr Ile Pro
 35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
 50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
 65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
 85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
 100 105 110

Glu His Gly His Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
 115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
 130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
 145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
 165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
 180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
 195 200 205

Val Phe Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Arg Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 63
<211> 236
<212> PRT
<213> Artificial

<220>
<223> Human nidogen G2 domain (truncated)

<400> 63

Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser
1 5 10 15

Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu His Ser Tyr Val Val
20 25 30

Met Asn His Gly Arg Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr
35 40 45

Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly
50 55 60

Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile
65 70 75 80

Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val Gly His
85 90 95

Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu His
100 105 110

Gly His Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile
115 120 125

Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His
130 135 140

Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val
145 150 155 160

Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr
165 170 175

Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser
180 185 190

Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser Val Phe
195 200 205

Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Arg Tyr Ala Leu Ser Asn
210 215 220

Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 64
<211> 238
<212> PRT
<213> Artificial

<220>
<223> Human nidogen G2 domain with H459A., R468N, F639S and R650A
mutations

<400> 64

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu His Gly His Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser

195

200

205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
 210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
 225 230 235

<210> 65

<211> 236

<212> PRT

<213> Artificial

<220>

<223> Human nidogen G2 domain with H459A., R468N, F639S and R650A
 mutations (truncated)

<400> 65

Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser
 1 5 10 15

Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val
 20 25 30

Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr
 35 40 45

Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly
 50 55 60

Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile
 65 70 75 80

Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val Gly His
 85 90 95

Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu His
 100 105 110

Gly His Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile
 115 120 125

Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His
 130 135 140

Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val
 145 150 155 160

Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr
 165 170 175

Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser
 180 185 190

Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser
195 200 205

Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn
210 215 220

Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 66
<211> 270
<212> PRT
<213> Artificial

<220>
<223> T22-G2-H6

<400> 66

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu His Ser Tyr Val Val Met Asn His Gly Arg
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu His Gly His Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Phe Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Arg Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 67
<211> 210
<212> PRT
<213> Human

<400> 67

Met Ala Ser Gly Gln Gly Pro Gly Pro Pro Arg Gln Glu Cys Gly Glu
1 5 10 15

Pro Ala Leu Pro Ser Ala Ser Glu Glu Gln Val Ala Gln Asp Thr Glu
20 25 30

Glu Val Phe Arg Ser Tyr Val Phe Tyr Arg His Gln Gln Glu Gln Glu
35 40 45

Ala Glu Gly Val Ala Ala Pro Ala Asp Pro Glu Met Val Thr Leu Pro
50 55 60

Leu Gln Pro Ser Ser Thr Met Gly Gln Val Gly Arg Gln Leu Ala Ile
65 70 75 80

Ile Gly Asp Asp Ile Asn Arg Arg Tyr Asp Ser Glu Phe Gln Thr Met
85 90 95

Leu Gln His Leu Gln Pro Thr Ala Glu Asn Ala Tyr Glu Tyr Phe Thr
100 105 110

Lys Ile Ala Thr Ser Leu Phe Glu Ser Gly Ile Asn Trp Gly Arg Val
115 120 125

Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu Ala Leu His Val Tyr Gln
130 135 140

His Gly Leu Thr Gly Phe Leu Gly Gln Val Thr Arg Phe Val Val Asp
145 150 155 160

Phe Met Leu His His Cys Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly
165 170 175

