

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 June 2011 (23.06.2011)

PCT

(10) International Publication Number
WO 2011/073209 A1

(51) International Patent Classification:
C07K 14/00 (2006.01) *C40B 40/02* (2006.01)
C12N 15/10 (2006.01)

(21) International Application Number:
PCT/EP2010/069666

(22) International Filing Date:
14 December 2010 (14.12.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09179147.5 14 December 2009 (14.12.2009) EP
10162264.5 7 May 2010 (07.05.2010) EP
10186980.8 8 October 2010 (08.10.2010) EP

(71) Applicant (for all designated States except US): **SCIL PROTEINS GMBH**; Heinrich-Damerow-Strasse 1, 06120 Halle/Saale (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **STEUERNAGEL, Arnd** [DE/DE]; Am Kirschberge 4, 37085 Goettingen (DE). **FIEDLER, Erik** [DE/DE]; Hardenberg Str. 14, 06114 Halle/Saale (DE). **FIEDLER, Markus** [DE/DE]; Kreuzvorwerk 60, 06120 Halle/Saale (DE). **KUNERT, Anja** [DE/DE]; Schwetschkestrasse 15, 06110 Halle/Saale (DE). **NERKAMP, Joerg** [DE/DE]; Beyschlagstrasse 30, 06110 Halle/Saale (DE). **GOETTLER, Thomas** [DE/DE]; Steinweg 16, 06110 Halle/Saale (DE). **GLOSER, Manja** [DE/DE]; Neue Siedlung 13, 06179 Teutschenthal (DE). **HAENBGEN, Ilka** [DE/DE]; Zoeberitzer Weg 9, 06116 Halle/Saale (DE).

(74) Agent: **BEHNISCH, Werner**; Friedrichstrasse 31, 80801 München (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))



WO 2011/073209 A1

(54) Title: MODIFIED UBIQUITIN PROTEINS HAVING A SPECIFIC BINDING ACTIVITY FOR THE EXTRADOMAIN B OF FIBRONECTIN

(57) Abstract: The present invention refers to novel recombinant proteins obtained from modified ubiquitin capable of binding the extradomain B of fibronectin (ED-B). Furthermore, the invention refers to fusion proteins comprising said recombinant protein fused to a pharmaceutically and/or diagnostically active component.

MODIFIED UBIQUITIN PROTEINS HAVING A SPECIFIC BINDING ACTIVITY FOR
THE EXTRADOMAIN B OF FIBRONECTIN

5

FIELD OF THE INVENTION

The present invention refers to novel proteins, in particular hetero-multimeric proteins, capable of binding the extradomain B of fibronectin (ED-B). Furthermore, the invention
10 refers to fusion proteins comprising said binding protein fused to a pharmaceutically and/or diagnostically active component. The invention is further directed to a method for the generation of such binding protein or fusion protein and to pharmaceutical/diagnostic compositions containing the same. In addition, the invention refers to libraries which are based on a scaffold protein comprising linear polyubiquitin chains with at least two
15 interacting binding determining regions (BDR).

In further embodiments, the invention is directed polynucleotides coding for said binding protein or fusion protein, vectors comprising said polynucleotide and host cells comprising said protein, fusion protein, vector and/or polynucleotide. In a preferred embodiment, said
20 binding protein or fusion protein is included in a medicament or a diagnostic agent. Additionally, methods for producing said recombinant protein or fusion protein as well as use of said proteins in medical treatment methods are described.

BACKGROUND OF THE INVENTION

25

There is a growing demand for binding molecules consisting of amino acids which are not immunoglobulins. While until now antibodies represent the best-established class of binding molecules there is still a need for new binding molecules in order to target ligands with high affinity and specificity since immunoglobulin molecules suffer from major drawbacks.
30 Although they can be produced quite easily and may be directed to almost any target, they have a quite complex molecular structure. There is an ongoing need to substitute antibodies by smaller molecules which can be handled in an easy way. These alternative binding agents

can be beneficially used for instance in the medical fields of diagnosis, prophylaxis and treatment of diseases.

5 Proteins having relatively defined 3-dimensional structures, commonly referred to as protein scaffolds, may be used as starting material for the design of said alternative binding agents. These scaffolds typically contain one or more regions which are amenable to specific or random sequence variation, and such sequence randomisation is often carried out to produce a library of proteins from which the specific binding molecules may be selected. Molecules with a smaller size than antibodies and a comparable or even better affinity towards a target
10 antigen are expected to be superior to antibodies in terms of pharmacokinetic properties and immunogenicity.

A number of previous approaches do use protein scaffolds as starting material of binding proteins. For example, in WO 99/16873 modified proteins of the lipocalin family (so-called
15 Anticalins) exhibiting binding activity for certain ligands were developed. The structure of peptides of the lipocalin family is modified by amino acid replacements in their natural ligand binding pocket using genetic engineering methods. Like immunoglobulins, the Anticalins can be used to identify or bind molecular structures. In a manner analogously to antibodies, flexible loop structures are modified; these modifications enable the recognition of ligands
20 different from the natural ones.

WO 01/04144 describes the artificial generation of a binding domain on the protein surface in beta sheet structural proteins per se lacking a binding site. By means of this de novo generated artificial binding domain e.g. variations in γ -crystallin - an eye lens structural protein - can be
25 obtained which interact with ligands with high affinity and specificity. In contrast to the modification of binding sites which are already present and formed from flexible loop structures as mentioned above for Anticalins, these binding domains are generated de novo on the surface of beta sheets. However, WO 01/04144 only describes the alteration of relatively large proteins for the generation of novel binding properties. Due to their size the proteins
30 according to WO 01/04144 can be modified on the genetic engineering level only by methods which require some effort. Furthermore, in the proteins disclosed so far only a relatively small proportion by percentage of the total amino acids was modified in order to maintain the overall structure of the protein. Therefore, only a relatively small region of the protein surface

is available which can be utilized for the generation of binding properties that did not exist previously. Moreover, WO 01/04144 discloses only the generation of a binding property to γ -crystallin.

5 WO 04/106368 describes the generation of artificial binding proteins on the basis of ubiquitin proteins. Ubiquitin is a small, monomeric, and cytosolic protein which is highly conserved in sequence and is present in all known eukaryotic cells from protozoans to vertebrates. In the organism, it plays a crucial role in the regulation of the controlled degradation of cellular proteins. For this purpose, the proteins destined for degradation are covalently linked to
10 ubiquitin or polyubiquitin chains during their passage through a cascade of enzymes and are selectively degraded because of this label. According to recent results, ubiquitin or the labelling of proteins by ubiquitin, respectively, plays an important role also in other cellular processes such as the import of several proteins or the gene regulation thereof.

15 Besides the clarification of its physiological function, ubiquitin is a research object primarily because of its structural and protein-chemical properties. The polypeptide chain of ubiquitin consists of 76 amino acids folded in an extraordinarily compact α/β structure (Vijay-Kumar, 1987): almost 87% of the polypeptide chain is involved in the formation of the secondary structural elements by means of hydrogen bonds. Secondary structures are three and a half
20 alpha-helical turns as well as an antiparallel β sheet consisting of four strands. The characteristic arrangement of these elements - an antiparallel β sheet exposed of the protein surface onto the back side of which an alpha helix is packed which lies vertically on top of it - is generally considered as so-called ubiquitin-like folding motif. A further structural feature is a marked hydrophobic region in the protein interior between the alpha helix and the β sheet.

25 Because of its small size, artificial preparation of ubiquitin can be carried out both by chemical synthesis and by means of biotechnological methods. Due to the favourable folding properties, ubiquitin can be produced by genetic engineering using microorganisms such as *Escherichia coli* in relatively large amounts either in the cytosol or in the periplasmic space.
30 Because of the oxidizing conditions predominating in the periplasm the latter strategy generally is reserved for the production of secretory proteins. Due to the simple and efficient bacterial preparation ubiquitin can be used as a fusion partner for other foreign proteins to be

prepared for which the production is problematic. By means of fusion to ubiquitin an improved solubility and thereby an improved production yield can be achieved.

5 Compared to antibodies or other alternative scaffolds, artificial binding proteins on the basis of ubiquitin proteins (also referred to as Affilin[®]) have the advantages of a small size and high stability, high affinity high specificity, cost effective microbial manufacturing, and adjustment of serum half life. However, there is still a need to further develop those proteins in terms of new therapeutic approaches with high affinities to specific targets. While WO 05/05730 generally describes the use of ubiquitin scaffolds in order to obtain artificial binding proteins,
10 no solution is provided on how to modify an ubiquitin protein in order to obtain a specific and high affinity binding to the ED-B of fibronectin.

WO 2008/022759 describes recombinant binding protein wherein the Src homology 3 domain (SH3) of the FYN kinase is used for obtaining new binding proteins. It was found that the
15 target specificity can be designed by mutating the RT loop and/or the Src loop in order to develop protein therapeutics and/or diagnostics. Like in lipocalins used as scaffold, the amino acid residues to be mutagenized lie within the variable and flexible loop regions mimicking the principle underlying the antibody/antigen binding function. This overall flexibility of the interaction site by which antibodies bind the epitope is a mainly enthalpically driven process;
20 this process, however, leads to an unfavorable entropic contribution by loss of mobility upon association of the flexible complementarity determining region. Contrary thereto, using ubiquitin as a scaffold, the present inventors did not change amino acid residues primarily within the flexible loop regions but within the rigid and inflexible β strands of a β sheet region or closely adjacent to the beta strands. The advantage of selecting amino acid residues within
25 the inflexible and rigid β strands or closely adjacent to the beta strands of ubiquitin as binding regions for ED-B is *inter alia* the following: The binding partners are thought to already present a complementary geometry appropriate for tight binding. Consequently, these interactions involve complementarity in shape, charge and hydrophilic/hydrophobic elements of the more rigid structures of the binding partners. These rigid body interactions optimize the
30 interface and accommodate biological function.

Fibronectins (FN) are an important class of high molecular weight extracellular matrix glycoproteins abundantly expressed in healthy tissues and body fluids. Their main role consists in facilitating the adhesion of cells to a number of different extracellular matrices.

The presence of fibronectins on the surface of non-transformed cells in culture as well as their absence in the case of transformed cells resulted in the identification of fibronectins as important adhesion proteins. They interact with numerous various other molecules, e.g. collagen, heparan sulphate-proteoglycans and fibrin and thus regulate the cell shape and the creation of the cytoskeleton. In addition, they are responsible for cell migration and cell differentiation during embryogenesis. They also play an important role in wound healing, in which they facilitate the migration of macrophages and other immune cells and in the formation of blood clots by enabling the adhesion of blood platelets to damaged regions of the blood vessels.

10

The extra-domain B (ED-B) of fibronectin is a small domain which is inserted by alternative splicing of the primary RNA transcript into the fibronectin molecule. The molecule is either present or omitted in fibronectin molecules of the extracellular matrix and represents a one of the most selective markers associated with angiogenesis and tissue remodeling, as it is abundantly expressed around new blood vessels, but undetectable in virtually all normal adult tissues (except for uterus and ovaries). ED-B is known to be involved in cancer and in psoriasis. High levels of ED-B expression were detected in almost all human solid cancer entities, including breast, colorectal, pancreatic, non-small cell lung, hepatocellular, intracranial meningioma, human skin, and glioblastoma (Menrad u. Menssen, 2005). Furthermore, ED-B can be bound to diagnostic agents and be favorably used as diagnostic tool. One example is its use in molecular imaging of e.g. atherosclerotic plaques and detection of cancer, e.g. by immunoscintigraphy of cancer patients. Plenty of further diagnostic uses are conceivable.

15

20

The amino acid sequence of 91 amino acids of human extra-domain B (ED-B) of fibronectin is shown in SEQ ID NO: 2. For expression of the protein, a start methionin has to be added. ED-B is conserved in mammals, e.g. in rodents, cattle, primates, carnivore, human etc. Examples of animals in which there is a 100% sequence identity to human ED-B are *Rattus norvegicus*, *Bos taurus*, *Mus musculus*, *Equus caballus*, *Macaca mulatta*, *Canis lupus familiaris*, and *Pan troglodytes*.

25

30

ED-B specifically accumulates in neo-vascular structures and represents a target for molecular intervention in cancer. A number of antibodies or antibody fragments to the ED-B domain of fibronectin are known in the art as potential therapeutics for cancer and other indications (see, for example, WO 97/45544, WO 07/054120, WO 99/58570, WO 01/62800). Human single chain Fv antibody fragment ScFvL19 (also referred to as L19) is specific to the ED-B domain of fibronectin and has been verified to selectively target tumor neovasculature, both in experimental tumor models and in patients with cancer. Furthermore, conjugates comprising an anti-ED-B antibody or an anti-ED-B antibody fragment with cytokines such as IL-12, IL-2, IL-10, IL-15, IL-24, or GM-CSF have been described for targeting drugs for the manufacture of a medicament for inhibiting particularly cancer, angiogenesis, or neoplastic growth (see, for example, WO06/119897, W007/128563, WO01/62298). The selective targeting of neovasculature of solid tumors with anti-ED-B antibodies or anti-ED-B antibody fragments such as L19 conjugated to an appropriate effector function such as a cytotoxic or an immunostimulating agent has proven to be successful in animal experiments. For the therapy of pancreatic cancer, fusion proteins comprising an Interleukin-2 part (IL-2) and an anti-ED-B antibody part were combined with the small molecule Gemcitabine (2'-deoxy-2',2'-difluorocytidine) (see for example WO 07/115837).

The above-discussed prior art documents describe the use of various protein scaffolds including antibodies to generate new ED-B binding proteins.

Targeting ED-B with currently available compounds has certain disadvantages. Smaller molecules (such as ubiquitin-based ED-B binding proteins of this invention) with a comparable or even higher affinity towards the ED-B antigen are expected to have significant advantages to antibodies or other binding proteins.

Since cancer represents one of the leading causes for death worldwide, there is a growing need for improved agents for treating cancer. Current chemotherapeutic agents and radiation treatment suffer from poor selectivity and most chemotherapeutic agents do not accumulate at the tumor site and thus fail to achieve adequate levels within the tumor. There is a strong medical need to effectively treat cancer.

It is thus an object of the present invention to provide new binding proteins based on ubiquitin being able to bind specifically with very high affinity to the extracellular domain of fibronectin (ED-B). It is a further object of the present invention to identify and provide novel binding proteins with very high binding specificity to ED-B for example, for use in the treatment of cancer. Furthermore, a method shall be provided in order to produce said binding molecules.

The above-described objects are solved by the subject-matter of the enclosed independent claims. Preferred embodiments of the invention are included in the dependent claims as well as in the following description, examples and figures.

DESCRIPTION OF THE INVENTION

More specifically, the inventors provide a protein capable of binding the ED-B of human fibronectin, comprising a modified ubiquitin protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 60%, wherein at least 4 amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66, and 68 of SEQ ID NO: 1 are modified in order to obtain a modified ubiquitin protein with a detectable binding to said ED-B of fibronectin with a specific binding affinity of $K_d = 10^{-6} - 10^{-12}$ M.

In a preferred embodiment, the protein is recombinant.

In further embodiments of the invention, 4, 5, 6, 7, 8, 9 or all of the amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66, and 68 of SEQ ID NO: 1 are modified.

Definitions of important Terms used in the Application

The term "extra-domain Bof fibronectin" or briefly designated as "ED-B" comprises all proteins which show a sequence identity to SEQ ID NO: 2 of at least 70%, optionally 75%,
5 further optionally 80%, 85%, 90%, 95%, 96% or 97% or more, or 100% and having the above defined functionality of ED-B.

The terms "protein capable of binding" or "binding protein" refer to an ubiquitin protein comprising a binding domain to ED-B as further defined below. Any such binding protein
10 based on ubiquitin may comprise additional protein domains that are not binding domains, such as, for example, multimerization moieties, polypeptide tags, polypeptide linkers and/or non- proteinaceous polymer molecules. Some examples of non-proteinaceous polymer molecules are hydroxyethyl starch, polyethylene glycol, polypropylene glycol, or polyoxyalkylene.

15 Antibodies and fragments thereof are well known to the person skilled in the art. The binding protein of the invention is **not** an antibody or a fragment thereof, such as Fab or scFv fragments. Further, the binding domain of the invention does not comprise an immunoglobulin fold as present in antibodies.

20 In the present specification, the terms "ligand" and "target" and "binding partner" are used synonymously and can be exchanged. A ligand is any molecule capable of binding with an affinity as defined herein to the hetero-multimeric modified ubiquitin protein.

25 The term "ubiquitin protein" covers the ubiquitin in accordance with SEQ ID NO: 1 and modifications thereof according to the following definition. Ubiquitin is highly conserved in eukaryotic organisms. For example, in all mammals investigated up to now ubiquitin has the identical amino acid sequence. Particularly preferred are ubiquitin molecules from humans, rodents, pigs, and primates. Additionally, ubiquitin from any other eukaryotic source can be
30 used. For instance ubiquitin of yeast differs only in three amino acids from the sequence of SEQ ID NO: 1. Generally, the ubiquitin proteins covered by said term "ubiquitin protein" show an amino acid identity of more than 70%, preferably more than 75% or more than 80%,

of more than 85%, of more than 90%, of more than 95%, of more than 96% or up to a sequence identity of 97% to SEQ ID NO: 1.