Trp Val Ala Ala Leu Asn Leu Gly Asn Gly Pro Ile Leu Asn Val Leu
180 185 190

Val Val Leu Gly Val Val Leu Leu Gly Gln Phe Val Val Arg Arg Phe
195 200 205

Phe Lys
210

<210> 68
<211> 15
<212> PRT
<213> Artificial

<220>
<223> BH3 domain

<400> 68

Val Gly Arg Gln Leu Ala Ile Ile Gly Asp Asp Ile Asn Arg Arg
1 5 10 15

<210> 69
<211> 193
<212> PRT
<213> Human

<400> 69

Met Ala Arg Ala Arg Gln Glu Gly Ser Ser Pro Glu Pro Val Glu Gly
1 5 10 15

Leu Ala Arg Asp Gly Pro Arg Pro Phe Pro Leu Gly Arg Leu Val Pro
20 25 30

Ser Ala Val Ser Cys Gly Leu Cys Glu Pro Gly Leu Ala Ala Ala Pro
35 40 45

Ala Ala Pro Thr Leu Leu Pro Ala Ala Tyr Leu Cys Ala Pro Thr Ala
50 55 60

Pro Pro Ala Val Thr Ala Ala Leu Gly Gly Ser Arg Trp Pro Gly Gly
65 70 75 80

Pro Arg Ser Arg Pro Arg Gly Pro Arg Pro Asp Gly Pro Gln Pro Ser
85 90 95

Leu Ser Leu Ala Glu Gln His Leu Glu Ser Pro Val Pro Ser Ala Pro
100 105 110

Gly Ala Leu Ala Gly Gly Pro Thr Gln Ala Ala Pro Gly Val Arg Gly
115 120 125

Glu Glu Glu Gln Trp Ala Arg Glu Ile Gly Ala Gln Leu Arg Arg Met
130 135 140

Ala Asp Asp Leu Asn Ala Gln Tyr Glu Arg Arg Arg Gln Glu Glu Gln
145 150 155 160

Gln Arg His Arg Pro Ser Pro Trp Arg Val Leu Tyr Asn Leu Ile Met
165 170 175

Gly Leu Leu Pro Leu Pro Arg Gly His Arg Ala Pro Glu Met Glu Pro
180 185 190

Asn

<210> 70
<211> 187
<212> PRT
<213> Corynebacterium diphtheriae

<400> 70

Gly Ala Asp Asp Val Val Asp Ser Ser Lys Ser Phe Val Met Glu Asn
1 5 10 15

Phe Ser Ser Tyr His Gly Thr Lys Pro Gly Tyr Val Asp Ser Ile Gln
20 25 30

Lys Gly Ile Gln Lys Pro Lys Ser Gly Thr Gln Gly Asn Tyr Asp Asp
35 40 45

Asp Trp Lys Gly Phe Tyr Ser Thr Asp Asn Lys Tyr Asp Ala Ala Gly
50 55 60

Tyr Ser Val Asp Asn Glu Asn Pro Leu Ser Gly Lys Ala Gly Gly Val
65 70 75 80

Val Lys Val Thr Tyr Pro Gly Leu Thr Lys Val Leu Ala Leu Lys Val
85 90 95

Asp Asn Ala Glu Thr Ile Lys Lys Glu Leu Gly Leu Ser Leu Thr Glu
100 105 110

Pro Leu Met Glu Gln Val Gly Thr Glu Glu Phe Ile Lys Arg Phe Gly
115 120 125

Asp Gly Ala Ser Arg Val Val Leu Ser Leu Pro Phe Ala Glu Gly Ser
130 135 140

Ser Ser Val Glu Tyr Ile Asn Asn Trp Glu Gln Ala Lys Ala Leu Ser
145 150 155 160

Val Glu Leu Glu Ile Asn Phe Glu Thr Arg Gly Lys Arg Gly Gln Asp
165 170 175

Ala Met Tyr Glu Tyr Met Ala Gln Ala Cys Ala
180 185

<210> 71
<211> 209
<212> PRT
<213> Pseudomonas aeruginosa

<400> 71

Gly Asp Ile Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu
1 5 10 15

Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe
20 25 30

Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe
35 40 45

Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly
50 55 60

Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp
65 70 75 80

Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg
85 90 95

Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Gly Leu
100 105 110

Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly
115 120 125

His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu
130 135 140

Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr
145 150 155 160

Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly
165 170 175

Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala
180 185 190

Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu
195 200 205

Lys

<210> 72
<211> 1247
<212> PRT
<213> Human

<400> 72

Met Leu Ala Ser Ser Ser Arg Ile Arg Ala Ala Trp Thr Arg Ala Leu
1 5 10 15

Leu Leu Pro Leu Leu Leu Ala Gly Pro Val Gly Cys Leu Ser Arg Gln
20 25 30

Glu Leu Phe Pro Phe Gly Pro Gly Gln Gly Asp Leu Glu Leu Glu Asp
35 40 45

Gly Asp Asp Phe Val Ser Pro Ala Leu Glu Leu Ser Gly Ala Leu Arg
50 55 60

Phe Tyr Asp Arg Ser Asp Ile Asp Ala Val Tyr Val Thr Thr Asn Gly
65 70 75 80

Ile Ile Ala Thr Ser Glu Pro Pro Ala Lys Glu Ser His Pro Gly Leu
85 90 95

Phe Pro Pro Thr Phe Gly Ala Val Ala Pro Phe Leu Ala Asp Leu Asp
100 105 110

Thr Thr Asp Gly Leu Gly Lys Val Tyr Tyr Arg Glu Asp Leu Ser Pro
115 120 125

Ser Ile Thr Gln Arg Ala Ala Glu Cys Val His Arg Gly Phe Pro Glu
130 135 140

Ile Ser Phe Gln Pro Ser Ser Ala Val Val Val Thr Trp Glu Ser Val
145 150 155 160

Ala Pro Tyr Gln Gly Pro Ser Arg Asp Pro Asp Gln Lys Gly Lys Arg
165 170 175

Asn Thr Phe Gln Ala Val Leu Ala Ser Ser Asp Ser Ser Ser Tyr Ala
180 185 190

Ile Phe Leu Tyr Pro Glu Asp Gly Leu Gln Phe His Thr Thr Phe Ser
195 200 205

Lys Lys Glu Asn Asn Gln Val Pro Ala Val Val Ala Phe Ser Gln Gly
210 215 220

Ser Val Gly Phe Leu Trp Lys Ser Asn Gly Ala Tyr Asn Ile Phe Ala
225 230 235 240

Asn Asp Arg Glu Ser Val Glu Asn Leu Ala Lys Ser Ser Asn Ser Gly
245 250 255

Gln Gln Gly Val Trp Val Phe Glu Ile Gly Ser Pro Ala Thr Thr Asn
260 265 270

Gly Val Val Pro Ala Asp Val Ile Leu Gly Thr Glu Asp Gly Ala Glu
275 280 285

Tyr Asp Asp Glu Asp Glu Asp Tyr Asp Leu Ala Thr Thr Arg Leu Gly
290 295 300

Leu Glu Asp Val Gly Thr Thr Pro Phe Ser Tyr Lys Ala Leu Arg Arg
305 310 315 320

Gly Gly Ala Asp Thr Tyr Ser Val Pro Ser Val Leu Ser Pro Arg Arg
325 330 335

Ala Ala Thr Glu Arg Pro Leu Gly Pro Pro Thr Glu Arg Thr Arg Ser
340 345 350

Phe Gln Leu Ala Val Glu Thr Phe His Gln Gln His Pro Gln Val Ile
355 360 365

Asp Val Asp Glu Val Glu Glu Thr Gly Val Val Phe Ser Tyr Asn Thr
370 375 380

Asp Ser Arg Gln Thr Cys Ala Asn Asn Arg His Gln Cys Ser Val His
385 390 395 400

Ala Glu Cys Arg Asp Tyr Ala Thr Gly Phe Cys Cys Ser Cys Val Ala
405 410 415

Gly Tyr Thr Gly Asn Gly Arg Gln Cys Val Ala Glu Gly Ser Pro Gln
420 425 430

Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln
435 440 445

Val Pro Ile Val Phe Glu Asn Thr Asp Leu His Ser Tyr Val Val Met
450 455 460

Asn His Gly Arg Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val
465 470 475 480

Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp
485 490 495

Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr
500 505 510

Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro
515 520 525

Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu His Gly
530 535 540

His Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro
545 550 555 560

Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr
565 570 575

Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr
580 585 590

Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln
595 600 605

Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg
610 615 620

Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser Val Phe Val
625 630 635 640

Leu Tyr Asn Gln Glu Glu Lys Ile Leu Arg Tyr Ala Leu Ser Asn Ser
645 650 655

Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala Leu Gln Asn Pro Cys
660 665 670