5 The term "a modified ubiquitin protein" refers to modifications of the ubiquitin protein by any one of substitutions, insertions, or deletions of amino acids, or a combination thereof. The modified ubiquitin proteins of the invention are engineered proteins with novel binding affinities to targets.

10 For determining the extent of sequence identity of a derivative of the ubiquitin to the amino acid sequence of SEQ ID NO: 1, for example, the SIM Local similarity program (Xiaoquin Huang and Webb Miller, " Advances in Applied Mathematics, vol. 12: 337- 357, 1991) or Clustal, W. can be used (Thompson et al., Nucleic Acids Res., 22(22): 4673-4680, 1994.). Preferably, the extent of the sequence identity of the modified protein to SEQ ID NO: 1 is determined relative to the complete sequence of SEQ ID NO: 1.

15

The "hetero-dimeric fusion protein" or "hetero-dimeric protein" of the invention is considered as a protein which comprises two differently modified monomeric ubiquitin proteins with two interacting binding domain regions providing together a monovalent binding property (binding domain) for ED-B as the specific binding partner.. A hetero-dimer is accomplished
20 by fusing two monomeric ubiquitin molecules wherein both of these molecules are differently modified as described herein.

An advantage of multimerization of differently modified ubiquitin monomers in order to generate hetero-multimeric binding proteins (here: hetero-dimeric proteins) with monovalent
25 binding activity lies in the increase of the total number of amino acid residues that can be modified to generate a new high affinity binding property to ED-B. The main advantage is that while even more amino acids are modified, the protein-chemical integrity is maintained without decreasing the overall stability of the scaffold of said newly created binding protein to ED-B. The total number of residues which can be modified in order to generate a novel
30 binding site for ED-B is increased as the modified residues can be allocated to two monomeric ubiquitin proteins. The number of modifications can so be two corresponding to the number of modified monomeric ubiquitin molecules. A modular structure of the ubiquitin-based ED-B binding protein allows increasing the overall number of modified amino acids as

said modified amino acids are included on two monomeric ubiquitin molecules. The present method provides for the identification of hetero-dimeric ubiquitin molecules having one monovalent specificity for ED-B.

5 Thus, the use of hetero-dimers having a common binding site for binding partners opens up the possibility to introduce an increased number of modified residues which do not unduly influence the protein-chemical integrity of the final binding molecule, since the overall amount of those modified residues is distributed over the two monomeric units which form the dimer. Said hetero-dimeric modified ubiquitin proteins binding to ED-B are present in a
10 library of proteins.

"Monovalent" has to be understood as the capability that both binding regions created in the first and the second monomeric unit of the modified dimeric ubiquitin together bind ED-B in a synergistic and combined manner, i.e. both binding regions act together to form a
15 monovalent binding activity. Taking each binding region of both the first and the second modified ubiquitin in said hetero-dimeric molecule separately will apparently bind ED-B with a much lower efficiency and affinity than the dimeric molecule. Both binding regions form a unique binding site which is formed as a contiguous region of amino acids on the surface of the hetero-dimeric modified ubiquitin protein so that said modified ubiquitin is feasible to
20 bind much more efficient to ED-B than each monomeric protein taken alone. It is particularly important that according to the present invention the two monomeric proteins are not linked together after having screened the most potent binding ubiquitin molecules but that already the screening process is performed in the presence of the hetero-dimeric ubiquitins. After having received the sequence information on the most potent binding ubiquitin molecules,
25 these molecules may be obtained by any other method, e.g. by chemical synthesis or by genetic engineering methods, e.g. by linking the two already identified monomeric ubiquitin units together.

According to the invention, the at least two differently modified ubiquitin monomers which
30 bind to one ligand are to be linked by head-to-tail fusion to each other using e.g. genetic methods. The differently modified fused ubiquitin monomers bind in a monovalent manner and are only effective if both "binding domain regions" ("BDR") act together. A "binding domain region" is defined herein as region on a ubiquitin monomer that has modified amino

acids in at least 4, preferably 6 amino acids of positions 2, 4, 6, 8, 62, 63, 64, 65, 66, 68 of SEQ ID NO:1 which are involved in binding the target.

5 The modified and linked ubiquitin monomers which form the hetero-dimeric protein bind to the same epitope via a single contiguous binding region. This contiguous region of the heteromer is formed by both binding determining regions of the two modules formed by two differently modified ubiquitin monomers.

10 A "head to-tail fusion" is to be understood as fusing two proteins together by connecting them in the direction N-C-N-C- depending on the number of units contained in the dimer. In this head-to-tail fusion, the ubiquitin monomers may be connected directly without any linker. Alternatively, the fusion of ubiquitin monomers can be performed via linkers, for example, a linker having at least the amino acid sequence GIG or having at least the amino acid sequence SGGGG or any other linker, for example GIG, SGGGG, SGGGGIG, SGGGGSGGGGIG or
15 SGGGGSGGGG. Also other linkers for the genetic fusion of two ubiquitin monomers are known in the art and can be used.

The modified ubiquitin proteins of the invention are engineered proteins with novel binding affinities to ED-B as target or ligand (which expressions are used herein interchangeably).
20 The term "substitution" comprises also the chemical modification of amino acids by e.g. substituting or adding chemical groups or residues to the original amino acid. The substitution of amino acids in at least one surface-exposed region of the protein comprising amino acids located in at least one beta sheet strand of the beta sheet region or positioned up to 3 amino acids adjacent to the beta sheet strand is crucial.

25 The substitution of amino acids for the generation of the novel binding domain specific to the ED-B can be performed according to the invention with any desired amino acid, i.e. for the modification to generate the novel binding property to ED-B it is not mandatory to take care that the amino acids have a particular chemical property or a side chain, respectively, which is
30 similar to that of the amino acids substituted so that any amino acid desired can be used for this purpose.

The step of modification of the selected amino acids is performed according to the invention preferably by mutagenesis on the genetic level by random mutagenesis, i.e. a random substitution of the selected amino acids. Preferably, the modification of ubiquitin is carried out by means of methods of genetic engineering for the alteration of a DNA belonging to the
5 respective protein. Preferably, expression of the ubiquitin protein is then carried out in prokaryotic or eukaryotic organisms.

Substitutions are performed particularly in surface-exposed amino acids of the four beta strands of the beta sheets or surface exposed amino acids up to 3 amino acids adjacent to the
10 beta sheet strand of ubiquitin protein. Each beta strand consists usually of 5-7 amino acids. With reference to SEQ ID NO: 1, for example, the beta strands usually cover amino acid residues 2 - 7, 12 - 16, 41 - 45 and 65 - 71. Regions which may be additionally and preferably modified include positions up to 3 amino acids (i.e. 1, 2, or 3) adjacent to the beta sheet strand. The preferred regions which may be additionally and preferably modified include in
15 particular amino acid residues 8-11, 62-64 and 72-75. The preferred regions include beta turns which link two beta strands together. One preferred beta-turn includes amino residues 62 - 64. A most preferred amino acid which is closely adjacent to the beta sheet strand is the amino acid in position 8. In addition, further preferred examples for amino acid substitutions are positions 36, 44, 70, and/or 71. For example, those regions which may be additionally and
20 preferably modified include amino acids 62, 63, and 64 (3 amino acids), or 72, 73 (2 amino acids), or 8 (1 amino acid).

In preferred embodiments, the amino acid residues are altered by amino acid substitutions. However, also deletions and insertions are allowable. The number of amino acids which may
25 be added or deleted is limited to 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in a monomeric ubiquitin subunit, and accordingly 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 24, 26 or 28 amino acids with respect to the dimeric ubiquitin protein, generally x-times the number of modifications in the monomeric protein. In one embodiment, no amino acid insertions are made. In a still further embodiment, no deletions have been performed.

30

Provided that the modified ubiquitin protein of the present invention comprises substitutions, deletions and/or additions of one or more amino acids, the amino acid positions given for wildtype human ubiquitin (SEQ ID NO: 1) have to be aligned with the modified ubiquitin in

order to allot the corresponding proteins to each other. In case of fusion proteins (see below), the numbering (and alignment) of each of the monomeric ubiquitin subunits is done in the same way, i.e. an alignment of, for example, a dimer is started at amino acid position 1 for each respective subunit.

5

In monomeric ubiquitin, preferably from mammals, e.g. human, at least 10% of the amino acids present in beta strands or positions up to 3 amino acids adjacent to the beta sheet strand, preferably at least 20%, further preferably at least 25%, can be modified, preferably substituted, according to the present invention to generate a binding property that did not exist previously. At a maximum, preferably about 50% of the amino acids present in beta strands or positions up to 3 amino acids adjacent to the beta sheet strand, further preferably at a maximum about 40% or about 35% or up to about 30% or up to about 25% are modified, preferably substituted. In one beta strand, generally one to four amino acids are modified. In one embodiment, three of six amino acids in preferably the first and the fourth beta strand, e.g. region of amino acid residues 2-7 or 65-71, are modified.

15

A modified monomeric ubiquitin according to the invention used as building unit for a heterodimer accounts for in total up to 20% of amino acids. Considering this, there is a sequence identity to SEQ ID NO:1 of the modified ubiquitin protein to at least 60%. In further embodiments of the invention, the sequence identity on amino acid level is at least 60%, 70%, at least 80% and furthermore at least 90% or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1. The invention covers also amino acid sequence identities of more than 65%, 75%, 85% or 97% of the modified ubiquitin protein compared to the amino acid sequence of SEQ ID NO: 1.

20

In a further embodiment of the invention, an ubiquitin is modified in 3 or 4 or 5 or 6 or 7 amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66, and/or 68 of SEQ ID NO: 1. In another embodiment, the ubiquitin to be modified in these positions, was already pre-modified. For example, further modifications could comprise modifications at amino acids 74 and 75 or at amino acid 45 to generate better stability or protein-chemical properties. A modified ubiquitin monomer is obtainable wherein in total up to 9, 10, 11, 12, 13, 14 and a maximum of 15 amino acids of the ubiquitin of SEQ ID NO: 1 are modified, preferably substituted. According to an example, a modified monomeric ubiquitin could be obtained having 14

25

30

substitutions and a deletion. Based on the total number of amino acids of ubiquitin this corresponds to a percentage of about 20%. This was extraordinarily surprising and could not be expected since usually a much lower percentage is already sufficient to disturb the folding of the protein.

5

In one embodiment of the invention, those amino acids are modified for the generation of a region having the novel binding properties which form a contiguous region on the surface of the protein. In this manner, a contiguous region can be generated which has a binding property to the ED-B. "Contiguous region" according to the invention refers to the following:
10 due to the charge, the spatial structure and the hydrophobicity/hydrophilicity of their side chains, amino acids interact with their environment in the corresponding manner. The environment can be the solvent, generally water, or other molecules, e.g. spatially close amino acids. By means of structural information about the protein as well as the respective software the surface of the proteins can be characterized. For example, the interface region between the
15 atoms of the protein and the solvent can be visualized in this way including the information about how this interface region is structured, which surface areas are accessible to the solvent or how the charges are distributed on the surface. A contiguous region can be revealed for example by visualization of this type using suitable software. Such methods are known to those skilled in the art. According to the invention, basically, also the whole surface-exposed
20 region can be used as the contiguous region on the surface to be modified for the generation of novel binding properties. In one embodiment, for this purpose a modification can also comprise the α -helical region. In a hetero-dimeric modified ubiquitin protein, a binding-determining region comprises two of the surface-exposed regions forming together one contiguous region which comprises two times the length of one binding determining region.

25

The modification of amino acids in at least one surface-exposed region of the protein comprising at least one beta strand of the beta sheet region or positions up to 3 amino acids adjacent to the beta sheet strand is crucial. The "beta sheet structure" is defined by being essentially sheet-like and almost completely stretched. In contrast to alpha helices which are
30 formed from an uninterrupted segment of the polypeptide chain, beta sheets can be formed by different regions of the polypeptide chain. In this way, regions spaced further apart in the primary structure can get into close proximity with each other. A beta strand typically has a length of 5-10 amino acids (usually 5-6 residues in ubiquitin) and has an almost completely

stretched conformation. The beta strands come so close to each other that hydrogen bonds form between the C-O group of one strand and the NH group of the other strand and vice versa. Beta-sheets can be formed from several strands and have a sheet-like structure wherein the position of the C alpha atoms alternates between above or below the sheet-like plane. The amino acid side chains follow this pattern and, thus, alternatively point towards the top or towards the bottom. Depending on the orientation of the beta strands the sheets are classified into parallel and antiparallel sheets. According to the invention both can be mutated and used for the preparation of the proteins claimed.

For the mutagenesis of the beta strands and the beta-sheet structure, a beta strand or positions up to 3 amino acids adjacent to the beta strand (which is a strand of the beta sheet) are selected in the ubiquitin that are close to the surface. Surface-exposed amino acids can be identified with respect to the available x-ray crystallographic structure. If no crystal structure is available attempts can be made by means of computer analysis to predict surface-exposed beta sheet regions and the accessibility of individual amino acid positions with respect to the available primary structure or to model the 3d protein structure and to obtain information about potential surface-exposed amino acids in this manner. Further disclosure thereof can be taken e.g. from J. Mol. Biol., 1987 Apr 5; 194(3):531-44. Vijay-Kumar S, Bugg C.E., Cook W.J.

It is, however, also possible to carry out modifications in the beta sheet or of positions up to 3 amino acids adjacent to the beta strand for which the time-consuming pre-selection of amino acid positions to be mutagenized can be omitted. Those DNA regions encoding the beta sheet structures or up to 3 amino acids adjacent to the beta sheet strand are isolated from their DNA environment, subjected to random mutagenesis and are afterwards re-integrated into the DNA coding for the protein from which they were removed previously. This is followed by a selection process for mutants with the desired binding properties.

In another embodiment of the invention the beta strands or up to 3 amino acids adjacent to the beta strand close to the surface are selected as already explained above and the amino acid positions to be mutagenized within these selected regions are identified. The amino acid positions selected in this way can then be mutagenized on the DNA level either by site-directed mutagenesis, i.e. a codon coding for a specific amino acid is substituted by a codon encoding another previously selected specific amino acid, or this substitution is carried out in

the context of a random mutagenesis wherein the amino acid position to be substituted is defined but not the codon encoding the novel, not yet determined amino acid.

5 Surface-exposed amino acids are amino acids that are accessible to the surrounding solvent. If the accessibility of the amino acids in the protein is more than 8% compared to the accessibility of the amino acid in the model tripeptide Gly-X-Gly, the amino acids are called "surface-exposed". These protein regions or individual amino acid positions, respectively, are also preferred binding sites for potential binding partners for which a selection shall be carried out according to the invention. In addition, reference is made to Caster et al., 1983 Science, 10 221, 709 - 713, and Shrake & Rupley, 1973 J. Mol. Biol. 79(2):351-371, which for complete disclosure are incorporated by reference in this application.

Variations of ubiquitin protein scaffold differing by amino acid substitutions in the region of the de novo generated artificial binding site from the parental protein and from each other can be generated by a targeted mutagenesis of the respective sequence segments. In this case, 15 amino acids having certain properties such as polarity, charge, solubility, hydrophobicity or hydrophilicity can be replaced or substituted, respectively, by amino acids with respective other properties. Besides substitutions, the terms "mutagenesis" and "modified" and "replaced" comprises also insertions and deletions. On the protein level the modifications can also be carried out by chemical alteration of the amino acid side chains according to methods 20 known to those skilled in the art.

Methods of mutagenesis of ubiquitin

25 As a starting point for the mutagenesis of the respective sequence segments, for example the cDNA of ubiquitin which can be prepared, altered, and amplified by methods known to those skilled in the art can be used. For site-specific alteration of ubiquitin in relatively small regions of the primary sequence (about 1-3 amino acids) commercially available reagents and methods are on hand ("Quick Change", Stratagene; "Mutagene Phagemid in vitro 30 Mutagenesis Kit", Biorad). For the site-directed mutagenesis of larger regions specific embodiments of e.g. the polymerase chain reaction (PCR) are available to those skilled in the art. For this purpose a mixture of synthetic oligodeoxynucleotides having degenerated base pair compositions at the desired positions can be used for example for the introduction of the

mutation. This can also be achieved by using base pair analogs which do not naturally occur in genomic DNA, such as e.g. inosine.

5 Starting point for the mutagenesis of one or more beta strands of the beta sheet region or positions up to 3 amino acids adjacent to the beta sheet strand can be for example the cDNA of ubiquitin or also the genomic DNA. Furthermore, the gene coding for the ubiquitin protein can also be prepared synthetically.

10 Different methods known per se are available for mutagenesis, e.g. methods for site-specific mutagenesis, methods for random mutagenesis, mutagenesis using PCR or similar methods.

In a preferred embodiment of the invention the amino acid positions to be mutagenized are predetermined. The selection of amino acids to be modified is carried out to meet the limitations of present claim 1 with respect to those amino acids which have to be modified. In
15 each case, a library of different mutants is generally established which is screened using methods known *per se*. Generally, a pre-selection of the amino acids to be modified can be particularly easily performed as sufficient structural information is available for the ubiquitin protein to be modified.

20 Methods for targeted mutagenesis as well as mutagenesis of longer sequence segments, for example by means of PCR, by chemical mutagenesis or using bacterial mutator strains also belong to the prior art and can be used according to the invention.

In one embodiment of the invention the mutagenesis is carried out by assembly of DNA
25 oligonucleotides carrying the amino acid codon NNK. It should be understood, however, that also other codons (triplets) can be used. The mutations are performed in a way that the beta sheet structure is preferably maintained. Generally, the mutagenesis takes place on the outside of a stable beta sheet region exposed on the surface of the protein. It comprises both site-specific and random mutagenesis. Site-specific mutagenesis comprising a relatively small
30 region in the primary structure (about 3-5 amino acids) can be generated with the commercially available kits of Stratagene (QuickChange) or Bio-Rad (Mutagene phagemid in vitro mutagenesis kit) (cf. US 5,789,166; US 4,873,192).

If more extended regions are subjected to site-specific mutagenesis a DNA cassette must be prepared wherein the region to be mutagenized is obtained by the assembly of oligonucleotides containing the mutated and the unchanged positions (Nord et al., 1997 Nat. Biotechnol. 8, 772-777; McConell and Hoess, 1995 J. Mol. Biol. 250, 460-470.). Random mutagenesis can be introduced by propagation of the DNA in mutator strains or by PCR amplification (error-prone PCR) (e.g. Pannekoek et al., 1993 Gene 128, 135-140). For this purpose, a polymerase with an increased error rate is used. To enhance the degree of the mutagenesis introduced or to combine different mutations, respectively, the mutations in the PCR fragments can be combined by means of DNA shuffling (Stemmer, 1994 Nature 370, 389-391). A review of these mutagenesis strategies with respect to enzymes is provided in the review of Kuchner and Arnold (1997) TIBTECH 15, 523-530. To carry out this random mutagenesis in a selected DNA region also a DNA cassette must be constructed which is used for mutagenesis.

Random modification is performed by methods well-established and well-known in the art. A "randomly modified nucleotide or amino acid sequence" is a nucleotide or amino acid sequence which in a number of positions has been subjected to insertion, deletion or substitution by nucleotides or amino acids, the nature of which cannot be predicted. In many cases the random nucleotides (amino acids) or nucleotide (amino acid) sequences inserted will be "completely random" (e. g. as a consequence of randomized synthesis or PCR-mediated mutagenesis). However, the random sequences can also include sequences which have a common functional feature (e. g. reactivity with a ligand of the expression product) or the random sequences can be random in the sense that the ultimate expression product is of completely random sequence with e. g. an even distribution of the different amino acids.

In order to introduce the randomized fragments properly into the vectors, it is according to the invention preferred that the random nucleotides are introduced into the expression vector by the principle of site directed PCR-mediated mutagenesis. However, other options are known to the skilled person, and it is e. g. possible to insert synthetic random sequence libraries into the vectors as well.

To generate mutants or libraries by fusion PCR, for example three PCR reactions may be carried out. Two PCR reactions are performed to generate partially overlapping intermediate fragments. A third PCR reaction is carried out to fuse the intermediate fragments.

- 5 The method for construction of the library or mutant variants may include constructing a first set of primers around a desired restriction site (restriction site primer), a forward and reverse restriction primer and a second set of primers around, e. g. , upstream and downstream of the codon of interest (the mutagenic primers), a forward and reverse mutagenic primer. In one embodiment, the primers are constructed immediately upstream and downstream respectively
- 10 of the codon of interest. The restriction and mutagenic primers are used to construct the first intermediate and second intermediate fragments. Two PCR reactions produce these linear intermediate fragments. Each of these linear intermediate fragments comprises at least one mutated codon of interest, a flanking nucleotide sequence and a digestion site. The third PCR reaction uses the two intermediate fragments and the forward and reverse restriction primers
- 15 to produce a fused linear product. The opposite, here to for unattached ends of the linear product are digested with a restriction enzyme to create cohesive ends on the linear product. The cohesive ends of the linear product are fused by use of a DNA ligase to produce a circular product, e. g. a circular polynucleotide sequence.
- 20 To construct the intermediate fragments, the design and synthesis of two sets of forward and reverse primers are performed by providing a first set containing a restriction enzyme digestion site together with its flanking nucleotide sequence, and a second set containing at least one variant codon of interest (mutagenic primers). Those skilled in the art will recognize that the number of variants will depend upon the number of variant amino acid modifications
- 25 desired. It is contemplated by the inventor that if other restriction enzymes are used in the process, the exact location of this digestion site and the corresponding sequence of the forward and reverse primers may be altered accordingly. Other methods are available in the art and may be used instead.
- 30 Apart from having the randomized fragment of the expression product introduced into a scaffold in accordance with the present invention, it is often necessary to couple the random sequence to a fusion partner by having the randomized nucleotide sequence fused to a nucleotide sequence encoding at least one fusion partner. Such a fusion partner can e. g.

facilitate expression and/or purification/isolation and/or further stabilization of the expression product.

5 Random substitution of amino acids according to one example of the present invention of at least 3 or 4 amino acids at positions 2, 4, 6, 8, 62, 63, 64, 65, 66 and/or 68 of monomeric ubiquitin can be performed particularly easily by means of PCR since the positions mentioned are localized close to the amino or the carboxy terminus of the protein. Accordingly, the codons to be manipulated are at the 5' and 3' end of the corresponding cDNA strand. Thus, the first oligodeoxynucleotide used for a mutagenic PCR reaction apart from the codons at
10 positions 2, 4, 6, and/or 8 to be mutated - corresponds in sequence to the coding strand for the amino terminus of ubiquitin. Accordingly, the second oligodeoxynucleotide - apart from the codons of positions 62, 63, 64, 65, 66, and/or 68 to be mutated - at least partially corresponds to the non-coding strand of the polypeptide sequence of the carboxy terminus. By means of both oligodeoxynucleotides a polymerase chain reaction can be performed using the DNA
15 sequence encoding the monomeric ubiquitin as a template.

Furthermore, the amplification product obtained can be added to another polymerase chain reaction using flanking oligodeoxynucleotides which introduce for example recognition sequences for restriction endonucleases. It is preferred according to the invention to introduce
20 the gene cassette obtained into a vector system suitable for use in the subsequent selection procedure for the isolation of ubiquitin variations having binding properties to a predetermined hapten or antigen.

Regions to be modified in ubiquitin

25

The regions for modification can be basically selected as to whether they can be accessible for ED-B as binding partner and whether the overall structure of the protein will presumably show tolerance to a modification.

Besides modifications in surface-exposed beta strands also modifications in other surface-
30 exposed regions of the protein can be carried out, preferably in positions up to 3 amino acids adjacent to the beta strand. These modified regions are involved in the newly generated binding with high affinity to ED-B.

According to another preferred embodiment of the present invention at least 3 or 4 or 6, optionally at least 8, 10, 12 and maximal 15 surface-exposed amino acids of ubiquitin, preferably mammalian or human ubiquitin, can be modified wherein a substitution is preferred as the modification. This comprises the modification of 3,, 4, 5, 6, 7, 8, 9, 10, 11, 5 12, 13, 14, or 15 surface-exposed amino acids of ubiquitin. These at least 4 and maximal 15 surface-exposed modified amino acids then form the region with binding affinity to the predetermined binding partner. This region is defined herein as “binding domain region” (BDR). In this respect, it is particularly preferred that at least 2, optionally at least 4, further optionally at least 6, 8, 10, 12 and maximal 15 of the surface-exposed amino acids are in a 10 beta sheet region, i.e. in a beta sheet strand or distributed on several beta strands or positions up to 3 amino acids adjacent to a beta sheet strand. It is further preferred that at least 3 of all modified, preferably substituted, amino acids are directly adjacent to each other in the primary sequence.

15 In another optional embodiment of the present invention amino acids in one or two, preferably two of the four beta strands in the protein or positions up to 3 amino acids adjacent to preferably two of the four beta strands are modified to generate a novel binding property. Also optional is a modification in three or four of the four beta strands or positions up to 3 amino acids adjacent to three or four of the beta strands for the generation of an ED-B 20 binding.

It is particularly preferred that amino acids in the amino-terminal and carboxy-terminal strand or in positions up to 3 amino acids adjacent to the amino-terminal and carboxy-terminal strand are modified, preferably substituted, to generate a novel binding site to ED-B. In this 25 respect, it is particularly preferred that up to 4 amino acids adjacent to the carboxy-terminal beta sheet strand are modified, preferably substituted, and up to 1 amino acid adjacent to the amino-terminal beta sheet strand is modified, preferably substituted.

Particularly preferred is a modification, preferably a substitution, in at least three surface- 30 exposed amino acids of the following positions of a mammalian ubiquitin, preferably human ubiquitin: 2, 4, 6, 8, 62, 63, 64, 65, 66, 68. These at least four amino acids from said group of amino acids form a contiguous surface-exposed region on the surface of ubiquitin which was found to be particularly suitable for the generation of modified proteins having a binding

affinity that did not exist previously with respect to the ED-B as binding partner. At least 3 of these amino acid residues have to be modified. Optionally 3, 4, 5, 6, 7, 8, 9 or 10 of said amino acid residues are modified, optionally in combination with additional amino acid residues.

5

After having made the modifications above, the inventors have found the amino acid modified ubiquitin sequences described in the examples which bind ED-B with very high affinity (Kd values up to 10^{-9}).

10 Fusion proteins

In another preferred embodiment, the invention relates to a fusion protein comprising a binding protein of the invention fused to a pharmaceutically and/or diagnostically active component.

15

In a still further aspect, the invention relates to a fusion protein comprising a heterodimeric binding protein of the invention fused to a pharmaceutically and/or diagnostically active component. A fusion protein of the invention may comprise non-polypeptide components, e.g. non-peptidic linkers, non-peptidic ligands, e.g. for therapeutically or diagnostically relevant radionuclides. It may also comprise small organic or non-amino acid based compounds, e.g. a sugar, oligo- or polysaccharide, fatty acid, etc. In one preferred embodiment of the invention, the heteromeric ubiquitin-based ED-B binding molecule is covalently or non-covalently conjugated to a protein or peptide having therapeutically or diagnostically relevant properties.

25

The following gives some examples on how to obtain ubiquitin-based fusion proteins with ED-B binding capacity.

30

- a) conjugation of the protein via Lysine residues present in ubiquitin;
- b) conjugation of the heterodimeric ubiquitin-based binding protein via Cysteine residues – can be located C-terminal, or at any other position (e.g. amino acid residue 24 or 57); conjugation with maleimid selectable components;
- c) peptidic or proteinogenic conjugations – genetic fusions (preferred C- or N-terminal);

- d) "Tag"-based fusions - A protein or a peptide located either at the C- or N- terminus of the target protein ED-B. Fusion "tags", e.g. poly-histidine (particularly relevant for radiolabeling).

- 5 These and other methods for covalently and non-covalently attaching a protein of interest to a support are well known in the art, and are thus not described in further detail here.

Optionally, said active component is a cytokine, preferably a cytokine selected from the group consisting of tumor necrosis factors (e.g. TNF alpha, TNF beta), interleukins (e.g. IL-2, IL-10, IL-15, IL-24, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, IL-8, IL-1 alpha, IL-1 beta), interferons (e.g. IFN alpha, IFN beta, IFN gamma), GM-CSF, GRO (GRO alpha, GRO beta, GRO gamma), MIP (MIP-1-alpha, MIP-1 beta, MIP-3 alpha, MIP-3 beta), TGF-beta, LIF, CD80, CD-40 ligand, B70, LT-beta, Fas-ligand, ENA-78, LDGF-PBP, GCP-2, PF4, Mig, IP-10, SDF-1 alpha/beta, BUNZO/STRC33, I-TAC, BLC/BCA-1, MDC, TECK, TARC, RANTES, HCC-1, HCC-4, DC-CK1, MCP-1-5, Eotaxin, Eotaxin-2, I-309, MPIF-1, 6Ckine, CTACK, MEC, Lymphotactin, Fractalkine, and others.

One of the most preferred cytokines for use in the present invention is TNF. The inflammatory cytokine TNF has multiple activities in the mammalian body including an anti-tumor effect that is currently clinically irrelevant due to unacceptable toxicity of effective doses in humans. Currently, TNF is therapeutically used in combination with cytostatic substances like Melphalan.

Further optionally, said active component that can be conjugated to the hetero-multimeric ubiquitin-based binding protein is a toxic compound, preferably a small organic compound or a polypeptide, optionally a toxic compound, for example, selected from the group consisting of saporin, truncated Pseudomonas exotoxin A, recombinant gelonin, Ricin-A chain, calicheamicin, neocarzinostatin, esperamicin, dynemicin, kedarcidin, maduropeptin, doxorubicin, daunorubicin, auristatin, cholera toxin, modeccin, diphtheria toxin.

30

In a further embodiment of the invention the ubiquitin-based binding protein according to the invention may contain artificial amino acids.

In further embodiments of the fusion protein of the present invention said active component is a fluorescent dye, preferably a component selected from the groups of a radionuclide either from the group of gamma-emitting isotopes, preferably ^{99}Tc , ^{123}I , ^{111}In , or from the group of positron emitters, preferably ^{18}F , ^{64}Cu , ^{68}Ga , ^{86}Y , ^{124}I , or from the group of beta-emitter, preferably ^{131}I , ^{90}Y , ^{177}Lu , ^{67}Cu , or from the group of alpha-emitter, preferably ^{213}Bi , ^{211}At ; Alexa Fluor or Cy dyes (Berlier et al., J. Histochem. Cytochem. 51 (12): 1699-1712, 2003.); a photosensitizer; a pro-coagulant factor, preferably tissue factor (e.g. tTF truncated tissue factor); an enzyme for pro-drug activation, preferably an enzyme selected from the group consisting of carboxy-peptidases, glucuronidases and glucosidases; and/or a functional Fc domain, preferably a human functional Fc domain.

A further embodiment relates to fusion proteins according to the invention, further comprising a component modulating serum half-life, preferably a component selected from the group consisting of polyethylene glycol albumin-binding peptides, and immunoglobulin.

15

Binding specificities

The binding specificities (dissociation constants) of the fusion proteins according to the invention are as defined above for the non-fusion protein given in Kd. In accordance with the invention, the term "Kd" defines the specific binding affinity which is in accordance with the invention in the range of 10^{-5} - 10^{-12} M. A value of 10^{-5} M and below can be considered as a quantifiable binding affinity. Depending on the application a value of 10^{-6} M to 10^{-12} M is preferred, further preferably 10^{-6} M to 10^{-11} M for e.g. chromatographic applications or 10^{-9} to 10^{-12} M for e.g. diagnostic or therapeutic applications. Further preferred binding affinities are in the range of 10^{-7} to 10^{-10} M, preferably to 10^{-11} M.

The methods for determining the binding affinities are known per se and can be selected for instance from the following methods: ELISA, Surface Plasmon Resonance (SPR) based technology (offered for instance by Biacore®), fluorescence spectroscopy, isothermal titration calorimetry (ITC), analytical ultracentrifugation, FACS. Other methods available in the art can be used also by the expert within his general knowledge.

30

After having made the modifications above, the inventors have found the amino acid modified ubiquitin sequences described in the examples which bind their targets with very high affinity (Kd values up to 10^{-12} M).

5 **Multimerization of ubiquitin**

In a further embodiment of the invention said ubiquitin protein capable of binding ED-B is fused with at least one second ubiquitin protein capable of binding ED-B to obtain a multimer, optionally a dimer or trimer of said ubiquitin protein which is optionally fused with
10 a pharmaceutically active component, optionally a cytokine, or a diagnostic component. Alternatively, said ubiquitin protein capable of binding ED-B is fused with at least one second ubiquitin protein capable of binding ED-B to obtain a multimer, optionally a dimer or trimer of said ubiquitin protein wherein multimer, dimer or trimer is formed via said
15 pharmaceutically active component which is optionally TNF-alpha, or via said diagnostic component.

The "multimer" is considered as a protein herein which might comprise the same or one or more different monomeric ubiquitin proteins. If the multimer contains the same monomeric ubiquitin units, then a homomer or homo-multimer (for example homodimer or -trimer) will
20 be formed. If the dimer comprises two differently modified monomers, it is called a "heteromeric-dimer" or "hetero-dimer". If more than one different monomeric ubiquitin units are present, a heteromer or hetero-multimer will be formed. In a most preferred embodiment of this invention, the heteromer with ED-B binding capability consists of at least two different modified ubiquitin monomers. A "hetero-dimer" of the invention is considered as a fusion of
25 modified ubiquitin proteins consisting out of two different monomeric ubiquitin proteins having each a monovalent binding property for a specific binding partner.

Thus, the "hetero-multimer", optionally "hetero-dimer" of the invention is considered as a fusion of a least two differently modified (or at least one modified and one not modified)
30 monomeric ubiquitin proteins exhibiting a combined monovalent binding property for the specific binding partner ED-B. It is emphasized that the modified hetero-dimeric ED-B binding ubiquitin protein of the invention is not obtained by separately screening each monomeric ubiquitin protein and combining two of them afterwards but by screening for

hetero-dimeric proteins consisting of a first and a second monomeric unit which exhibit together a monovalent binding activity of said ED-B ligand. It is to be expected that each of said subunits exhibit a quite limited binding affinity towards ED-B while only the combined dimeric modified ubiquitin protein will have the excellent binding properties described herein (see, for example, Figure 4).

According to the invention two differently modified ubiquitin monomers (or at least one modified and one not modified) genetically linked by head-to-tail fusion bind to the same epitope of ED-B and are only effective if both binding domain regions act together. The BDRs of the monomers form a single contiguous binding region.