Tyr Ile Gly Thr His Gly Cys Asp Thr Asn Ala Ala Cys Arg Pro Gly
675 680 685

Pro Arg Thr Gln Phe Thr Cys Glu Cys Ser Ile Gly Phe Arg Gly Asp
690 695 700

Gly Arg Thr Cys Tyr Asp Ile Asp Glu Cys Ser Glu Gln Pro Ser Val
705 710 715 720

Cys Gly Ser His Thr Ile Cys Asn Asn His Pro Gly Thr Phe Arg Cys
725 730 735

Glu Cys Val Glu Gly Tyr Gln Phe Ser Asp Glu Gly Thr Cys Val Ala
740 745 750

Val Val Asp Gln Arg Pro Ile Asn Tyr Cys Glu Thr Gly Leu His Asn
755 760 765

Cys Asp Ile Pro Gln Arg Ala Gln Cys Ile Tyr Thr Gly Gly Ser Ser
770 775 780

Tyr Thr Cys Ser Cys Leu Pro Gly Phe Ser Gly Asp Gly Gln Ala Cys
785 790 795 800

Gln Asp Val Asp Glu Cys Gln Pro Ser Arg Cys His Pro Asp Ala Phe
805 810 815

Cys Tyr Asn Thr Pro Gly Ser Phe Thr Cys Gln Cys Lys Pro Gly Tyr
820 825 830

Gln Gly Asp Gly Phe Arg Cys Val Pro Gly Glu Val Glu Lys Thr Arg
835 840 845

Cys Gln His Glu Arg Glu His Ile Leu Gly Ala Ala Gly Ala Thr Asp
850 855 860

Pro Gln Arg Pro Ile Pro Pro Gly Leu Phe Val Pro Glu Cys Asp Ala
865 870 875 880

His Gly His Tyr Ala Pro Thr Gln Cys His Gly Ser Thr Gly Tyr Cys
885 890 895

Trp Cys Val Asp Arg Asp Gly Arg Glu Val Glu Gly Thr Arg Thr Arg
900 905 910

Pro Gly Met Thr Pro Pro Cys Leu Ser Thr Val Ala Pro Pro Ile His
915 920 925

Gln Gly Pro Ala Val Pro Thr Ala Val Ile Pro Leu Pro Pro Gly Thr
930 935 940

His Leu Leu Phe Ala Gln Thr Gly Lys Ile Glu Arg Leu Pro Leu Glu
945 950 955 960

Gly Asn Thr Met Arg Lys Thr Glu Ala Lys Ala Phe Leu His Val Pro
965 970 975

Ala Lys Val Ile Ile Gly Leu Ala Phe Asp Cys Val Asp Lys Met Val
980 985 990

Tyr Trp Thr Asp Ile Thr Glu Pro Ser Ile Gly Arg Ala Ser Leu His
995 1000 1005

Gly Gly Glu Pro Thr Thr Ile Ile Arg Gln Asp Leu Gly Ser Pro
1010 1015 1020

Glu Gly Ile Ala Val Asp His Leu Gly Arg Asn Ile Phe Trp Thr
1025 1030 1035

Asp Ser Asn Leu Asp Arg Ile Glu Val Ala Lys Leu Asp Gly Thr
1040 1045 1050

Gln Arg Arg Val Leu Phe Glu Thr Asp Leu Val Asn Pro Arg Gly
1055 1060 1065

Ile Val Thr Asp Ser Val Arg Gly Asn Leu Tyr Trp Thr Asp Trp

1070					1075					1080				
Asn	Arg	Asp	Asn	Pro	Lys	Ile	Glu	Thr	Ser	Tyr	Met	Asp	Gly	Thr
1085						1090					1095			
Asn	Arg	Arg	Ile	Leu	Val	Gln	Asp	Asp	Leu	Gly	Leu	Pro	Asn	Gly
1100						1105					1110			
Leu	Thr	Phe	Asp	Ala	Phe	Ser	Ser	Gln	Leu	Cys	Trp	Val	Asp	Ala
1115						1120					1125			
Gly	Thr	Asn	Arg	Ala	Glu	Cys	Leu	Asn	Pro	Ser	Gln	Pro	Ser	Arg
1130						1135					1140			
Arg	Lys	Ala	Leu	Glu	Gly	Leu	Gln	Tyr	Pro	Phe	Ala	Val	Thr	Ser
1145						1150					1155			
Tyr	Gly	Lys	Asn	Leu	Tyr	Phe	Thr	Asp	Trp	Lys	Met	Asn	Ser	Val
1160						1165					1170			
Val	Ala	Leu	Asp	Leu	Ala	Ile	Ser	Lys	Glu	Thr	Asp	Ala	Phe	Gln
1175						1180					1185			
Pro	His	Lys	Gln	Thr	Arg	Leu	Tyr	Gly	Ile	Thr	Thr	Ala	Leu	Ser
1190						1195					1200			
Gln	Cys	Pro	Gln	Gly	His	Asn	Tyr	Cys	Ser	Val	Asn	Asn	Gly	Gly
1205						1210					1215			
Cys	Thr	His	Leu	Cys	Leu	Ala	Thr	Pro	Gly	Ser	Arg	Thr	Cys	Arg
1220						1225					1230			
Cys	Pro	Asp	Asn	Thr	Leu	Gly	Val	Asp	Cys	Ile	Glu	Gln	Lys	
1235						1240					1245			

<210> 73
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 <213> Artificial

<220>
 <223> Hexahistidine tag

<400> 73

His His His His His His
 1 5

<210> 74
 <211> 7
 <212> PRT
 <213> Artificial

<220>
 <223> IL-6 ligand

<400> 74

Leu Ser Leu Ile Thr Arg Leu
1 5

<210> 75

<211> 31

<212> PRT

<213> Artificial

<220>

<223> IL-6 ligand

<400> 75

Trp Gln Asp Pro His Ser Trp Asn Ser Ser Phe Tyr Arg Leu Arg Phe
1 5 10 15

Glu Leu Arg Tyr Arg Ala Glu Arg Ser Lys Thr Phe Thr Thr Trp
20 25 30

<210> 76

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> linker region

<400> 76

Gly Gly Ser Ser Arg Ser Ser Ser
1 5

<210> 77

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> positively charged peptide region

<400> 77

Arg Lys Arg Lys Arg Lys
1 5

<210> 78

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> positively charged peptide region

<400> 78

Arg Arg Arg Arg Arg Arg
1 5

<210> 79

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> positively charged peptide region

<400> 79

Lys Lys Lys Lys Lys Lys
1 5

<210> 80

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Positively charged peptide region

<400> 80

His His His His His His
1 5

<210> 81

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> positively charged peptide region

<400> 81

Arg His Arg His Arg His
1 5

<210> 82

<211> 8

<212> PRT

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<220>

<223> positively charged peptide region

<400> 82

Arg Lys Arg Lys Arg Lys Arg Lys
1 5

<210> 83

<211> 6

<212> PRT

<213> Artificial Sequence

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<223> positively charged peptide region

<400> 83

Arg Lys Arg His Arg Lys
1 5

<210> 84

<211> 6

<212> PRT
<213> Artificial Sequence

<220>
<223> positively charged peptide region

<400> 84

Arg Lys Arg His Arg His
1 5

<210> 85
<211> 6
<212> PRT
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<220>
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<400> 85

Arg His Arg His Arg His
1 5

<210> 86
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
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<400> 86

Arg Lys Arg Lys Arg Lys Arg
1 5

<210> 87
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3

<400> 87

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 88
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_V45T

<400> 88

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 89
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_V121Q