In one embodiment of the invention the modified ubiquitin protein provided according to the invention can be linked to an ubiquitin protein of a different specificity in a preferably site-specific manner thereby obtaining a modularly composed ubiquitin with monovalent binding properties, respectively, with respect to a binding partner.

Thus, for example two variations of ubiquitin obtained by the procedure described above can be linked to each other in a site-specific manner. Thus, if multimers are formed, this can be done by producing the monomeric ubiquitin units and linking them in a head-to-tail-manner. That is to say, the monomeric units are linked N-C-N-C- depending on the number of units contained in the multimer. The multimerization can be done either directly using no linkers or via linkers.

In another embodiment of the invention, two variations of modified ubiquitin monomers which bind to ED-B can be genetically linked to each other by appropriate methods so that exclusively highly specific binding molecules are obtained. Most preferred, two variations of modified ubiquitin monomers genetically linked by head-to-tail fusion bind to the same epitope and are only effective if both binding domain regions act together. Or in other words, they bind to the same epitope via a single contiguous binding region which is formed by the acting together of both binding regions of the two modules.

Thus, the ubiquitin protein modified in accordance with the invention to efficiently bind ED-B of fibronectin may be multimerized, particularly dimerized or trimerized. The fusion of

ubiquitin monomers can be performed via linkers, for example, a linker having at least the sequence GIG or any other linker, for example SGGGGIG or SGGGSGGGGIG. Another linker for the genetic fusion of two or more ubiquitin monomers is possible. Preferably, such an ubiquitin protein consists of at least two binding determining regions (BDR) wherein each BDR shows modifications in at least one of amino acids 2, 4, 6, 8, 62, 63, 64, 65, 66, 68 and wherein the BDR are genetically fused to each other. The binding to the target is mediated by both BDRs in collaboration.

A further multimerization of the modified ubiquitin protein can be performed for example by fusing the modified ubiquitin protein to effector molecules having a multimerization domain like a cytokine for example TNF- α . A fusion of one or more modified ubiquitin monomers, such as dimers, preferably hetero-dimers, with TNF-alpha can be done via linkers. In a still further embodiment, said multimerization is performed by genetically fusing two or more modified ubiquitin molecules or by using a polyethylene glycol (PEG) linker. In a still further embodiment said multimerization domain also acts as pharmaceutically active component; one example is TNF-alpha acting both as multimerization domain and pharmaceutical component.

In a further embodiment of the invention, the multimerized modified ubiquitin protein of the invention consists of heteromers. The term "heteromer" means that ubiquitin monomers of different compositions are included in the multimerized molecule. Particularly preferred are hetero-dimers consisting of two genetically fused ubiquitin monomers with different binding domain regions but having one binding site. These hetero-dimers can be multimerized by the use of for example TNF, most preferred, TNFalpha. One advantage of using ubiquitin hetero-dimers is to target ED-Bs with high binding specificities.

A further advantage of dimerization or generally multimerization lies in the increase of the number of amino acid residues to be modifiable maintaining at the same time protein-chemical integrity without decreasing the overall stability of the scaffold of said newly created binding protein to ED-B. On the one hand the total number of residues which can be modified in order to generate the binding site for ED-B is increased as the modified residues can be allocated to the scaffolds of two or three ubiquitin proteins; on the other hand the number of residues on the scaffold of one ubiquitin protein is decreased maintaining at the

same time the protein-chemical stability of the modified binding molecule. Summarizing, a modular structure of the ubiquitin-based ED-B binding protein allows increasing the overall number of modified amino acids as said modified amino acids are included on two or three ubiquitin molecules. The number of modifications as described above is given per one molecule of modified ubiquitin.

Further, multimerisation may provide modified ubiquitin molecules having two (or more) binding sites for target substances, having one monovalent specificity (for one single epitope), but may also provide bi- or multispecific (thus recognizing two or more different epitopes at one time).

If two or more modified ubiquitin molecules are fused, preferably as genetic head-to-tail fusion, the number of modifications can be two times or x-times corresponding to the number of modified ubiquitin molecules. As an alternative, the fusion can be post-translationally.

Regarding the use of monomeric ubiquitin proteins in modified form, the advantage of using multimeric structures such as dimers, in particular homo- or hetero-dimers, resides in the increase of the number of modifiable residues which do not affect the proteinchemical integrity of the underlying scaffold.

Modified ubiquitin proteins bind to ED-B

According to the invention, modified ubiquitin monomers were identified that bind to ED-B. Preferred modified ubiquitin hetero-dimers with specific binding to ED-B are as follows:

1. The binding determining region (BDR1) of the first ubiquitin molecule: modified amino acids 2, 4, 6, 62 to 68, preferably genetically fused to a second ubiquitin molecule with modified amino acids 6, 8, 62 to 68 defining the BDR2.
2. The binding determining region (BDR1) of the first ubiquitin molecule: modified amino acids 6, 8, 62 to 68, preferably genetically fused to a second ubiquitin molecule: modified amino acids 6, 8, 62 to 68 defining the BDR2.

3. The binding determining region (BDR1) of the first ubiquitin molecule: modified amino acids 2, 4, 6, 8, 62 to 68, preferably genetically fused to a second ubiquitin molecule: modified amino acids 2, 4, 6, 8, 62 to 68 defining the BDR2.

4. The binding determining region (BDR1) of the first ubiquitin molecule: modified amino acids 6, 8, 63 to 66, preferably genetically fused to a second ubiquitin molecule: modified amino acids 2, 6, 8, 62 to 68 defining the BDR2.

In an embodiment, the fusion protein is a genetically fused dimer of said ubiquitin protein having amino acids substitutions in positions 6, 8, 63-66 of the first ubiquitin monomer and substitutions in amino acid residues in positions 6, 8, 62-66, and optionally in position 2 of the second ubiquitin monomer, preferably

Lysine (K) to Tryptophane (W) or Phenylalanine (F) in position 6,

Leucine (L) to Tryptophane or Phenylalanine (W, F) in position 8,

Lysine (K) to Arginine (R) or Histidine (H) in Position 63,

15 Glutamic acid (E) to Lysine (K), Arginine (R) or Histidine (H) in position 64,

Serine (S) to Phenylalanine (F) or Tryptophane (W) in position 65 and

Threonine (T) to Proline (P) in position 66;

- in the the second ubiquitin monomer, the substitutions

Lysine (K) to Threonine (T), Asparagine (N), Serine (S) or Glutamine (Q) in position 6,

20 Leucine (L) to Glutamine (Q) or Threonine (T) or Asparagine (N) or Serine (S) in position 8,

Glutamine (Q) to Tryptophane (W) or Phenylalanine (F) in position 62,

Lysine (K) to Serine (S), Threonine (T), Asparagine (N) or Glutamine (Q) in position 63,

Glutamic acid (E) to Asparagine (N), Serine (S), Threonine (T), or Glutamine (Q) in position 64,

25 Serine (S) to Phenylalanine (F) or Tryptophane (W) in position 65, and

Threonine (T) to Glutamic acid (E) or Aspartic acid (D) in position 66, and

Optionally Glutamine (Q) to Arginine (R), Histidine (H) or Lysine (K) in position 2 are preferred.

30

Most preferred are the following substitutions:

(1) in the first monomeric unit at least- K6W, L8W, K63R, E64K, S65F, and T66P;

and in the second monomeric unit at least- K6T, L8Q, Q62W, K63S, E64N, S65W, and T66E; optionally additionally Q2R, or

(2) in the first monomeric unit at least Q2T, F4W, K6H, Q62N, K63F, E64K, S65L, and
5 T66S;

and in the second monomeric unit at least K6X, L8X, Q62X, K63X, E64X, S65X, and T66X; optionally additionally Q2X, wherein X can be any amino acid.

Particularly preferred are the following substitutions in the first ubiquitin monomer to
10 generate binding proteins for ED-B:

2: Q→T, 4: F→W, 6: K→H, 62: Q→N, 63: K→F, 64: E→K, 65: S→L, 66: T→S

Either no linker or any linker can be used to connect the two monomers head-to-tail. Preferred
linkers are those of SEQ ID NO: 32 or the sequence GIG or SGGGGIG or
15 SGGGGSGGGGIG.

In a preferred embodiment, a ubiquitin hetero-dimer with two binding determining regions
(BDR) acting together for binding ED-B comprises the amino acid sequence of SEQ ID NO:
33 or 34. A preferred fusion protein of the invention comprising TNF-alpha as a
20 pharmaceutically active component has the sequence of SEQ ID NO: 35 or 36. A further
preferred protein is provided by the following sequence wherein XXXX may be any amino
acid (SEQ ID NO: 47). As linker, SGGGGSGGGGIG was used here (shown in italics). It is to
be understood that also other kind of linkers or no linker are feasible alternatives.

25 :MTIIVVHTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQORLIWAGKQLEDGRTLSDYININ
FKLSLHLVLRRLRGGSGGGGSGGGGIG
MQIFVXTXFGKTITLEVEPSDTIENVKAKIQDKEGIPPDQORLIWAGKQLEDGRTLSDYINIX
XXXXLHLVLRRLRGG

The consensus sequences of examples of proteins with these sequences are shown in Figure
30 19.

In a still further embodiment, the fusion protein of the present invention is a trimer of a fusion protein of a ubiquitin hetero-dimer fused to TNF-alpha, wherein the fusion protein preferably has the sequence of SEQ ID NO: 35 or 36 or has an identity of at least 90%, preferably of more than 95%, of more than 96% or up to a sequence identity of 97% to SEQ SEQ ID NO: 35 or 36, and, at the same time, maintains or improves the capability of the original fusion protein of high affinity binding the ED-B domain of fibronectin.

In a further aspect of the invention, the present invention covers also polynucleotides which encode for a protein or fusion protein as described before. Additionally, vectors comprising said polynucleotide are covered by the invention.

In an additional aspect of the present invention, host cells are covered which comprise a protein or a fusion protein described herein and/or a polynucleotide coding for said recombinant protein or fusion protein of the invention or a vector containing said polynucleotide.

Uses of the proteins of the invention, e.g. hetero-dimeric ubiquitin based binding proteins specifically for ED-B fused to an effector such as TNF alpha

The modified ubiquitin ED-B binding proteins of the invention are to be used for instance for preparing diagnostic means for *in vitro* or *in vivo* use as well as therapeutic means. The proteins according to the invention can be used e.g. as direct effector molecules (modulator, antagonist, agonist) or antigen-recognizing domains. Examples of tumors with abundant appearance of ED-B antigen are shown in the table of Figure 1.

Depending on the selected fusion partner the pharmaceutical composition of the invention is adapted to be directed to the treatment of cancer, e.g. breast and colorectal cancers, or any other tumor diseases in which ED-B is abundant (cf. examples thereof listed in Figure 1).

The compositions are adapted to contain a therapeutically effective dose. The quantity of the dose to be administered depends on the organism to be treated, the type of disease, the age and weight of the patient and further factors known per se.

The compositions contain a pharmaceutically or diagnostically acceptable carrier and optionally can contain further auxiliary agents known per se. These include for example but not limited to stabilizing agents, surface-active agents, salts, buffers, colouring agents etc.

- 5 The pharmaceutical composition can be in the form of a liquid preparation, a cream, a lotion for topical application, an aerosol, in the form of powders, granules, tablets, suppositories, or capsules, in the form of an emulsion or a liposomal preparation. The compositions are preferably sterile, non-pyrogenic and isotonic and contain the pharmaceutically conventional and acceptable additives known per se. Additionally, reference is made to the regulations of
10 the U.S. Pharmacopoeia or Remington's Pharmaceutical Sciences, Mac Publishing Company (1990).

In the field of human and veterinary medical therapy and prophylaxis pharmaceutically effective medicaments containing at least one ED-B binding ubiquitin protein modified in
15 accordance with the invention can be prepared by methods known per se. Depending on the galenic preparation these compositions can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, transdermally or by other methods of application. The type of pharmaceutical preparation depends on the type of disease to be treated, the severity of the disease, the patient to be treated and other factors known to those skilled in the art of
20 medicine. The administration can either be parentally by injection or infusion, systemically, rectally, transdermally or by any other methods conventionally employed.

In an embodiment, the pharmaceutical composition contains a protein or a fusion protein of the invention or a combination thereof and further comprises one or more chemotherapeutic
25 agents, preferably selected from the following table:

Substance Class	Examples
Alkylating agents (ATC L01A)	melphalan, cyclophosphamide
Antimetabolites (ATC L01B)	5-fluorouracil, gemcitabine

Taxanes (ATC L01CD)	Paclitaxel
Cytotoxic antibiotics (ATC L01D)	doxorubicin, liposomal doxorubicin
Platinum compounds (ATC L01XA)	Cisplatin

In a preferred embodiment, the chemotherapeutic agent is selected from melphalan, doxorubicin, cyclophosphamide, dactinomycin, fluorodesoxyuracil, cisplatin, paclitaxel, and gemcitabine; or from the group of kinase inhibitors.

A “pharmaceutical composition” according to the invention may be present in the form of a composition, wherein the different active ingredients and diluents and/or carriers are in admixed with each other, or may take the form of a combined preparation, where the active ingredients are present in partially or totally distinct form. An example for such a combination or combined preparation is a kit-of-parts.

A “composition” according to the present invention comprises at least two pharmaceutically active compounds. These compounds can be administered simultaneously or separately with a time gap of one minute to several days. The compounds can be administered via the same route or differently; e.g. oral administration of one active compound and parenteral administration of another are possible. Also, the active compounds may be formulated in one medicament, e.g. in one infusion solution or as a kit comprising both compounds formulated separately. Also, it is possible that both compounds are present in two or more packages.

A particularly preferred combination is a fusion protein according to the invention and melphalan, and/or (liposomal) doxorubicin. Apart from antineoplastic agents from the ATC class L01, the TNF-fusion protein of the invention can be combined with other antineoplastic substances including cytokines and derivatives thereof, radiopharmaceuticals, cell based therapeutics and nanoparticles.

Due to its tumor permeabilisation activity, the TNF-fusion protein of the invention (but also the other recombinant proteins/fusion proteins of the present invention) can be combined with all antineoplastic agents as listed under L01 in the Anatomical Therapeutic Chemical

Classification System (ATC) provided by the World Health Organisation. Here, it surprisingly turned out that a fusion protein of a ubiquitin hetero-dimer fused to TNF-alpha, wherein the fusion protein preferably has the sequence of SEQ ID NO: 35 or 36, can be advantageously applied in therapy. TNF-alpha is highly toxic and, thus, may only be administered in low dosages, which usually lie below the minimum therapeutic threshold (and thus are therapeutically inactive). Due to this toxicity of TNF-alpha, in order to reach a therapeutically effective concentration, the isolated limb perfusion approach is presently selected when using TNF-alpha. Limb perfusion is a medical technique that may be used to deliver anticancer drugs directly to an arm or leg. The flow of blood to and from the limb is temporarily stopped with a tourniquet, and anticancer drugs are put directly into the blood of the limb. This allows the patient to receive a high dose of TNF-alpha in the area where the cancer occurred.

However, by applying the TNF-alpha fusion proteins of the present invention, it is possible to administer TNF-alpha in a non-toxic, but still therapeutically effective concentration. Since TNF-alpha is coupled to the (binding) fusion protein of the present invention, it can be directly active at the disease site (for example, tumor site) and, thus, the amount of "free" TNF-alpha can be drastically reduced.

Systemic side effects of TNF-alpha can be remarkably reduced by administering TNF-alpha as a fusion protein according to the present invention. By using a TNF-alpha fusion protein of the invention, the overall dosage of TNF-alpha to reach a therapeutic effect thus can be reduced to a large extent and can be advantageously used for systemic tumor treatment (without the necessity and restrictions of limb perfusion) in particular in combination with chemotherapeutic agents (see above).

In a further embodiment, the pharmaceutical composition is in the form of a kit of parts, providing separated entities for the recombinant protein/fusion protein of the invention and for the one or more chemotherapeutic agents.

Method of production of the ED-B binding proteins of the invention

ED-B binding proteins according to the invention may be prepared by any of the many conventional and well known techniques such as plain organic synthetic strategies, solid phase-assisted synthesis techniques or by commercially available automated synthesizers. On the other hand, they may also be prepared by conventional recombinant techniques alone or in combination with conventional synthetic techniques.

In another aspect of the present invention, a method for generating a recombinant modified protein is provided. The method comprises at least the following steps:

- a) providing an ubiquitin protein;
- b) providing the ED-B of fibronectin;
- c) modifying said ubiquitin protein in order to obtain a protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 60% wherein at least 4 amino acids are modified by substitution, deletion or addition of amino acids in positions 2, 4, 6, 62, 63, 64, 65, 66, and/or 68 ;
- d) contacting said modified ubiquitin protein with said ED-B of fibronectin;
- e) screening for modified ubiquitin proteins which bind to said ED-B of fibronectin with a specific binding affinity of 10^{-5} - 10^{-12} M, and optionally
- f) isolating said modified ubiquitin proteins.

Optionally, the modification may be performed by genetic engineering on the DNA level and expression of the modified protein in prokaryotic or eukaryotic organisms or in vitro.

In a further embodiment, said modification step includes a chemical synthesis step.

In a still further embodiment, said method is adapted in order that said modified ubiquitin protein is fused with a pharmaceutically active component, optionally a cytokine, preferably TNF-alpha, or a diagnostic component, or wherein said recombinant protein is fused with at least one second recombinant ubiquitin protein to obtain a multimer, optionally a dimer or trimer of said recombinant ubiquitin protein which is optionally fused with a pharmaceutically active component, optionally a cytokine, or a diagnostic component, or

- wherein said recombinant protein is fused with at least one second recombinant ubiquitin protein to obtain a multimer, optionally a dimer or trimer of said recombinant ubiquitin protein wherein said multimer, dimer or trimer is formed via said pharmaceutically active component which is optionally TNF-alpha, or via said diagnostic component.

5

According to the invention, a modified protein can further be prepared by chemical synthesis. In this embodiment the steps c) to d) of claim 1 are then performed in one step.

In a further aspect of the invention, a method for generating a hetero-multimeric fusion protein is provided, comprising the following steps:

10

a) providing a multimeric ubiquitin protein comprising two or more modified ubiquitin monomers linked by a suitable linker, wherein each monomer of said multimeric ubiquitin protein was modified in order to obtain a protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 60% wherein at least 4 amino acids in each monomer are modified by substitution, deletion or addition of amino acids in positions 2, 4, 6, 62, 63, 64, 65, 66, and/or 68;

15

b) providing the ED-B of fibronectin;

20

c) contacting said hetero-multimeric modified ubiquitin protein with said ED-B of fibronectin;

d) screening for modified ubiquitin proteins which bind to said ED-B of fibronectin with a specific binding affinity of 10^{-5} - 10^{-12} M, and optionally

e) isolating said modified hetero-multimeric ubiquitin proteins.

In another aspect of the present invention, a method for generating a recombinant modified ubiquitin protein is provided. The method comprises at least the following steps:

25

a) providing a population of differently modified dimeric ubiquitin proteins originating from monomeric ubiquitin proteins, said population comprising dimeric ubiquitin proteins comprising two modified ubiquitin monomers linked together in a head-to-tail arrangement wherein each monomer of said dimeric protein is differently modified by substitutions of at least 6 amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66 and 68 of SEQ ID NO: 1

30

wherein said substitutions comprise

- (1) in the first monomeric unit substitutions at least in amino acid positions 6, 8, 63, 64, 65, and 66; and
in the second monomeric unit substitutions at least in amino acid positions 6, 8, 62, 63, 64, 65, and 66; optionally additionally 2; or
- 5 (2) in the first monomeric unit substitutions at least in amino acid positions 2, 4, 6, 62, 63, 64, 65, and 66; and
in the second monomeric unit substitutions at least in amino acid positions 6, 8, 62, 63, 64, 65, and 66; optionally additionally 2
- b) providing the extradomain B (ED-B) of fibronectin as potential ligand;
- 10 c) contacting said population of differently modified proteins with said extradomain B (ED-B) of fibronectin;
- d) identifying a modified dimeric ubiquitin protein by a screening process, wherein said modified dimeric ubiquitin protein binds to said the extradomain B (ED-B) of fibronectin with a specific binding affinity of K_d in a range of 10^{-7} - 10^{-12} M and
- 15 exhibits a monovalent binding activity with respect to said extradomain B (ED-B) of fibronectin, and optionally
- e) isolating said modified dimeric ubiquitin protein with said binding affinity.
- 20 In said preferred embodiment, the hetero-multimeric ubiquitin protein is a hetero-dimeric ubiquitin protein.

In a further aspect, the present invention is directed to a library based on linear polyubiquitin chains as mentioned above and being randomised at least in two BDR's.

25

In a still further aspect of the invention, a fusion library containing DNA obtained by fusing two libraries as specified above is provided each library encoding for differently modified monomeric ubiquitin protein units in order to obtain hetero-dimeric ubiquitin fusion proteins, the monomeric units thereof being linked together in a head-to-tail arrangement, said library

30 encoding for hetero-dimeric fusion proteins of ubiquitin exhibiting a monovalent binding activity with respect to said extradomain B (ED-B) of fibronectin. Said linking together is performed either by using anyone of the linkers known by the skilled artisan or a linker

described herein. In one embodiment of the invention TNF-alpha is used as linker acting simultaneously as pharmaceutically active compound.

5 Example 1 outlines the production of a complex library. However, care must be taken as regards the quality of such a library. The quality of a library in the antibody or scaffold technology is in the first place dependent from its complexity (number of individual variants) as well as functionality (structural and protein-chemical integrity of the resulting candidates). Both characteristics, however, may exert negative influences on each other: enhancing the complexity of a library by increasing the number of modified positions on the scaffold might
10 lead to a deterioration of the protein-chemical characteristics of the variants. This might result in a decreased solubility, aggregation and/or low yields. A reason for this is the larger deviation from native scaffolds having an energetically favourable protein packaging.

Therefore, it is a balancing act to construct such a scaffold library suitably between the
15 extreme positions of introducing as many variations as possible into the original sequence in order to optimize it for a target and, on the other hand, of conserving the original primary sequence as much as possible in order to avoid negative protein-chemical effects.

It is noted that the present disclosure encompasses also each conceivable combination of the
20 features described herein in view of the aspects or embodiments of the invention.

25 Selection of the modified ubiquitin proteins with binding affinity with respect to the target ED-B and determination of the modified amino acids responsible for the binding affinity

After e.g. at least two different DNA libraries encoding for hetero-dimeric modified ubiquitin proteins have been established by differently modifying selected amino acids in each of the monomeric ubiquitin units, these libraries are genetically fused by e.g. linker technology to
30 obtain DNA molecules encoding for hetero-dimeric modified ubiquitin proteins. The DNA of these libraries is expressed into proteins and the modified dimeric proteins obtained thereby are contacted according to the invention with the ED-B to optionally enable binding of the partners to each other if a binding affinity does exist.

It is a crucial aspect of the invention that the contacting and screening process is performed already with respect to the hetero-dimeric ubiquitin protein. This process enables screening on those ubiquitin proteins which provide a monovalent binding activity to ED-B.

5

Contacting according to the invention is preferably performed by means of a suitable presentation and selection method such as the phage display, ribosomal display, mRNA display or cell surface display, yeast surface display or bacterial surface display methods, preferably by means of the phage display method. For complete disclosure, reference is made also to the following references: Hoess, *Curr. Opin. Struct. Biol.* 3 (1993), 572-579; Wells and Lowmann, *Curr. Opin. Struct. Biol.* 2 (1992), 597-604; Kay et al., *Phage Display of Peptides and Proteins-A Laboratory Manual* (1996), Academic Press. The methods mentioned above are known to those skilled in the art and can be used according to the invention including modifications thereof.

15

The determination whether the modified protein has a quantifiable binding affinity with respect to a predetermined binding partner can be performed according to the invention preferably by one or more of the following methods: ELISA, plasmon surface resonance spectroscopy, fluorescence spectroscopy, FACS, isothermal titration calorimetry and analytical ultracentrifugation.

20

Phage display selection method

One type of phage display procedure adapted to this application is described in the following as an example for a selection procedure according to the invention with respect to variations of ubiquitin which show binding properties. In the same manner e.g. methods for the presentation on bacteria (bacterial surface display; Daugherty et al., 1998, *Protein Eng.* 11(9):825-832) or yeast cells (yeast surface display; Kieke et al., 1997 *Protein Eng.* 10(11):1303-10) or cell-free selection systems such as the ribosome display (Hanes and Plückthun, 1997 *Proc Natl Acad Sci U S A.* 94(10):4937-4942; He and Taussig, 1997 *Nucleic Acids Res.* 25(24):5132-5134) or the cis display (Odegrip et al., 2004 *Proc Natl Acad Sci U S A.* 101(9):2806-2810) or the mRNA display can be applied. In the latter case a transient

30

physical linkage of genotype and phenotype is achieved by coupling of the protein variation to the appropriate mRNA via the ribosome.

5 In the phage display procedure described herein recombinant variations of ubiquitin are presented on a filamentous phage while the coding DNA of the presented variation is present at the same time packed in a single-stranded form in the phage envelope. Thus, in the frame of an affinity enrichment variations having certain properties can be selected from a library and their genetic information can be amplified by infection of suitable bacteria or added to another cycle of enrichment, respectively. Presentation of the mutated ubiquitin on the phage
10 surface is achieved by genetic fusion to an amino-terminal signal sequence-preferably the PelB signal sequence-and a capsid or surface protein of the phage-preferred is the carboxyterminal fusion to the capsid protein pIII or a fragment thereof. Furthermore, the encoded fusion protein can contain further functional elements such as e.g. an affinity tag or an antibody epitope for detection and/or purification by affinity chromatography or a protease
15 recognition sequence for specific cleavage of the fusion protein in the course of the affinity enrichment. Furthermore, an amber stop codon can be present for example between the gene for the ubiquitin variation and the coding region of the phage capsid protein or the fragment thereof which is not recognized during translation in a suitable suppressor strain partially due to the introduction of one amino acid.

20

The bacterial vector suitable for the selection procedure in the context of the isolation of ubiquitin variations with binding properties to a predetermined hapten or antigen and into which the gene cassette for the fusion protein described is inserted is referred to as phagemid. Among others, it contains the intergenic region of a filamentous phage (e.g. M13 or f1) or a
25 portion thereof which in the case of a superinfection of the bacterial cell carrying the phagemid by means of helper phages such as e.g. M13K07 results in the packaging of a closed strand of phagemid DNA into a phage capsid. The phagemids generated in this manner are secreted by the bacterium and present the respective ubiquitin variation encoded-due to its fusion to the capsid protein pIII or the fragment thereof-on their surface. Native pIII capsid
30 proteins are present in the phagemid so that its ability to re-infect suitable bacterial strains and therefore the possibility to amplify the corresponding DNA is retained. Thus, the physical linkage between the phenotype of the ubiquitin variation - i.e. its potential binding property - and its genotype is ensured.

Phagemids obtained can be selected with respect to the binding of the ubiquitin variation presented thereon to predetermined haptens or antigens by means of methods known to those skilled in the art. For this purpose, the presented ubiquitin variations can be transiently immobilized to target substance bound e.g. on microtiter plates and can be specifically eluted after non-binding variations have been separated. The elution is preferably performed by basic solutions such as e.g. 100 mM triethylamine. Alternatively, the elution can be performed under acidic conditions, by proteolysis or direct addition of infected bacteria. The phagemids obtained in this manner can be re-amplified and enriched by successive cycles of selection and amplification of ubiquitin variations with binding properties to a predetermined hapten or antigen.

Further characterization of the ubiquitin variations obtained in this way can be performed in the form of the phagemid, i.e. fused to the phage, or after cloning of the corresponding gene cassette into a suitable expression vector in the form of a soluble protein. The appropriate methods are known to those skilled in the art or described in the literature. The characterization can comprise e.g. the determination of the DNA sequence and thus of the primary sequence of the variations isolated. Furthermore, the affinity and specificity of the variations isolated can be detected e.g. by means of biochemical standard methods such as ELISA or plasmon surface resonance spectroscopy, fluorescence spectroscopy, FACS, isothermal titration calorimetry, analytical ultracentrifugation or others. In view of the stability analysis, for example spectroscopic methods in connection with chemical or physical unfolding are known to those skilled in the art.

25

Ribosomal display selection method

In a further embodiment of the invention ribosomal display procedure variations of ubiquitin are prepared by means of a cell-free transcription/translation system and presented as a complex with the corresponding mRNA as well as the ribosome. For this purpose, a DNA library as described above is used as a basis in which the genes of variations are present in form of fusions with the corresponding regulatory sequences for expression and protein biosynthesis. Due to the deletion of the stop codon at the 3' end of the gene library as well as

30

suitable experimental conditions (low temperature, high Mg^{2+} concentration) the ternary complex consisting of the nascent protein, the mRNA and the ribosome is maintained during in vitro transcription/translation.

- 5 After a protein library containing hetero-dimeric modified ubiquitin proteins has been established by differently modifying of selected amino acids in each of the monomeric ubiquitin units, the modified dimeric proteins are contacted according to the invention with the ED-B to enable binding of the partners to each other if a binding affinity does exist. These protein libraries may be in the form of a display method library displaying or using any other
- 10 method presenting the modified proteins in a manner enabling the contact between the modified proteins and the ED-B target protein, wherein said display method is optionally a phage display, ribosomal display, TAT phage display, yeast display, bacterial display or mRNA display method.
- 15 Selection of the modified ubiquitin variations with respect to their binding activities to ED-B with a specific binding affinity of K_d in a range of 10^{-7} - 10^{-12} M can be performed by means of methods known to those skilled in the art. For this purpose, the ubiquitin variations presented e.g. on the ribosomal complexes can be transiently immobilized to target substance bound e.g. on microtiter plates or can be bound to magnetic particles after binding in solution,
- 20 respectively. Following separation of non-binding variations the genetic information of variations with binding activity can be specifically eluted in the form of the mRNA by destruction of the ribosomal complex. The elution is preferably carried out with 50 mM EDTA. The mRNA obtained in this manner can be isolated and reverse transcribed into DNA using suitable methods (reverse transcriptase reaction), and the DNA obtained in this manner
- 25 can be re-amplified.
- By means of successive cycles of in vitro transcription/translation, selection, and amplification ubiquitin variations with binding properties for a predetermined hapten or antigen can be enriched.

Characterization of the EDB-binding proteins

The further characterization of the ubiquitin variations obtained in this manner can be performed in the form of a soluble protein as detailed above after cloning of the corresponding gene cassette into a suitable expression vector. The appropriate methods are known to those skilled in the art or described in the literature.

Preferably, the step of detection of the proteins having a binding affinity with respect to a predetermined binding partner is followed by a step of isolation and/or enrichment of the detected protein.

Following the expression of the ubiquitin protein modified according to the invention, it can be further purified and enriched by methods known per se. The selected methods depend on several factors known per se to those skilled in the art, for example the expression vector used, the host organism, the intended field of use, the size of the protein and other factors. For simplified purification the protein modified according to the invention can be fused to other peptide sequences having an increased affinity to separation materials. Preferably, such fusions are selected that do not have a detrimental effect on the functionality of the ubiquitin protein or can be separated after the purification due to the introduction of specific protease cleavage sites. Such methods are also known per se to those skilled in the art.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a Table listing the occurrence of ED-B in various tumors.

Figure 2A shows a concentration dependent ELISA of the binding of modified ubiquitin ED-B binder 5E1 to human ED-B (filled circles) with an affinity of 23 nM compared to no binding to BSA (open circles).

30

Figure 2B shows a concentration dependent ELISA of the binding of modified ubiquitin ED-B binder 1H4 to human ED-B (filled circles) with an affinity of 7,8 nM. The binding to BSA is plotted as a control (open circles).

Figure 3 shows that tetramerization leads to an increase in affinity.

The table shows the K_d values of modified ubiquitin monomers compared to tetramers consisting of modified ubiquitin monomers. Shown are ubiquitin variants 5E1 and 1H4 as examples. The ED-B binding is compared to binding to c-FN (cellular fibronectin). The figures demonstrate the significant higher affinity in binding of the tetrameric variant (for example, 56 nM for 5E1 or 1.4 nM for 1H4) to the target ED-B compared to the monomer (4,51 microM for 5E1 or 9,98 microM for 1H4).

10 **Figure 4** shows that the recombination of a front (first) modified ubiquitin monomer (having a BDR1) with a different modified rear (second) ubiquitin monomer (having a BDR2) to generate a hetero-dimer results in an significant increase of affinity as well as specificity. The modified ubiquitin molecules are analyzed via Biacore, fluorescence anisotropy, binding on cells and tissue sections. Shown are concentration dependent ELISAs (conc-ELISA) of the
15 binding of several variants to human ED-B.

Figure 4 A shows a binding affinity of $K_d = 9.45 \mu\text{M}$ for the monomer 41B10.

Figure 4 B shows that a binding affinity of a $K_d = 131 \text{ nM}$ for 41B10 combined with a
20 different second monomer resulting in 46H9.

Figure 5 shows specific variants fused to a cytokine (for example, TNFalpha). The fusion proteins trimerize the modified ubiquitin monomer and are biologically active molecules.

25 **Figure 5 A** is a schematic drawing of a modified ubiquitin based ED-B binding-effector-fusion protein; in green (structure on top) – effector, e.g. a cytokine, preferably TNF-alpha; brown: light brown: structure of the modified ubiquitin monomers (Affilin®).

Figure 5 B shows that the modified ubiquitin effector conjugate 5E1-TNF-conjugate has pro-
30 apoptotic activity (as measured in an L929 apoptosis assay).

Figure 5 C shows high affinity binding of 1H4-TNFalpha-fusion to ED-B ($K_d=15,1$ nM) (closed circles connected by a fitted line). The binding to BSA is plotted as a control (closed circles not connected by a line).

5 **Figure 6** shows the affinity and activity of a modified ubiquitin based ED-B binding hetero-dimer molecule fused to a cytokine, for example, TNFalpha.

- Apoptosis inducing activity of modified ubiquitin based ED-B binding cytokine fusion:
EC₅₀ 0.78 ± 0.24 pM

10 • Apoptosis inducing activity of free cytokine: EC₅₀ 3.14 ± 3.59 pM

Figure 6A shows the affinity of modified ubiquitin based ED-B binding hetero-dimer 24H12 (K_d 50.7 nM).