<400> 89

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe

65		70		75		80
Ser Ile Thr Gly	Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Gln					
	85		90		95	
Gly His Pro Gly	Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp					
	100		105		110	
Glu Lys Gly	Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro					
	115		120		125	
Gln Ile Pro Phe Gly	Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu					
	130		135		140	
Tyr His Tyr Ser Thr	Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr					
	145		150		155	
Thr Val Thr Glu Pro Glu Arg Asp Gly	Ala Ser Pro Ser Arg Ile Tyr					
	165		170		175	
Thr Tyr Gln Trp Arg Gln Thr Ile Thr	Phe Gln Glu Cys Val His Asp					
	180		185		190	
Asp Ser Arg Pro Ala Leu Pro Ser Thr	Gln Gln Leu Ser Val Asp Ser					
	195		200		205	
Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile	Leu Ala Tyr Ala Leu					
	210		215		220	
Ser Asn Ser Ile Gly Pro Val Arg Glu Gly	Ser Pro Asp Ala					
	225		230		235	
<210>	90					
<211>	238					
<212>	PRT					
<213>	Artificial Sequence					
<220>						
<223>	NID0mut3_F157E					
<400>	90					
Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly						
1	5		10		15	
Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr						
	20		25		30	
Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro						
	35		40		45	
Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile						
	50		55		60	

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 91
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_V215T

<400> 91

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Thr His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 92
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> Seq_93

<400> 92

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Gln
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Thr His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 93
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut4_T215V

<400> 93

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile

50

55

60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Gln
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 94

<211> 238

<212> PRT

<213> Artificial Sequence

<220>

<223> NID0mut5

<400> 94

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Gln
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Ser Thr His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 95
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_V176T

<400> 95

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Thr Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 96
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_I200T

<400> 96

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Thr Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 97
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_V236Y

<400> 97

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro

35

40

45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Tyr Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 98

<211> 238

<212> PRT

<213> Artificial Sequence

<220>

<223> NID0mut3_L237T

<400> 98

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Thr Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 99
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_S65I

<400> 99

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ile Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 100
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_R114I

<400> 100

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Ile Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 101
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut3_C214S

<400> 101

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr

20

25

30

Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro
 35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
 50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
 65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val
 85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
 100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
 115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
 130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
 145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
 165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Ser Val His Asp
 180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
 195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
 210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
 225 230 235

<210> 102
 <211> 238
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> NID0mut3_S65I_R114I

<400> 102

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
 1 5 10 15

Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ile Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Ile Gln Ala Glu Val Thr Phe Val
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 103
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut5_S65I_R114I

<400> 103

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ile Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Ile Gln Ala Glu Val Thr Phe Gln
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Ser Thr His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 104
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut5_S51I

<400> 104

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly
1 5 10 15

Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr
20 25 30

Val Val Met Asn His Gly Asn Ile Tyr Thr Ala Ile Ser Thr Ile Pro
35 40 45

Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile
50 55 60

Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe
65 70 75 80

Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Gln
85 90 95

Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp
100 105 110

Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro
115 120 125

Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu
130 135 140

Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr
145 150 155 160

Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr
165 170 175

Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Ser Thr His Asp
180 185 190

Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser
195 200 205

Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu
210 215 220

Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala
225 230 235

<210> 105
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> NID0mut5_R114I

<400> 105

Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly

1	5	10	15
Ser Ser Gln Thr Pro Ile Val Phe Glu Asn Thr Asp Leu Ala Ser Tyr	20	25	30
Val Val Met Asn His Gly Asn Ser Tyr Thr Ala Ile Ser Thr Ile Pro	35	40	45
Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile	50	55	60
Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe	65	70	75
Ser Ile Thr Gly Gly Glu Phe Thr Ile Gln Ala Glu Val Thr Phe Gln	85	90	95
Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp	100	105	110
Glu Lys Gly Asn Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro	115	120	125
Gln Ile Pro Glu Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu	130	135	140
Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr	145	150	155
Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr	165	170	175
Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln Glu Ser Thr His Asp	180	185	190
Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser	195	200	205
Val Ser Val Leu Tyr Asn Gln Glu Glu Lys Ile Leu Ala Tyr Ala Leu	210	215	220
Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala	225	230	235

<210> 106
 <211> 270
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> T22-NID0mut3-H6

<400> 106

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 107

<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_V45T-H6

<400> 107

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 108
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_V121Q-H6

<400> 108

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 109
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_F157E-H6

<400> 109

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile	Thr	Ser	Ser	Thr	Arg	Glu	Tyr	Thr	Val	Thr	Glu	Pro	Glu	Arg	
		180					185					190			
Asp	Gly	Ala	Ser	Pro	Ser	Arg	Ile	Tyr	Thr	Tyr	Gln	Trp	Arg	Gln	Thr
		195					200					205			
Ile	Thr	Phe	Gln	Glu	Cys	Val	His	Asp	Asp	Ser	Arg	Pro	Ala	Leu	Pro
		210				215					220				
Ser	Thr	Gln	Gln	Leu	Ser	Val	Asp	Ser	Val	Ser	Val	Leu	Tyr	Asn	Gln
		225			230					235					240
Glu	Glu	Lys	Ile	Leu	Ala	Tyr	Ala	Leu	Ser	Asn	Ser	Ile	Gly	Pro	Val
				245					250					255	
Arg	Glu	Gly	Ser	Pro	Asp	Ala	Lys	His	His	His	His	His	His		
			260					265						270	
<210>	110														
<211>	270														
<212>	PRT														
<213>	Artificial Sequence														
<220>															
<223>	T22-NID0mut3_V215T-H6														
<400>	110														
Arg	Arg	Trp	Cys	Tyr	Arg	Lys	Cys	Tyr	Lys	Gly	Tyr	Cys	Tyr	Arg	Lys
1				5					10					15	
Cys	Arg	Gly	Gly	Ser	Ser	Arg	Ser	Ser	Ser	Pro	Gln	Arg	Val	Asn	Gly
			20					25					30		
Lys	Val	Lys	Gly	Arg	Ile	Phe	Val	Gly	Ser	Ser	Gln	Val	Pro	Ile	Val
		35				40						45			
Phe	Glu	Asn	Thr	Asp	Leu	Ala	Ser	Tyr	Val	Val	Met	Asn	His	Gly	Asn
	50					55					60				
Ser	Tyr	Thr	Ala	Ile	Ser	Thr	Ile	Pro	Glu	Thr	Val	Gly	Tyr	Ser	Leu
65				70						75					80
Leu	Pro	Leu	Ala	Pro	Val	Gly	Gly	Ile	Ile	Gly	Trp	Met	Phe	Ala	Val
				85					90					95	
Glu	Gln	Asp	Gly	Phe	Lys	Asn	Gly	Phe	Ser	Ile	Thr	Gly	Gly	Glu	Phe
			100					105					110		
Thr	Arg	Gln	Ala	Glu	Val	Thr	Phe	Val	Gly	His	Pro	Gly	Asn	Leu	Val
		115					120					125			
Ile	Lys	Gln	Arg	Phe	Ser	Gly	Ile	Asp	Glu	Lys	Gly	Asn	Leu	Thr	Ile