15 **Figure 6B** shows the affinity of modified ubiquitin based ED-B binding heterodimer 24H12 genetically fused to cytokine TNFalpha to result in a multimerisation of the hetero-dimer 24H12 (K_d = 5.6 nM)

Figure 6C shows an analysis of exemplary candidates from a hetero-dimeric modified ubiquitin library selection, for example hetero-dimer clones 9E12, 22D1, 24H12, 41B10. The
20 Kd ELISA values are increased for the target ED-B compared to cytosolic fibronectin used as control, confirming a specific binding to the target.

Figure 6D shows results of an analysis of the modified hetero-dimeric ubiquitin molecule 9E12 via label-free interaction assays using Biacore®. Different concentrations of the hetero-dimeric ubiquitin variants were analyzed (see figure legend: 0-15 microM of 9E12) for
25 binding to ED-B immobilized on a chip (Biacore) to analyze the interaction between the hetero-dimeric variant 9E12 and ED-B. A K_d could not be determined from analyzing the association and dissociation curves.

30 **Figure 6E** shows results of an analysis of the modified hetero-dimeric ubiquitin molecule 41B10 via label-free interaction assays using Biacore®. Different concentrations of the hetero-dimeric ubiquitin variants were analyzed (see figure legend: 0-15 microM of 41B10)

for binding to ED-B immobilized on a chip (Biacore) to analyze the interaction between the hetero-dimeric variant 41B10 and ED-B. Analyzing the association and dissociation curves resulted in a Kd of 623 nM (623×10^{-9} M, 6.2×10^{-7} M).

5 **Figure 7** shows the contribution of different modified ubiquitin based variants to binding affinity and specificity. The different variants share common sequence modules which are marked with lower case letters. The variants were analyzed with respect to their ED-B binding. Figure 3 shows different combinations of monomers resulting in modified ubiquitin-heterodimers. Hetero-dimeric variants 46-A5, 50-G11 and 46-H4 have all the same first
10 (front) modified monomer with BDR1 (labeled with the letter “a” in the figure), but a second (rear) ubiquitin monomer modified in different positions with BDR2. Variants 52-D10 and 52-B3 have a different first (front) modified monomer compared to 46-H9 with BDR1, but the same second (rear) ubiquitin monomer with BDR2 (labeled with the letter “e”).

15 The modified ubiquitin hetero-dimers have the following sequences:

46-H4: SEQ ID NO: 25, 45-H9: SEQ ID NO: 26, 46-A5: SEQ ID NO: 27, 50-G11: SEQ ID NO: 28, 52-B3: SEQ ID NO: 29, 52-D10: SEQ ID NO: 30

The above described sequences were modified in the course of the experiments by adding a
20 His-Tag with the sequence LEHHHHHH (SEQ ID NO: 31).

As can be seen from Figure 7, 46-H4 has an excellent binding affinity to ED-B ($K_d=189\text{nM}$); 46-A5 and 52-D10 have no binding activity while other modified ubiquitin proteins provide a minor binding activity compared 46-H4 to ED-B. Thus it can be concluded that both
25 monomers in a hetero-dimeric variant are required for a high affinity binding to a target; both monomers show a monovalent binding to the target.

The modified ubiquitin hetero-dimer with high ED-B binding activity named 46 H9 is identified by the following amino acid replacements in both binding domain region in the two
30 monomers as compared to wild type ubiquitin monomers:

in the first module (BDR1) (a) Q2G, F4V, K6R, Q62P, K63H, E64A, S65T, T66L

in the second module (BDR2) (e) K6H, L8M, Q62K, K63P, E64I, S65A, T66E

50G11

in the first module (46H9)(a) Q2G, F4V, K6R, Q62P, K63H, E64P, S65T, T66L

in the second module (c) K6M L8R, Q62M, K63N, E64A, S65R, T66L

5 46H4

in the first module (46H9)(a) Q2G, F4V, K6R, Q62P, K63H, E64P, S65T, T66L

in the second module (d) K6G, L8W, Q62T, K63Q, E64Q, S65T, T66R

52B3

10 in the first module (g) Q2R, F4P, K6Y, Q62P, K63P, E64F, S65A, T66R

in the second module (46H9) K6H, L8M, Q62K, K63P, E64I, S65A, T66E

52D10 (non-ED-B binder)

in the first module Q2V, F4C, K6R, Q62T, K63A, E64P, S65G, T66D

15 in the second module (46H9) (e) K6H, L8M, Q62K, K63P, E64I, S65A, T66E

46A5 (non-ED-B binder)

in the first module (46H9)(a) Q2G, F4V, K6R, Q62P, K63H, E64P, S65T, T66L

in the second module (b) K6L, L8M, Q62L, K63A, E64F, S65A,

20

Figure 8 shows a sequence alignment. Line 1: Two monomers of the wild type ubiquitin protein (1st line) are linked with a 12-amino acid linker SGGGGSGGGGIG starting at Position 77 and ending at Position 88; the second monomer with BDR2 starts at position 89 with a Methionine. This dimeric wild-type ubiquitin protein is aligned with the modified ubiquitin hetero-dimeric variant 46-H9 (2nd line) with different modifications in the first and in the second monomer resulting in two BDR's. Both BDRs act together in the binding of the target due to a monovalent binding to the target..

30 **Figure 9** shows a sequence alignment of modified ubiquitin hetero-dimeric variant 1041-D11 (1st line) to "Ub2_TsX9" (ubiquitin modified in position 45 in both monomers to Tryptophane, showing the linker GIG between the two monomers (position 77 to 79; the second monomer starts with a Methionine at Position 80), and an exchange from Glycine to Alanine at the last c-terminal amino acids of the 2nd monomer. The third line shows "Ubi-
35 Dimer wt", the wildtype ubiquitin as dimer; showing no linker alignment (thus, the second

monomer starts at position 77 with a Methionin). The 4th line shows the “Ubi-Monomer wt” which is the human wild type ubiquitin.

5 **Figure 10** shows a concentration dependent ELISA of the binding of the hetero-dimeric ubiquitin variant 1041-D11 to human ED-B. Variant 1041-D11 shows very high affinity binding to ED-B ($K_d = 6.9 \text{ nM} = 6.9 \times 10^{-9} \text{ M}$). The closed dots show the affinity of the binding of hetero-dimeric ubiquitin variant 1041-D11 to an ED-B containing fibronectin fragment (referred to as 67B89-t0) compared to no binding of this variant to negative control (referred to as 6789-t0) (open circles).

10

Figure 11 shows competitive concentration dependent ELISAs of the binding of hetero-dimeric ubiquitin variant 1041-D11 to immobilized ED-B containing fibronectin fragment (67B89) in the presence of increasing amounts of free target. Hetero-dimeric ubiquitin variant 1041-D11 shows a very high affinity binding to ED-B ($IC_{50} = 140 \text{ nM}$).

15

Figure 12 shows a result of an analysis of the modified hetero-dimeric ubiquitin molecule 1041-D11 in label-free interaction assays using Biacore®. Different concentrations of the hetero-dimeric ubiquitin variant were analyzed (see figure legend: 0-200 nM of 1041-D11) for binding to an ED-B containing fibronectin fragment (referred to as 67B89) immobilized on a SA-chip (Biacore). Analyzing the association and dissociation curves resulted in a K_d of 20 1 nM ($1 \times 10^{-9} \text{ M}$) and a k_{off} rate of $7.7 \times 10^{-4} \text{ s}^{-1}$ which indicates a long half time of an complex of 1041-D11 and ED-B.

25

Figure 13 shows the binding of hetero-dimeric ubiquitin variant 1041-D11 to ED-B in a concentration dependent ELISA simultaneously analyzing the serum-stability of binding activity. Shown are different conditions, such as pre-incubation for 1 h at 37°C of the variant in mouse or rat serum or in PBST as control. The K_d -values are all between 10 and 20 nM. Thus, it can be concluded that the binding of the hetero-dimer 1041-D11 to ED-B is not significantly influenced by blood serum.

30

Figure 14 shows an analysis of the complex-formation of hetero-dimeric ubiquitin variant 1041-D11 with fibronectin fragments by SE-HPLC.

Fig. 14 A shows the complex formation of 1041-D11 with ED-B. Three HPLC runs are overlaid: the blue peak with a retention time of 21.651 min originates from pure 1041-D11; the black peak with a retention time of 26.289 min represents the fibronectin fragment 67B89; a mixture of 1041-D11 and 67B89 results in the red peak with a retention time of 21.407 min after SE-HPLC. The shift of the 1041-D11 peak to a lower retention time as well as the disappearance of the 67B89 peak indicates formation of a complex of 1041-D11 and soluble ED-B.

Fig. 14 B shows the overlay of three SE-HPLC runs of 1041-D11 (blue, 21.944 min), fibronectin fragment 6789 without ED-B (black, 26.289 min) and a mixture of 1041-D11 and 6789 (red line with peaks at 21.929 min and 26.289 min). Almost no shift of the 1041-D11 peak is observed. This fact together with a lack of disappearance of the 6789 peak indicates no significant binding of the ED-B free fibronectin fragment 6789.

Figure 15 shows the binding of hetero-dimeric ubiquitin variant 1041-D11 to cell culture cells.

Figure 15A shows binding of the hetero-dimeric ubiquitin variant 1041-D11 on human fetal lung fibroblast cells (Wi38) which were fixed. The first column in Figure 15 shows the control using anti-ED-B antibodies; the second column shows the incubation of the variant at a protein concentration of 58.7 nM, the third column a ten-fold higher concentration of 1041-D11 protein (587 nM), and the fourth column is a negative control with PBS. In the first row, human Wi38 fibroblast cells are shown in phase contrast; the second row shows the immunofluorescence and the third row a DAPI staining the nuclei. It can be concluded that the variant 1041-D11 binds to Wi38 with high specificity to ED-B containing extracellular matrix. A control using NHDF cells which express low level of EDB was performed (data not shown). The variants do not bind to those cells.

Figure 15B shows the binding on vital human fetal lung fibroblast cells (Wi38). The negative control cells type NHDF are primary normal fibroblast cells, which express low levels of EDB-fibronectin. The first and third line shows the variant at different protein concentration and the negative control. The second and fourth line shows the incubation of the control using EDB antibodies. The first 2 lines show the variant and positive control on Wi38-cell line. The

third and fourth line shows the incubation of NHDF-cells. It can be seen that the variant 1041-D11 binds to Wi38 with high specificity to ED-B containing extracellular matrix.

Figure 15C shows the binding on fixed murine Balb 3T3-cells. Three different protein concentrations (1, 10, 50nM) of the variant were tested. The first rows shows the variant (SPVF-28-1041-411-TsX9) on cells, the second row shows the positive control (Fv28-EDB-Antibodies), the third row shows the incubation with the negative control (UB2_TsS9; unmodified ubiquitin corresponding to SEQ ID NO:1). It can be seen that the variant 1041-D11 binds to murine Balb 3T3 cells with high specificity to ED-B containing extracellular matrix.

Figure 15D shows the binding on fixed murine ST-2-cells. Three different protein concentrations (1, 10, 50nM) of the variant were tested. The first rows shows the variant (SPVF-28-1041-411-TsX9) on cells, the second row shows the positive control (Fv28-EDB-Antibodies), the third row shows the incubation with the negative control (UB2_TsS9; unmodified ubiquitin corresponding to SEQ ID NO: 1). It can be seen that the hetero-dimeric ubiquitin variant 1041-D11 binds to murine Balb ST-2 cells with high specificity to ED-B containing extracellular matrix.

Figure 16 A shows the specificity of hetero-dimeric ubiquitin variant 1041-D11 to the target in mammalian tissue sections. F9 tumor tissues from seven samples were evaluated. Immunohistochemistry with different concentrations between 10 nM and 100 nM of hetero-dimeric ubiquitin variant 1041-D11 resulted in ED-B specific vascular staining on F9 tumors from mice. ED-B is a highly specific marker for tumor vasculature. The target protein ED-B is located on the abluminal side of the vessels. Variant 1041-D11 specifically decorates the vasculature in tissue sections from F9 tumors. The obtained results are comparable to the antibody fragment L19. In addition, 48 tissues were tested; no unspecific staining in any out of 48 tissues in FDA relevant panel was observed.

Figure 16 B shows the accumulation of 1041-D11 in tumor tissue in comparison to wild type ubiquitin (in the figure, Ub2 (NCP2)). F9 tumor tissues were analyzed for the presence of 1041-D11 and wildtype ubiquitin at different time points between 30 min and 16 h. The highest accumulation of 1041-D11 in tumor tissue is observed 30 min and 16 h after

administration whereas the accumulation of wildtype ubiquitin in F9 tumor tissues is low. The variant is enriched in tumors expressing ED-B when compared to wildtype ubiquitin. This is an evidence for the directed targeting of 1041-D11 to tumor tissues. Further, the tumour to blood-ratio of 1041-D11 in a cancer model clearly demonstrates *in vivo* activity of 1041-D11 variant in animals (data not shown).

Figure 17 shows the high selectivity and specificity 1041-D11-TNF-alpha fusion protein for ED-B.

Figures 17A and 17B: Apoptosis inducing TNF-alpha activity of the 1041-D11-TNF α fusion protein was tested in a cell based assay (L929 cells). The figures clearly show that the 1041-D11-TNF-alpha fusion protein (FIG. 17B) is as active as free TNF-alpha (FIG. 17B) in cell culture.

Figure 17C demonstrates the high selectivity of the hetero-dimeric ubiquitin 1041-D11 TNF-alpha fusion protein to the target ED-B. The human ED-B fibronectin domain 67B89 is bound with an apparent KD value of 1.8 nM to variant 1041-D11 (closed circles), showing the high affinity for the target. Human fibronectin lacking the ED-B domain (h6789) is not bound by 1041-D11 TNFalpha (open circles).

Figure 17D shows the binding analysis of modified ubiquitin-based ED-B binding 1041-D11-TNF-alpha fusion protein by Biacore assays. The results demonstrate the high affinity of 1041-D11 TNF-alpha fusion protein with a KD value of 1.13 nM.

Figure 17E shows the high binding specificity observed with variant 1041-D11 in cell culture is preserved when the 1041-D11 is fused to TNF-alpha. The fusion protein specifically binds to EDB expressing cells. Thus, 1041-D11 TNF-alpha fusion protein binds with very high affinity and specificity to the target ED-B ("target(+)"). In serum without ED-B ("target(-)"), no cross reaction can be observed.

Figure 18 shows the relative tumor growth *in vivo* during the time of treatment of mice for 7 days with variant 1041-D11 fused to TNFalpha in combination with Melphalan. The data clearly show that 1041-D11-TNFalpha in combination with the cytostatic agent Melphalan

reduces the relative tumor growth more efficiently than mTNF-alpha in combination with Melphalan or Melphalan alone. The tumor growth kinetic 7 days after treatment shows the efficient reduction of tumors by 1041-D11-mTNFa. This is a clear evidence for the efficacy of a treatment of tumors with fusion protein 1041-D11-TNF-alpha in combination with Melphalan. ED-B is identical in several mammalian species, including mice and human, and thus, the results are predictive of the performance of variant 1041-D11-TNFalpha in humans.

Figure 19 shows the consensus positions and amino acid substitutions of 16 further sequences which have been found to have surprisingly strong binding affinities to ED-B. The consensus amino acid positions are in the first monomeric binding determining region 2, 4, 6, 62, 63, 64, 65, 66 while the consensus amino acid substitutions are Q2T, F4W, K6H, Q62N, K63F, E64K, S65L, and T66S. As can be taken from Fig. 2, 4 families of sequences could be enriched (consensus sequences, size of the letters correspond to the frequency of occurrence of the amino acids). Positions 85 and 87 are positions in the hetero-dimeric protein; with reference to the second monomer, the corresponding positions are 6 and 8; 141 - 145 correspond to positions 62 - 64). TWH NFKLS depicted in dark-blue colour originates from 1071-C12. Residues marked with red colour belong to one of the said four families of sequences. Residues marked in red have been enriched predominantly (178/457 sequences) and include according to HIT ELISAs the strongest binding molecules.

20

EXAMPLES

The following Examples are provided for further illustration of the invention. The invention is particularly demonstrated with respect to the modification of ubiquitin as an example. The invention, however, is not limited thereto, and the following Examples merely show the practicability of the invention on the basis of the above description. For a complete disclosure of the invention reference is made also to the literature cited in the application and in the annex which are all incorporated in their entirety into the application by reference.

30

Example 1. Identification of ED-B binding proteins based on modified ubiquitin proteins

Library Construction for monomeric binding proteins and Cloning

Unless otherwise indicated, established recombinant genetic methods were used, for example as described in Sambrook et al. A random library of human ubiquitin monomers with high complexity was prepared by concerted mutagenesis of in total up to 10 selected amino acid positions. The modified amino acids, which were substituted by NNK triplets, comprised at least 3 amino acids selected from positions 2, 4, 6, 8, 62, 63, 64, 65, 66, 68 within the ubiquitin monomer.

Library Construction for hetero-dimeric binding proteins and Cloning

Unless otherwise indicated, established recombinant genetic methods were used, for example as described in Sambrook et al. A random library of human ubiquitin hetero-dimers with high complexity was prepared by concerted mutagenesis of in total 15 selected amino acid positions. The modified amino acids, which were substituted by NNK triplets, comprised at least 3 amino acids selected from positions 2, 4, 6, 8, 62, 63, 64, 65, 66, 68 within the proximal (first) ubiquitin monomer and at least 3 amino acids selected from positions 2, 4, 6, 8, 62, 63, 64, 65, 66, 68 within the distal (second) ubiquitin monomer. Both ubiquitin monomers were genetically linked (head to tail) by a Glycine/Serine linker with at least the sequence GIG or by Glycine/Serine linker with at least the sequence SGGGG, for example GIG, SGGGG, SGGGGIG, SGGGGSGGGGIG (SEQ ID NO: 32) or SGGGGSGGGG, but any other linker is possible.

20

TAT Phage Display Selection

The ubiquitin library was enriched against the target using, for example, TAT phage display as selection system. Other selection methods known in the art can be used. The target can be immobilized nonspecifically onto protein binding surfaces or via biotinylated residues which were covalently coupled to the protein. The immobilization via biotin onto streptavidin beads or neutravidin strips is preferred. The target-binding phages are selected either in solution or on immobilized target; for example, the biotinylated and immobilized target with phage was incubated followed by washing of the phages bound to the matrix and by elution of matrix-bound phages. In each cycle following target incubation, the beads were magnetically separated from solution and washed several times. In the first selection cycle the biotinylated target was immobilized to neutravidin strips whereas in cycles two to four selections in solution was performed followed by immobilization of target-phage complexes on Streptavidin-coated Dynabeads® (Invitrogen). After washing in the first two selection cycles

30

the phages of target-binding modified ubiquitin molecules were released by elution with acidic solution. In selection cycles three and four elution of phages was carried out by competitive elution with excess target. The eluted phages were reamplified. To direct specificity of binders a protein similar to the target can be included during selection.

5

Alternatively to TAT phage display selection: Ribosome Display Selection

The ubiquitin library was enriched against the target using, for example, ribosome display as selection system (Zahnd et al., 2007), Ohashi et al., 2007). Other selection methods known in the art can be used. The target was biotinylated according to standard methods and immobilized on Streptavidin-coated Dynabeads® (Invitrogen). Ternary complexes comprising ribosomes, mRNA and nascent ubiquitin polypeptide were assembled using the PURExpress™ *In Vitro* Protein Synthesis Kit (NEB). Two primary rounds of selection were performed, wherein ternary complexes were incubated followed by two similar rounds of selection. In each cycle following target incubation, the beads were magnetically separated from solution and washed with ribosome display buffer with increasing stringency. After washing in the first two selection cycles, the beads were again magnetically separated from solution and mRNA of target-binding modified ubiquitin molecules was released from ribosomes by addition of 50 mM EDTA. In selection cycles three and four elution of mRNA was carried out by competitive elution with excess target (Lipovsek and Pluckthun, 2004). After each cycle, RNA purification and cDNA synthesis were performed using RNeasy MinElute Cleanup Kit (Qiagen, Germany), Turbo DNA-free Kit (Applied Biosystems, USA) and Transcriptor Reverse Transcriptase (Roche, Germany).

25

Cloning of Enriched Pools

After the fourth selection cycle the synthesized cDNA was amplified by PCR via primers F1 (GGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG) (SEQ ID NO: 9) and WUBI(co)RD_xho (AAAAAAAAAACTCGAGACCGCCACGCAGACGCAGAACCAG) (SEQ ID NO: 10), cut with restriction nucleases *NdeI* and *XhoI* (Promega, USA) and ligated into expression vector pET-20b(+) (Merck, Germany) via compatible cohesive ends.

30

Single Colony Hit Analysis

After transformation into NovaBlue(DE3) cells (Merck, Germany) ampicillin-resistant single colonies were grown for 6 h at 37 °C in 200 µl SOBAG medium (SOB medium containing 100 µg/ml ampicillin and 20 g/l glucose). expression of the ED-B-binding modified ubiquitin was achieved by cultivation for 16 h at 37 °C in 96-well deep well plates (Genetix, UK) using 500 µl auto induction medium ZYM-5052 (Studier, 2005). Cells were harvested by 15 min of centrifugation at 4 °C and 3600 g and subsequently lysed by incubation for 30 min at 37 °C with 300 µl lysis buffer per well, containing 0.2 x BugBuster® (Merck, Germany), 0.3 mg/ml lysozyme (VWR, Germany) 0,2 mM PMSF (Roth, Germany), 3 mM MgCl₂ and 0.2 U/ml Benzonase (VWR, Germany) in 50 mM NaH₂PO₄, 300 mM NaCl, pH8. After centrifugation for 30 min at 4°C and 3600 g the resulting supernatants were screened by ELISA using Nunc MediSorp plates (Thermo Fisher Scientific, USA) coated with 4 µg/ml ED-B and a ubiquitin-specific Fab fragment conjugated with horseradish peroxidase (POD). As detecting reagent TMB-Plus (Biotrend, Germany) was used and the yellow colour was developed using 50 µl/well 0.2 M H₂SO₄ solution and measured in a plate reader at 450 nm versus 620 nm.

Several cycles of selection display versus ED-B were carried out. In the last two cycles of selection binding molecules were eluted with an excess of free ED-B. These ED-B-binding variants were identified, among others:

1H4:

MWIKVHTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSD
YNITLSRSLHLVLRRLRGG (SEQ ID NO: 3)

4B10:

MLILVLTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDY
NIATKPILHLVLRRLRGG (SEQ ID NO: 4)

30

5E1:

MVINVFTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDY
NIRSTSKLHLVLRRLRGG (SEQ ID NO: 5)

Sequence of 46H9 (linker between the different monomers shown in italics)

MGIVVRTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSD
 YNIPHPTLLHLVLRLRGGSGGGGSGGGGIGMQIFVHTMTGKTITLEVEPSDTIENVKA
 5 KIQDKEGIPPDQQRLIWAGKQLEDGRTLSDYNIKPIAELHLVLRLRGG (SEQ ID NO: 6)

Sequence of 9E12 (linker between the different monomers shown in italics)

MRIPVYTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDY
 10 NIPPFARLHLVLRLRGGSGGGGSGGGGIGMQIFVMTRTGKTITLEVEPSDTIENVKAKI
 QDKEGIPPDQQRLIWAGKQLEDGRTLSDYNIMNARLLHLVLRLRGG (SEQ ID NO: 7)

Sequence of 22D1 (linker between the different monomers shown in italics)

MLILVRTLTDKTITLEVEPSDTIGNVKAKIQDKEGIPPDQQRLIWAGKQLEDGRTLSDY
 15 NISVGAMLHLVLRLRGGSGGGGSGGGGIGMQIFVLTWTGKTITLEVEPSDTIENVKAK
 IQDKEGIPPDQQRLIWAGKQLEDGRTLSDYNIRRLPPLHLVLRLRGG (SEQ ID NO: 8)

A sequence alignment of wild type ubiquitin monomer (Ubi monomer wt), with wild type
 ubiquitin dimer (ubi dimer wt) and wild type ubiquitin protein (Ub2-TsX in Figure 9, with an
 20 exchange in Position 45 of each monomer and with two substitutions at the C-terminus) with
 the modified ubiquitin hetero-dimeric variant 1041-D11 is shown in FIGURE 9. In Ub2-TsX
 the substitutions at the C-terminus (GG to AA) of the monomer increase the stability in serum
 because deubiquitinases cleave behind the GG of ubiquitin but not behind the AA. The
 secondary structure of the wild type ubiquitin compared to the ubiquitin with these
 25 substitutions at the C-terminus is almost identical.

The modified ubiquitins with superior ED-B binding activity referred to as 1041-D11 (shown
 in FIGURE 9; SEQ ID NO: 36) or 1045-D10 are identified by the following amino acid
 replacements as compared to the wild type: in the first module: K6W, L8W, K63R, E64K,
 30 S65F, T66P; in the second module: K6T, L8Q, Q62W, K63S, E64N, S65W, T66E; optionally
 Q2R (in variant 1041-D11, but not in variant 1045-D10). Suitable preferred linkers for the
 fusion protein are those of SEQ ID NO: 32 or the sequence GIG. However, there are many
 conceivable linkers which can be used instead.

As a further preferred example a protein is provided by the following sequence wherein X may be any amino acid (SEQ ID NO: 47) As linker, SGGGGSGGGGIG was used here (shown in italics). It is to be understood that also other kind of linkers or no linker are feasible alternatives.

MTI~~WVHTTETGKTITLEVEPSDTIENVKAKIQDKEGIPPDQORLIWAGKQLEDGRTLSDYNI~~
FKLSLHLLVLRRLRGGSGGGGSGGGGIG

MQIFVXTXTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQORLIWAGKQLEDGRTLSDYNI~~X~~
XXXXLHLLVLRRLRGG

Examples of proteins with these sequences are shown in Figure 19.

Example 2. Production of fusion proteins from ED-B-binding modified ubiquitin variants and TNFalpha (for example, human TNF α , referred to as TNF α)

The variants can be expressed as fusion proteins between the modified ubiquitins, for example heterodimeric variant1041-D11, and mammalian, for example mouse or human, TNF α in *E.coli*. Protein analysis of the fusion protein includes: protein expression and purity, no aggregation potential, TNF α activity in cell culture, affinity for target protein ED-B, Selectivity, specific binding in cell culture can be analyzed. A prerequisite for an animal experiment to induce tumor shrinkage in F9 tumor bearing mice is a fusion with mouse TNF α .

Step 1: Production of a vector for cloning of fusion proteins (pETSUMO- TNF α)

pETSUMOADAPT is a modified vector pETSUMO (Invitrogen), which was modified by insertion of an additional *multiple cloning site* (MCS). Starting from TNF α cloned in pETSUMOADAPT, restriction sites for the insertion of modified ubiquitin variants binding ED-B were introduced. The resulting construct has the structure His₆-SUMO- TNF α with the following DNA-sequence (SEQ ID NO: 11):

ATGGGCAGCAGCCATCATCATCATCACGGCAGCGGCCTGGTGCCGCGCGGCA
GCGCTAGCATGTCGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGC
CAGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGA
GATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTC
GCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTATTA

GAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATATTAT
 TGAGGCTCACAGAGAACAGATTGGTGGTGTGCGTAGCAGCAGCCGTACCCCGAG
 CGATAAACCGGTGGCGCATGTGGTGGCGAATCCGCAGGCGGAAGGCCAGCTGCA
 GTGGCTGAACCGTCGTGCGAATGCGCTGCTGGCCAACGGCGTGGAAGTGCCTGAT
 5 AATCAGCTGGTTGTGCCGAGCGAAGGCCTGTATCTGATTTATAGCCAGGTGCTGT
 TTAAAGGCCAGGGCTGCCCGAGCACCCATGTGCTGCTGACCCATAACCATTAGCCG
 TATTGCGGTGAGCTATCAGACCAAAGTGAACCTGCTGTCTGCGATTAAGCCCG
 TGCCAGCGTGAAACCCCGGAAGGCGCGGAAGCGAAACCGTGGTATGAACCGATT
 TATCTGGGCGGCGTGTTCAGCTGGAAAAAGGCGATCGTCTGAGCGCGGAAATTA
 10 ACCGTCCGATTATCTGGATTTTGC GGAAAGCGGCCAGGTGTATTTTGGCATTATT
 GCGCTGTAATAA

The TNF α sequence was amplified via PCR by introducing a BamHI- and XhoI-site. Primers used:

15 SUMO-EDB- TNF α -fw (SEQ ID NO: 12): TTT TTT **GGA TCC** GTG CGT AGC AGC
AGC
 SUMO-EDB- TNF α -rev (SEQ ID NO: 13): CTT GTC TCT **CGA GGC GGC CGC** TTA
TTA C

The fw-primer (SEQ ID NO: 12) recognizes the first 15 base pairs of TNF α (underlined
 20 region) and has a BamHI-sequence (shown in bold). The rev-primer (SEQ ID NO: 13)
 contains the last base pair of TNF α , the stop codons (underlined) and a XhoI-restriction site
 (bold).

PCR reaction mix (100 μ l):

25 84.5 μ l H₂O; 10 μ l 10x Pwo buffer + Mg; 2 μ l 10 mM dNTPs (=200 μ M); each 0.5 μ l 100
 μ M primer fw/rev (=each 0.5 μ M); 2 μ l DNA (=0,25 μ g); 0.5 μ l Pwo polymerase (=2.5 U;
 Roche)

PCR-program: 3 min 94°C, 30 s 94°C, 30 s 60°C, 2 min 72°C (steps 2 – 4: 30 cycles), 5 min
 30 72°C, followed by 4°C followed by purification of the PCR product with the Qiagen-
 MinElute-Kit (elution in 10 μ l EB). The PCR product is introduced in the MCS of the vector
 pETSUMOadapt via BamHI-XhoI-restriction and ligation.

Restriction mix (100 µl):

Vector: 83 µl H₂O; 10 µl 10x NE buffer 3; 1 µl 100x BSA; 3 µl BamHI (=30 U; NEB), 1.5 µl XhoI (=30 U; NEB); 1.65 µl vector; 3 h 37°C incubation.

5 PCR product: 76.5 µl H₂O; 10 µl 10x NE buffer 3; 1 µl 100x BSA; 3 µl BamHI (=30 U; NEB), 1.5 µl XhoI (=30 U; NEB); 8 µl insert; 3 h 37°C incubation

Separation of Restriction in 1% Agarosegel (100 V 60 min run); cut vector fragment (5659 bp) and insert (491 bp); Purification with Qiagen gel extraction kit (elution in 30 µl EB).

Ligation (20 µl):

10 15.2 µl H₂O; 2 µl 10x T4-DNA-ligasebuffer; 2.26 µl Vector (200 ng); 0.54 µl insert (40 ng)
5 min 65°C incubation; cool to 16°C; add 1 µl T4-DNA-ligase (=3 U; NEB); 16 h 16°C incubation.

NaAc/Isopropanol-precipitation:

15 Ligation-mixture (20 µl) + 2,2 µl 3 M NaAc (pH 5,0) + 22,2 µl Isopropanol; 30 min -20°C;
15 min 4°C 13000 Upm; resuspend Pellet in 500 µl 70% EtOH; spin; resuspend pellet in 10 µl H₂O.

Transformation:

20 Mix electro-competent Novablue(DE3)-cells (40 µl-aliquot) with 10 µl Ligationsproduct;
transfer to 0,1-cm-elektroporation cuvette; puls in elektroporator (1,8 kV, 50 µF, 100 Ohm);
incubate solution with 1 ml SOC-medium 45 min 37°C 220 Upm; 100 µl on LB-plate with
Kanamycin; incubation overnight 37°C.

25 **Step 2: Cloning of modified ubiquitin-based EDB-fusion proteins**

For the production of fusions of EDB-binding modified ubiquitin-based variants and TNF α , the EDB- modified ubiquitin-based sequence of interest is amplified from a pET20b-vector via PCR; BsaI- and BamHI-restriction sites are introduced. The method is suitable for monomeric and for dimeric EDB- modified ubiquitin-based variants. Primer for monomeric

30 WT-Ubiquitin (Wubi):

SUMO-EDB-WUBI-fw (SEQ ID NO: 14):: GTT CCA AGG TCT CAT GGT ATG CAG
ATC TTC GTG

SUMO-EDB-Linker-rev (SEQ ID NO: 15):: GTG GTG **GGA TCC** ACC GCC ACC ACC
AGA ACC GCC ACG CAG ACG

The fw-primer (SEQ ID NO: 14) recognizes the first 15 base pairs of modified ubiquitin (underlined region) and has a BsaI-sequence (shown in bold). The rev-Primer (SEQ ID NO: 15) recognizes the last 15 base pairs of modified ubiquitin and inserts an amino acid linker (sequence SGGGG) and a BamHI-restriction site (bold). For each modified ubiquitin-based - variant, a specific fw-primer is used. Primers monomeric EDB- modified ubiquitin-based variants 1H4, 5E1 and 4B10:

1H4 (MWIKV...): Primer (SUMO-EDB-1H4-fw) (SEQ ID NO: 16): GTT CCA **AGG TCT**
10 CAT GGT ATG TGG ATC AAG GTG

4B10 (MLILV): Primer (SUMO-EDB-4B10-fw) (SEQ ID NO: 17): GTT CCA **AGG TCT**
CAT GGT ATG TTG ATC CTG GTG

5E1 (MVINV...): Primer (SUMO-EDB-5E1-fw) (SEQ ID NO: 18): GTT CCA **AGG TCT**
CAT GGT ATG GTT ATC AAT GTG

15 The rev-primer is used for all monomeric modified ubiquitin-based variants. Rev-Primer for dimeric modified ubiquitin-based variants:

Dimer-t0a-rev (SEQ ID NO: 19): GTG GTG **GGA TCC** ACC GCC ACC ACC AGA ACC
ACC ACG TAA ACG

fw-primer for the cloning of dimeric WT-ubiquitins (WubiHubi) and for dimeric EDB-
20 modified ubiquitin-based variants:

WT (MQIFV...) Primer (SUMO-EDB-WUBI-fw) (SEQ ID NO: 20): GTT CCA **AGG TCT**
CAT GGT ATG CAG ATC TTC GTG

(note: fw-Primer for dimerics WT-ubiquitin is identical to fw-Primer for monomeric WT-ubiquitin.)