130		135		140	
Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser					
145		150		155	160
Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val					
	165		170		175
Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg					
	180		185		190
Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr					
	195		200		205
Ile Thr Phe Gln Glu Cys Thr His Asp Asp Ser Arg Pro Ala Leu Pro					
	210		215		220
Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln					
225		230		235	240
Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val					
	245		250		255
Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His					
	260		265		270
<210> 111					
<211> 270					
<212> PRT					
<213> Artificial Sequence					
<220>					
<223> T22-NID0mut4-H6					
<400> 111					
Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys					
1		5		10	15
Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly					
	20		25		30
Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val					
	35		40		45
Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn					
	50		55		60
Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu					
65		70		75	80
Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val					
	85		90		95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
 100 105 110
 Thr Arg Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
 115 120 125
 Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
 130 135 140
 Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
 145 150 155 160
 Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
 165 170 175
 Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
 180 185 190
 Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
 195 200 205
 Ile Thr Phe Gln Glu Cys Thr His Asp Asp Ser Arg Pro Ala Leu Pro
 210 215 220
 Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
 225 230 235 240
 Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
 245 250 255
 Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
 260 265 270

<210> 112

<211> 270

<212> PRT

<213> Artificial Sequence

<220>

<223> T22-NID0mut4_T215V-H6

<400> 112

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
 1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
 20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val
 35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
 50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 113
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut5-H6

<400> 113

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val
 35 40 45
 Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
 50 55 60
 Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
 65 70 75 80
 Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
 85 90 95
 Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
 100 105 110
 Thr Arg Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
 115 120 125
 Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
 130 135 140
 Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
 145 150 155 160
 Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
 165 170 175
 Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
 180 185 190
 Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
 195 200 205
 Ile Thr Phe Gln Glu Ser Thr His Asp Asp Ser Arg Pro Ala Leu Pro
 210 215 220
 Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
 225 230 235 240
 Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
 245 250 255
 Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
 260 265 270

<210> 114

<211> 270

<212> PRT

<213> Artificial Sequence

<220>

<223> T22-NID0mut3_V176T-H6

<400> 114

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Thr
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 115
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_I200T-H6

<400> 115

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Thr Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln

225		230		235		240									
Glu	Glu	Lys	Ile	Leu	Ala	Tyr	Ala	Leu	Ser	Asn	Ser	Ile	Gly	Pro	Val
			245					250					255		
Arg	Glu	Gly	Ser	Pro	Asp	Ala	Lys	His	His	His	His	His	His		
			260					265					270		
<210> 116 <211> 270 <212> PRT <213> Artificial Sequence <220> <223> T22-NID0mut3_V236Y-H6 <400> 116															
Arg	Arg	Trp	Cys	Tyr	Arg	Lys	Cys	Tyr	Lys	Gly	Tyr	Cys	Tyr	Arg	Lys
1			5					10					15		
Cys	Arg	Gly	Gly	Ser	Ser	Arg	Ser	Ser	Ser	Pro	Gln	Arg	Val	Asn	Gly
			20				25						30		
Lys	Val	Lys	Gly	Arg	Ile	Phe	Val	Gly	Ser	Ser	Gln	Val	Pro	Ile	Val
		35				40					45				
Phe	Glu	Asn	Thr	Asp	Leu	Ala	Ser	Tyr	Val	Val	Met	Asn	His	Gly	Asn
	50				55						60				
Ser	Tyr	Thr	Ala	Ile	Ser	Thr	Ile	Pro	Glu	Thr	Val	Gly	Tyr	Ser	Leu
65				70					75					80	
Leu	Pro	Leu	Ala	Pro	Val	Gly	Gly	Ile	Ile	Gly	Trp	Met	Phe	Ala	Val
			85					90						95	
Glu	Gln	Asp	Gly	Phe	Lys	Asn	Gly	Phe	Ser	Ile	Thr	Gly	Gly	Glu	Phe
			100					105					110		
Thr	Arg	Gln	Ala	Glu	Val	Thr	Phe	Val	Gly	His	Pro	Gly	Asn	Leu	Val
		115					120					125			
Ile	Lys	Gln	Arg	Phe	Ser	Gly	Ile	Asp	Glu	Lys	Gly	Asn	Leu	Thr	Ile
	130					135					140				
Asp	Thr	Glu	Leu	Glu	Gly	Arg	Val	Pro	Gln	Ile	Pro	Phe	Gly	Ser	Ser
145				150					155					160	
Val	His	Ile	Glu	Pro	Tyr	Thr	Glu	Leu	Tyr	His	Tyr	Ser	Thr	Ser	Val
			165					170					175		
Ile	Thr	Ser	Ser	Ser	Thr	Arg	Glu	Tyr	Thr	Val	Thr	Glu	Pro	Glu	Arg
			180					185					190		

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Tyr Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 117
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_L237T-H6

<400> 117

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Thr Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 118
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_S65I-H6

<400> 118

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ile Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 119
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_R114I-H6

<400> 119

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val

85

90

95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
 100 105 110

Thr Ile Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
 115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
 130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
 145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
 165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
 180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
 195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
 210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
 225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
 245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
 260 265 270

<210> 120

<211> 270

<212> PRT

<213> Artificial Sequence

<220>

<223> T22-NID0mut3_C214S-H6

<400> 120

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
 1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
 20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
 35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Ser Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 121
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut3_S65I_R114I-H6

<400> 121

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
 20 25 30
 Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
 35 40 45
 Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
 50 55 60
 Ile Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
 65 70 75 80
 Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
 85 90 95
 Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
 100 105 110
 Thr Ile Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
 115 120 125
 Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
 130 135 140
 Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
 145 150 155 160
 Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
 165 170 175
 Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
 180 185 190
 Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
 195 200 205
 Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
 210 215 220
 Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
 225 230 235 240
 Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
 245 250 255
 Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
 260 265 270

<210> 122
 <211> 270
 <212> PRT
 <213> Artificial Sequence

<220>

<223> T22-NID0mut5_S51I_R114I

<400> 122

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ile Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Ile Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Ser Thr His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 123
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut5_S65I-H6

<400> 123

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ile Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Ser Thr His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 124
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut5_R114I-H6

<400> 124

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Thr Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Ile Gln Ala Glu Val Thr Phe Gln Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Glu Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg

180

185

190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
 195 200 205

Ile Thr Phe Gln Glu Ser Thr His Asp Asp Ser Arg Pro Ala Leu Pro
 210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
 225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
 245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
 260 265 270

<210> 125

<211> 360

<212> PRT

<213> Artificial Sequence

<220>

<223> Human Nidogen G2 domain from Figure 13A

<400> 125

His Gln Gln His Pro Gln Val Ile Asp Val Asp Glu Val Glu Glu Thr
 1 5 10 15

Gly Val Val Phe Ser Tyr Asn Thr Asp Ser Arg Gln Thr Cys Ala Asn
 20 25 30

Asn Arg His Gln Cys Ser Val His Ala Glu Cys Arg Asp Tyr Ala Thr
 35 40 45

Gly Phe Cys Cys Ser Cys Val Ala Gly Tyr Thr Gly Asn Gly Arg Gln
 50 55 60

Cys Val Ala Glu Gly Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly
 65 70 75 80

Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val Phe Glu Asn Thr
 85 90 95

Asp Leu His Ser Tyr Val Val Met Asn His Gly Arg Ser Tyr Thr Ala
 100 105 110

Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala
 115 120 125

Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly
 130 135 140

Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala
145 150 155 160

Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val Ile Lys Gln Arg
165 170 175

Phe Ser Gly Ile Asp Glu His Gly His Leu Thr Ile Asp Thr Glu Leu
180 185 190

Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser Val His Ile Glu
195 200 205

Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val Ile Thr Ser Ser
210 215 220

Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg Asp Gly Ala Ser
225 230 235 240

Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln
245 250 255

Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro Ser Thr Gln Gln
260 265 270

Leu Ser Val Asp Ser Val Phe Val Leu Tyr Asn Gln Glu Glu Lys Ile
275 280 285

Leu Arg Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val Arg Glu Gly Ser
290 295 300

Pro Asp Ala Leu Gln Asn Pro Cys Tyr Ile Gly Thr His Gly Cys Asp
305 310 315 320

Thr Asn Ala Ala Cys Arg Pro Gly Pro Arg Thr Gln Phe Thr Cys Glu
325 330 335

Cys Ser Ile Gly Phe Arg Gly Asp Gly Arg Thr Cys Tyr Asp Ile Asp
340 345 350

Glu Cys Ser Glu Gln Pro Ser Val
355 360

<210> 126
<211> 360
<212> PRT
<213> Artificial Sequence

<220>
<223> Mouse Nidogen G2 domain from Figure 13A

<400> 126

Pro Gln His His Pro Gln Val Ile Asp Val Asp Glu Val Glu Glu Thr
1 5 10 15

Gly Val Val Phe Ser Tyr Asn Thr Gly Ser Gln Gln Thr Cys Ala Asn
 20 25 30

Asn Arg His Gln Cys Ser Val His Ala Glu Cys Arg Asp Tyr Ala Thr
 35 40 45

Gly Phe Cys Cys Arg Cys Val Ala Asn Tyr Thr Gly Asn Gly Arg Gln
 50 55 60

Cys Val Ala Glu Gly Ser Pro Gln Arg Val Asn Gly Lys Val Lys Gly
 65 70 75 80

Arg Ile Phe Val Gly Ser Ser Gln Val Pro Val Val Phe Glu Asn Thr
 85 90 95

Asp Leu His Ser Tyr Val Val Met Asn His Gly Arg Ser Tyr Thr Ala
 100 105 110

Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu Leu Pro Leu Ala
 115 120 125

Pro Ile Gly Gly Ile Ile Gly Trp Met Phe Ala Val Glu Gln Asp Gly
 130 135 140

Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe Thr Arg Gln Ala
 145 150 155 160

Glu Val Thr Phe Leu Gly His Pro Gly Lys Leu Val Leu Lys Gln Gln
 165 170 175

Phe Ser Gly Ile Asp Glu His Gly His Leu Thr Ile Ser Thr Glu Leu
 180 185 190

Glu Gly Arg Val Pro Gln Ile Pro Tyr Gly Ala Ser Val His Ile Glu
 195 200 205

Pro Tyr Thr Glu Leu Tyr His Tyr Ser Ser Ser Val Ile Thr Ser Ser
 210 215 220

Ser Thr Arg Glu Tyr Thr Val Met Glu Pro Asp Gln Asp Gly Ala Ala
 225 230 235 240

Pro Ser His Thr His Ile Tyr Gln Trp Arg Gln Thr Ile Thr Phe Gln
 245 250 255

Glu Cys Ala His Asp Asp Ala Arg Pro Ala Leu Pro Ser Thr Gln Gln
 260 265 270

Leu Ser Val Asp Ser Val Phe Val Leu Tyr Asn Lys Glu Glu Arg Ile
 275 280 285

Leu Arg Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val Arg Asp Gly Ser
290 295 300

Pro Asp Ala Leu Gln Asn Pro Cys Tyr Ile Gly Thr His Gly Cys Asp
305 310 315 320

Ser Asn Ala Ala Cys Arg Pro Gly Pro Gly Thr Gln Phe Thr Cys Glu
325 330 335

Cys Ser Ile Gly Phe Arg Gly Asp Gly Gln Thr Cys Tyr Asp Ile Asp
340 345 350

Glu Cys Ser Glu Gln Pro Ser Arg
355 360

<210> 127
<211> 1247
<212> PRT
<213> Artificial Sequence

<220>
<223> Human Nidogen (Uniprot P14534)

<400> 127

Met Leu Ala Ser Ser Ser Arg Ile Arg Ala Ala Trp Thr Arg Ala Leu
1 5 10 15

Leu Leu Pro Leu Leu Leu Ala Gly Pro Val Gly Cys Leu Ser Arg Gln
20 25 30

Glu Leu Phe Pro Phe Gly Pro Gly Gln Gly Asp Leu Glu Leu Glu Asp
35 40 45

Gly Asp Asp Phe Val Ser Pro Ala Leu Glu Leu Ser Gly Ala Leu Arg
50 55 60

Phe Tyr Asp Arg Ser Asp Ile Asp Ala Val Tyr Val Thr Thr Asn Gly
65 70 75 80

Ile Ile Ala Thr Ser Glu Pro Pro Ala Lys Glu Ser His Pro Gly Leu
85 90 95

Phe Pro Pro Thr Phe Gly Ala Val Ala Pro Phe Leu Ala Asp Leu Asp
100 105 110

Thr Thr Asp Gly Leu Gly Lys Val Tyr Tyr Arg Glu Asp Leu Ser Pro
115 120 125

Ser Ile Thr Gln Arg Ala Ala Glu Cys Val His Arg Gly Phe Pro Glu
130 135 140

Ile Ser Phe Gln Pro Ser Ser Ala Val Val Val Thr Trp Glu Ser Val
145 150 155 160

Ala Pro Tyr Gln Gly Pro Ser Arg Asp Pro Asp Gln Lys Gly Lys Arg
 165 170 175

Asn Thr Phe Gln Ala Val Leu Ala Ser Ser Asp Ser Ser Ser Tyr Ala
 180 185 190

Ile Phe Leu Tyr Pro Glu Asp Gly Leu Gln Phe His Thr Thr Phe Ser
 195 200 205

Lys Lys Glu Asn Asn Gln Val Pro Ala Val Val Ala Phe Ser Gln Gly
 210 215 220

Ser Val Gly Phe Leu Trp Lys Ser Asn Gly Ala Tyr Asn Ile Phe Ala
 225 230 235 240

Asn Asp Arg Glu Ser Val Glu Asn Leu Ala Lys Ser Ser Asn Ser Gly
 245 250 255

Gln Gln Gly Val Trp Val Phe Glu Ile Gly Ser Pro Ala Thr Thr Asn
 260 265 270

Gly Val Val Pro Ala Asp Val Ile Leu Gly Thr Glu Asp Gly Ala Glu
 275 280 285

Tyr Asp Asp Glu Asp Glu Asp Tyr Asp Leu Ala Thr Thr Arg Leu Gly
 290 295 300

Leu Glu Asp Val Gly Thr Thr Pro Phe Ser Tyr Lys Ala Leu Arg Arg
 305 310 315 320

Gly Gly Ala Asp Thr Tyr Ser Val Pro Ser Val Leu Ser Pro Arg Arg
 325 330 335

Ala Ala Thr Glu Arg Pro Leu Gly Pro Pro Thr Glu Arg Thr Arg Ser
 340 345 350

Phe Gln Leu Ala Val Glu Thr Phe His Gln Gln His Pro Gln Val Ile
 355 360 365

Asp Val Asp Glu Val Glu Glu Thr Gly Val Val Phe Ser Tyr Asn Thr
 370 375 380

Asp Ser Arg Gln Thr Cys Ala Asn Asn Arg His Gln Cys Ser Val His
 385 390 395 400

Ala Glu Cys Arg Asp Tyr Ala Thr Gly Phe Cys Cys Ser Cys Val Ala
 405 410 415

Gly Tyr Thr Gly Asn Gly Arg Gln Cys Val Ala Glu Gly Ser Pro Gln
 420 425 430

Arg Val Asn Gly Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln
435 440 445

Val Pro Ile Val Phe Glu Asn Thr Asp Leu His Ser Tyr Val Val Met
450 455 460

Asn His Gly Arg Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val
465 470 475 480

Gly Tyr Ser Leu Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp
485 490 495

Met Phe Ala Val Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr
500 505 510

Gly Gly Glu Phe Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro
515 520 525

Gly Asn Leu Val Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu His Gly
530 535 540

His Leu Thr Ile Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro
545 550 555 560

Phe Gly Ser Ser Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr
565 570 575

Ser Thr Ser Val Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr
580 585 590

Glu Pro Glu Arg Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln
595 600 605

Trp Arg Gln Thr Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg
610 615 620

Pro Ala Leu Pro Ser Thr Gln Gln Leu Ser Val Asp Ser Val Phe Val
625 630 635 640

Leu Tyr Asn Gln Glu Glu Lys Ile Leu Arg Tyr Ala Leu Ser Asn Ser
645 650 655

Ile Gly Pro Val Arg Glu Gly Ser Pro Asp Ala Leu Gln Asn Pro Cys
660 665 670

Tyr Ile Gly Thr His Gly Cys Asp Thr Asn Ala Ala Cys Arg Pro Gly
675 680 685

Pro Arg Thr Gln Phe Thr Cys Glu Cys Ser Ile Gly Phe Arg Gly Asp
690 695 700

Gly Arg Thr Cys Tyr Asp Ile Asp Glu Cys Ser Glu Gln Pro Ser Val

705																	
Cys	Gly	Ser	His	Thr	Ile	Cys	Asn	Asn	His	Pro	Gly	Thr	Phe	Arg	Cys		
				725					730					735			
Glu	Cys	Val	Glu	Gly	Tyr	Gln	Phe	Ser	Asp	Glu	Gly	Thr	Cys	Val	Ala		
			740					745					750				
Val	Val	Asp	Gln	Arg	Pro	Ile	Asn	Tyr	Cys	Glu	Thr	Gly	Leu	His	Asn		
		755					760					765					
Cys	Asp	Ile	Pro	Gln	Arg	Ala	Gln	Cys	Ile	Tyr	Thr	Gly	Gly	Ser	Ser		
	770					775					780						
Tyr	Thr	Cys	Ser	Cys	Leu	Pro	Gly	Phe	Ser	Gly	Asp	Gly	Gln	Ala	Cys		
785					790					795					800		
Gln	Asp	Val	Asp	Glu	Cys	Gln	Pro	Ser	Arg	Cys	His	Pro	Asp	Ala	Phe		
			805						810					815			
Cys	Tyr	Asn	Thr	Pro	Gly	Ser	Phe	Thr	Cys	Gln	Cys	Lys	Pro	Gly	Tyr		
			820					825					830				
Gln	Gly	Asp	Gly	Phe	Arg	Cys	Val	Pro	Gly	Glu	Val	Glu	Lys	Thr	Arg		
		835					840					845					
Cys	Gln	His	Glu	Arg	Glu	His	Ile	Leu	Gly	Ala	Ala	Gly	Ala	Thr	Asp		
	850					855					860						
Pro	Gln	Arg	Pro	Ile	Pro	Pro	Gly	Leu	Phe	Val	Pro	Glu	Cys	Asp	Ala		
865					870					875					880		
His	Gly	His	Tyr	Ala	Pro	Thr	Gln	Cys	His	Gly	Ser	Thr	Gly	Tyr	Cys		
				885					890					895			
Trp	Cys	Val	Asp	Arg	Asp	Gly	Arg	Glu	Val	Glu	Gly	Thr	Arg	Thr	Arg		
			900					905					910				
Pro	Gly	Met	Thr	Pro	Pro	Cys	Leu	Ser	Thr	Val	Ala	Pro	Pro	Ile	His		
		915					920					925					
Gln	Gly	Pro	Ala	Val	Pro	Thr	Ala	Val	Ile	Pro	Leu	Pro	Pro	Gly	Thr		
	930					935					940						
His	Leu	Leu	Phe	Ala	Gln	Thr	Gly	Lys	Ile	Glu	Arg	Leu	Pro	Leu	Glu		
945					950					955					960		
Gly	Asn	Thr	Met	Arg	Lys	Thr	Glu	Ala	Lys	Ala	Phe	Leu	His	Val	Pro		
				965					970					975			
Ala	Lys	Val	Ile	Ile	Gly	Leu	Ala	Phe	Asp	Cys	Val	Asp	Lys	Met	Val		
			980					985					990				

Tyr Trp Thr Asp Ile Thr Glu Pro Ser Ile Gly Arg Ala Ser Leu His
995 1000 1005

Gly Gly Glu Pro Thr Thr Ile Ile Arg Gln Asp Leu Gly Ser Pro
1010 1015 1020

Glu Gly Ile Ala Val Asp His Leu Gly Arg Asn Ile Phe Trp Thr
1025 1030 1035

Asp Ser Asn Leu Asp Arg Ile Glu Val Ala Lys Leu Asp Gly Thr
1040 1045 1050

Gln Arg Arg Val Leu Phe Glu Thr Asp Leu Val Asn Pro Arg Gly
1055 1060 1065

Ile Val Thr Asp Ser Val Arg Gly Asn Leu Tyr Trp Thr Asp Trp
1070 1075 1080

Asn Arg Asp Asn Pro Lys Ile Glu Thr Ser Tyr Met Asp Gly Thr
1085 1090 1095

Asn Arg Arg Ile Leu Val Gln Asp Asp Leu Gly Leu Pro Asn Gly
1100 1105 1110

Leu Thr Phe Asp Ala Phe Ser Ser Gln Leu Cys Trp Val Asp Ala
1115 1120 1125

Gly Thr Asn Arg Ala Glu Cys Leu Asn Pro Ser Gln Pro Ser Arg
1130 1135 1140

Arg Lys Ala Leu Glu Gly Leu Gln Tyr Pro Phe Ala Val Thr Ser
1145 1150 1155

Tyr Gly Lys Asn Leu Tyr Phe Thr Asp Trp Lys Met Asn Ser Val
1160 1165 1170

Val Ala Leu Asp Leu Ala Ile Ser Lys Glu Thr Asp Ala Phe Gln
1175 1180 1185

Pro His Lys Gln Thr Arg Leu Tyr Gly Ile Thr Thr Ala Leu Ser
1190 1195 1200

Gln Cys Pro Gln Gly His Asn Tyr Cys Ser Val Asn Asn Gly Gly
1205 1210 1215

Cys Thr His Leu Cys Leu Ala Thr Pro Gly Ser Arg Thr Cys Arg
1220 1225 1230

Cys Pro Asp Asn Thr Leu Gly Val Asp Cys Ile Glu Gln Lys
1235 1240 1245

<210> 128
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> T22-NID0mut2-H6 (Sequence in figure 13B)

<400> 128

Arg Arg Trp Cys Tyr Arg Lys Cys Tyr Lys Gly Tyr Cys Tyr Arg Lys
1 5 10 15

Cys Arg Gly Gly Ser Ser Arg Ser Ser Ser Pro Gln Arg Val Asn Gly
20 25 30

Lys Val Lys Gly Arg Ile Phe Val Gly Ser Ser Gln Val Pro Ile Val
35 40 45

Phe Glu Asn Thr Asp Leu Ala Ser Tyr Val Val Met Asn His Gly Asn
50 55 60

Ser Tyr Thr Ala Ile Ser Thr Ile Pro Glu Thr Val Gly Tyr Ser Leu
65 70 75 80

Leu Pro Leu Ala Pro Val Gly Gly Ile Ile Gly Trp Met Phe Ala Val
85 90 95

Glu Gln Asp Gly Phe Lys Asn Gly Phe Ser Ile Thr Gly Gly Glu Phe
100 105 110

Thr Arg Gln Ala Glu Val Thr Phe Val Gly His Pro Gly Asn Leu Val
115 120 125

Ile Lys Gln Arg Phe Ser Gly Ile Asp Glu Lys Gly Asn Leu Thr Ile
130 135 140

Asp Thr Glu Leu Glu Gly Arg Val Pro Gln Ile Pro Phe Gly Ser Ser
145 150 155 160

Val His Ile Glu Pro Tyr Thr Glu Leu Tyr His Tyr Ser Thr Ser Val
165 170 175

Ile Thr Ser Ser Ser Thr Arg Glu Tyr Thr Val Thr Glu Pro Glu Arg
180 185 190

Asp Gly Ala Ser Pro Ser Arg Ile Tyr Thr Tyr Gln Trp Arg Gln Thr
195 200 205

Ile Thr Phe Gln Glu Cys Val His Asp Asp Ser Arg Pro Ala Leu Pro
210 215 220

Ser Thr Gln Gln Leu Ser Val Asp Ser Val Ser Val Leu Tyr Asn Gln
225 230 235 240

Glu Glu Lys Ile Leu Ala Tyr Ala Leu Ser Asn Ser Ile Gly Pro Val
245 250 255

Arg Glu Gly Ser Pro Asp Ala Lys His His His His His His
260 265 270

<210> 129
<211> 279
<212> PRT
<213> Artificial Sequence

<220>
<223> EPI-X4-(RK)-GFP-H6

<400> 129

Ile Val Arg Trp Ser Lys Lys Val Pro Cys Val Ser Ile Val Arg Trp
1 5 10 15

Ser Lys Lys Val Pro Cys Val Ser Arg Lys Arg Lys Arg Lys Gly Gly
20 25 30

Ser Ser Arg Ser Ser Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val
35 40 45

Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser
50 55 60

Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu
65 70 75 80

Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
85 90 95

Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp
100 105 110

His Met Lys Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr
115 120 125

Val Gln Glu Arg Thr Ile Ser Phe Lys Asp Asp Gly Asn Tyr Lys Thr
130 135 140

Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu
145 150 155 160

Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys
165 170 175

Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Thr Ala Asp Lys
180 185 190

Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu

195

200

205

Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile
 210 215 220

Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln
 225 230 235 240

Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu
 245 250 255

Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu
 260 265 270

Tyr His His His His His His
 275

<210> 130

<211> 236

<212> PRT

<213> Artificial Sequence

<220>

<223> GFP

<400> 130

Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu
 1 5 10 15

Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly
 20 25 30

Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr
 35 40 45

Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr
 50 55 60

Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg His
 65 70 75 80

Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr
 85 90 95

Ile Ser Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys
 100 105 110

Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp
 115 120 125

Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr
 130 135 140

Asn Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn Gly Ile
145 150 155 160

Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln
165 170 175

Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val
180 185 190

Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys
195 200 205

Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr
210 215 220

Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr
225 230 235

<210> 131

<211> 30

<212> PRT

<213> Artificial Sequence

<220>

<223> Optimized EPI-X4 coupled to RKRRK positively charged peptide region

<400> 131

Ile Val Arg Trp Ser Lys Lys Val Pro Cys Val Ser Ile Val Arg Trp
1 5 10 15

Ser Lys Lys Val Pro Cys Val Ser Arg Lys Arg Lys Arg Lys
20 25 30

<210> 132

<211> 17

<212> PRT

<213> Artificial sequence

<220>

<223> EPI-X4

<400> 132

Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val Ser Thr Pro Thr
1 5 10 15

Leu

<210> 133

<211> 23

<212> PRT

<213> Artificial sequence

<220>

<223> EPI-X4 coupled to RKRRK positively charged peptide region

<400> 133

Leu	Leu	Val	Arg	Tyr	Thr	Lys	Lys	Val	Pro	Gln	Val	Ser	Thr	Pro	Thr
1				5					10					15	

Leu	Arg	Lys	Arg	Lys	Arg	Lys
			20			