25 9E12 (MRIPV...): Primer (9E12-t0a-fw) (SEQ ID NO: 21): GTT CCA **AGG TCT** CAT
GGT ATG CGT ATC CCT GTG

24H12 (MVIKV...): Primer (24H12-t0a-fw) (SEQ ID NO: 22):GTT CCA **AGG TCT** CAT
GGT ATG GTT ATC AAG GTG

15G7 (MEIGV...): Primer (15G7-t0a-fw) (SEQ ID NO: 23): GTT CCA **AGG TCT** CAT
30 GGT ATG GAG ATC GGT GTG

22D1 (MLILV...): Primer (22D1-t0a-fw) (SEQ ID NO: 24): GTT CCA **AGG TCT** CAT
GGT ATG CTT ATC TTG GTG

PCR-Mixture (100 µl):

84.5 µl H₂O; 10 µl 10x Pwo-buffer + Mg; 2 µl 10 mM dNTPs (=200 µM); each 0.5 µl 100 µM Primer fw/rev (=je 0,5 µM); 2 µl DNA (dependent on the variant); 0.5 µl Pwo-Polymerase (=2,5 U; Roche)

5

PCR-Program:

1. 3 min 94°C
2. 30 s 94°C
3. 30 s 60°C
- 10 4. 2 min 72°C (steps 2 – 4: 30 cycles)
5. 5 min 72°C, followed by 4°C

Purification of the PCR-products in agarose gel, cut required band and purify with Qiagen-gel extraction kit. Cloning of the PCR-product via BsaI-BamHI-restriction (in pETSUMO-TNF α)

- Restriction (100 µl): 75 µl H₂O; 10 µl 10x NEBuffer 3; 1 µl 100x BSA; 3 µl BsaI (=30 U; NEB); 8 µl DNA (Vector or PCR-Product) 2 h 50°C incubation, 10 min 65°C, add 3 µl BamHI (=30 U; NEB), 2 h 37°C Separation of restriction in 1% agarose gel; cut vector fragment and insert; purification with Qiagen- gel extraction kit (elution in 30 µl EB).

- Ligation (20 µl):
- 20 12.5 µl H₂O; 2 µl 10x T4-DNA-ligase buffer; 5 µl vector (66 ng); 0.5 µl Insert (variabel) 5 min 65°C incubation; cool to 16°C; add 1 µl T4-DNA-ligase (=3 U; NEB); 16 h 16°C incubation

NaAc/Isopropanol-precipitation (see Step 1)

- Transformation in elektrocompetent Novablue(DE3)-cells as described above. The result is the following fusion construct: EDB- modified ubiquitin and TNF α in pETSUMOadapt with der His₆-SUMO- modified ubiquitin-SGGGG-TNF α (359 amino acids with monomeric modified ubiquitin 447 amino acids with dimeric modified ubiquitin)

30

Example 3: Expression and Purification of Ubiquitin-based- TNF α Fusion Proteins

DNA sequence analysis showed the correct sequences of the SUMO- TNF α fusion proteins. For expression of the variants the clones were cultivated in a shaker flask by diluting a preculture 1:100 with LB/Kanamycin and agitating the culture at 200 rpm and 37°C up to an

optical density at 600 nm (OD₆₀₀) of 0.5. Expression was induced by adding IPTG (final concentration 1 mM). Culturing was continued for 4 hours at 30°C and 200rpm. The bacteria cells were harvested by centrifugation at 4°C, 6000 x g for 20 min. The cell pellet was suspended in 30ml of NPI-20 buffer including benzonase and lysozyme. Cells were disrupted
5 by ultrasonication (3x20 sec) on ice. The supernatant containing the soluble proteins was obtained after centrifugation of the suspension at 4°C and 40000 x g for 30 min. Both proteins were purified by affinity chromatography at room temperature. One column of Ni-Agarose (5 ml, GE Healthcare) was equilibrated with 50 ml of NPI-20. The supernatant containing the soluble proteins was applied to the column, followed by a washing step with NPI-20. The
10 bound protein was eluted with a linear gradient to 50 % NPI-500 in 100ml. Fractions were analyzed by SDS-PAGE with respect to their purity. Suitable fractions were pooled and applied to a gel filtration column (Superdex 75, 1.6 x 60 cm, GE Healthcare) equilibrated with SUMO-hydrolase cleavage buffer (50 mM Tris, 300 mM NaCl, pH 8.0) at a flow rate of 1ml/min.

15 The cleavage reaction was done according to the manufactures instruction (Invitrogen). After cleavage the protein was applied to a Ni-Agarose column (5ml, GE Healthcare). His-tagged SUMO-hydrolase and His-tagged SUMO were bound to the column and the correct fusion protein passed the column (His-tag free). Purity of the proteins was proofed by rpHPLC
20 analysis and gel electrophoresis. The correct molecular mass of the trimer (via TNFa) was confirmed using analytical SEC analysis (10/30 Superdex G75, GE Healthcare).

Example 4: Binding Analysis of modified Ubiquitin-based ED-B binding Variants to human ED-B

25 **Example 4A. Binding analysis of modified ubiquitin-based ED-B binding variants by concentration dependent ELISA.**

Binding of ubiquitin-based variants to human ED-B was assayed by a concentration dependent ELISA. Increasing amounts of purified protein applied to NUNC-medisorp plates coated with human ED-B, BSA and cellular fibronectin (cFN). Antigen coating with 50 µl (10
30 µg/ml) per well was performed at 4°C overnight. After washing the plates with PBS, 0.1 % Tween 20 pH 7.4 (PBST) the wells were blocked using blocking solution (PBS pH 7.4; 3 % BSA; 0.5% Tween 20) at 37°C for 2 h. Wells were washed again three times with PBST.

Different concentrations of modified ubiquitin based ED-B binding protein were then incubated in the wells at RT for 1 h (50 μ l volume)(in FIG. 10, as start concentration, 500 nM of 1041-D11 protein was used). After washing the wells with PBST, the anti-Ubi fab fragment (AbyD) POD conjugate was applied in an appropriate dilution (for example, 1:2000 or 1:6500) in PBST. The plate was washed three times with 300 μ l buffer PBST/well. 50 μ l TMB substrate solution (KEM-EN-Tec) were added to each well and was incubated for 15 min. The reaction was stopped by adding 50 μ l 0.2 M H₂SO₄ per well. The ELISA plates were read out using the TECAN Sunrise ELISA-Reader. The photometric absorbance measurements were done at 450 nm using 620 nm as a reference wavelength. Figure 1 shows clearly the specific binding of the 1H4 to ED-B with an apparent KD value of 11 nM. The variant 5E1 shows an apparent KD value of 7.7 μ M and 4B10 of 280 nM respectively. Figure 10 shows very high affinity binding of variant 1041-D11 to ED-B (KD=6,9 nM). Thus, only a few modifications (up to 8 substitutions in each monomer) in the ubiquitin-wildtype result in a very higher affinity binding to ED-B.

15

Example 4B. Binding analysis of modified ubiquitin-based ED-B binding variants by competitive concentration dependent ELISA.

Competitive concentration dependent ELISAs analyzed the binding of ubiquitin variant 1041-D11 to immobilized ED-B containing fibronectin fragment (67B89) in the presence of increasing amounts of free target. Conditions of the ELISA were as described for Example 5A, except that 1041-D11 protein was preincubated with ED-B (67B89) (0 μ M – 10 μ M) or also with negative control 6789 (0 μ M – 10 μ M) for 1 h and subsequently the mixture was given to the target 67B89 that was placed on a Medisorp-plate; following this, the variant was detected by the corresponding antibody (anti-Ubiquitin-Fab-POD; dilution 1:6500).

Figure 11 shows that variant 1041-D11 has a very high affinity binding to ED-B (IC₅₀ =140 nM). The result shown in Figure 10 is confirmed; only a few modifications (up to 8 substitutions in each monomer) in the ubiquitin-wildtype result in a very higher affinity binding to ED-B.

25

Example 4C. Binding analysis of modified ubiquitin-based ED-B binding variants by concentration dependent ELISA simultaneously analyzing the serum-stability of binding activity.

The ELISA is performed using procedures well known in the art and as described above (Example 5A and 5B). ED-B (here referred to as 67B89) is coated to microtiter plates, the variant is bound to ED-B and detected by a specific ubiquitin-antibody (Anti-Ubi-Fab-POD). The variant in this assay is treated in different ways: the variant is incubated in mouse serum for 1 h at 37°C (see in Fig. 13, circles in blue); the variant is incubated in rat serum for 1 h at 37°C (in Fig. 13, circles in red); or the variant is incubated PBS for 1 h at 37°C (in Fig. 13, circles in black). Figure 13 shows that all K_D s of variant 1041-D11 are between 10,3 nM (in PBS) to 20,74 nM (in mouse-serum).

Example 4D. Binding analysis of modified ubiquitin-based ED-B binding variants by Biacore assays.

Different concentrations of the variant were analyzed (for example, 0-200 nM of the variant, preferably 1041-D11) for binding to an ED-B containing fibronectin fragment (referred to as 67B89) immobilized on a CM5-chip (Biacore) using methods known to those skilled in the art. The obtained data were processed via the BIAevaluation software and 1:1-Langmuir-fitting. The K_D of variant 1041-D11 was 1.0 nM, as shown in Figure 12. The kinetic binding constants were $k_{on} = 7.6 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{off} = 7.7 \cdot 10^{-4} \text{ s}^{-1}$. The K_D of the fusion protein 1041-D11 – TNF α was 1.13 nM, as shown in Figure 17D. The kinetic binding constants were $k_{on} = 4,5 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{off} = 5,0 \cdot 10^{-4} \text{ s}^{-1}$.

Example 4E. Complex-formation analysis of modified ubiquitin-based ED-B binding variants by SE-HPLC.

For the analysis of complex formation, Tricorn Superdex 75 5/150 GL columns (GE-Healthcare) ($V = 3 \text{ ml}$) was used, protein amount of 50 μl was applied. Further conditions: buffer: 1x PBS, pH 7.3, flow-rate: 0,3 ml/min, run: 45 min (injection of sample: after 15 min). Condition: 0.72 nmol 1041-D11 protein + 0.72 nmol ED-B (herein referred to 67B89 or also as a negative control 6789) incubated for 1 h at RT; then applied to column for analysis of complex-formation. In Figure 14, only the variant is shown in black, only the target ED-B is shown in blue, the variant binding building a complex with ED-B in pink. Figure 14 A shows

ED-B with the variant; Figure 14 B is the variant without ED-B. The figure shows that variant 1041-D11 builds a complex together with ED-B (67B89), but it builds no complex with 6789.

Example 5: Biological Assay of TNF α

5

The physiological TNF α -activity of TNF α -modified ubiquitin based ED-B binding fusions has been determined using the L929 apoptosis assay (Flick et al., 1984 J. Immunol. Methods. 68:167-175). In this assay, TNF-alpha efficiently stimulates cell death in actinomycinD sensitized cells at EC₅₀ values in the picomolar range.

10

Cells have been resuspended in medium containing FBS and antibiotics. A cell suspension of 100 μ l of a density of 3.5×10^5 cells/ml has been seeded into the wells of a 96 well standard cell culture plate followed by over night incubation in a humidified CO₂ incubator. Thereafter, the culture medium has been removed and 50 μ l of medium containing FBS, ActinomycinD and antibiotics has been added to each well followed by a further 30 min incubation time. Thereafter, 50 μ l of the test items, TNF α - modified ubiquitin based ED-B binding -fusions or the human recombinant TNF α control, at an appropriate concentration range of between 10^{-7} and 10^{-18} M, have been added. After a further 48 h incubation time the metabolic activity as a measure of cell survival was determined using WST-1 reagent (Roche).

20

Per test item at least three independent experiments have been conducted, each of them in triplicates. Each testing of TNF α - modified ubiquitin based ED-B binding -fusion proteins was paralleled by testing a dose range of human recombinant TNF α to get information on the inter assay variability.

25

The quantitative evaluation is based on the EC₅₀-value, i.e. the value according to the test item concentration promoting the survival of half of the cells.

30 Table 2

TNF α - mub-Fusion	EC ₅₀ value	
	mub[®]- TNFαFusion	Corresponding TNFα
Wubi-TNF-alpha	5,18 \pm 2,84 pM	7,97 \pm 12,18 pM

Wubi-Hubi-TNF-alpha	32,58 ± 11,26 pM	5,02 ± 3,70 pM
SPWF-28_22-D1_TNF-alpha	26,15 ± 14,41 pM	2,32 ± 2,07 pM
SPWF-28_24-H12_TNF-alpha	0,78 ± 0,24 pM	3,01 ± 4,18 pM

mub: modified ubiquitin based ED-B binding

5 Of the TNF α - modified ubiquitin based ED-B binding -fusion one ubiquitin monomer (Wubi) and three ubiquitin dimer constructs have been analyzed. Depending on the modified ubiquitin based ED-B binding variant coupled to the TNF-alpha moiety the TNF-alpha associated activity has been increased (SPWF-28_24-H12_TNF-alpha) or decreased (SPWF-28_22-D1_TNF-alpha, Wubi-Hubi-TNF-alpha) by about one order of magnitude. See Figure 17 for variant 1041-D11 TNFalpha analysis.

10

Example 6. Binding analysis of ubiquitin variants in cell culture assays

15 The binding of, for example, variant 1041-D11 to cell culture cells was tested. Different cell culture cells were analysed, including normal human fetal lung fibroblast cells having high expression levels of ED-B (Wi38 cells), a mouse embryonic fibroblast cell line (Balb 3T3); a stromal cell line, derived from mouse bone marrow (ST-2) monocytes/macrophages (RAW 264.7), NHDF cells and murine fibroblast cells (LM).

20 The variant 1041-D11 (different concentrations) or an ED-B specific antibody (500nM FV28 CH4/F1 1x PBS were incubated (1h, 37°C) with Wi38 cells (60,000 cells/ml; from ATCC), followed by fixation with methanol (5 min, -20°C), blocking (5% Horse/PBS, 1h); incubation with rabbit-a-Strep-Tag-IgG (obtained from GenScript A00875, 1:500) for 1h and incubation with a-rabbit-IgG*Alexa488-AK (obtained from Invitrogen A11008, 1:1000) for 1h. The nuclei were stained with DAPI. The first column in Figure 15A shows the control using EDB antibodies, the second column shows the incubation of the variant at a protein concentration of 58,7 nM, the third column a ten-fold higher concentration of 1041-D11 protein (587 nM), the fourth column is a negative control with PBS. In the first row, human Wi38 fibroblast cells are shown in phase contrast, the second row shows the immunofluorescence and the third row a DAPI staining. It can be concluded from the pictures that the variant 1041-D11

binds to fixed Wi38 cells with high specificity to ED-B containing extracellular matrix. The negative control cell type NHDF are primary normal fibroblast cells, which express low levels of EDB-fibronectin (data not shown). The variants do not bind to those cells.

5 Figure 15 B shows the analysis of variant 1041-D11 on vital Wi38 cells. The negative control cell type NHDF is primary normal fibroblast cells, which express low levels of EDB-fibronectin. The cells were plated in chamber-slides (NUNC, 60000cells/ml). To analyse the binding potential the cells were fixed with 100% MeOH for 5 min at -20°C. To block unspecific binding, the cells were incubated with 5% Horse-serum 1h 37°C. The cells were
10 tested with the variant 1041-D11, an ED-B specific antibody FV28 CH4/F1 as positive control or UB_2 as negative control with different concentrations 1h RT. The proving occurred about an incubation with rabbit-a-Strep-Tag-IgG (obtained from GenScript A00875, 1:500) for 1h and incubation with a-rabbit-IgG*Alexa488-AK (obtained from Invitrogen A11008, 1:1000) for 1h. The nuclei were stained with DAPI. The first and third line in Figure
15 15B shows the variant at different protein concentration and the negative control. The second and fourth line shows the incubation of the control using EDB antibodies. The first 2 lines show the variant and positive control on Wi38-cell line. The third and fourth line shows the incubation of NHDF-cells. It can be seen from the pictures that the variant 1041-D11 binds to vital Wi38 cells with high specificity to ED-B containing extracellular matrix. A control using
20 NHDF cells which do not contain low EDB was performed (data not shown). The variants do not bind to those cells.

Similar experiments were performed using different cells types, for example Balb3T3 (ATCC, Kat-Nr. 30-2002), Raw (Lonza, Kat-Nr. BE12-115F/U1), ST-2 (Lonza, Kat-Nr.
25 BE12-115F/U1). Figures 15C and D show that the binding of ED-B is highly specific to murine Balb3T3 and ST-2 cells. No binding was observed to monocytes/macrophages (Raw)(data not shown).

As outlined above, Figure 16 A shows the specificity of 1041-D11 in tissue sections. F9
30 tumor tissues from seven samples were evaluated. Immune-histochemistry with 500 nM 1041-D11 resulted in ED-B specific vascular staining on F9 tumors from mice. ED-B is a highly specific marker for tumor vasculature. The target protein EDB is located on the abluminal side of the vessels. 1041-D11 specifically decorates the vasculature in tissue

sections from F9 tumors. The obtained results are comparable to tissue specificity of the antibody fragment L19. In addition, 48 tissues were tested; no unspecific staining in any out of 48 tissues in FDA relevant panel was observed. Figure 16 B shows the accumulation of 1041-D11 in tumor cells in comparison to wild type Ubiquitin. Thus, fusion proteins based on modified ubiquitin specifically binding to ED-B are suitable an ED-B based targeted therapy for cancer.

Example 7: Efficacy in vivo study of 1041D11- TNF α

10 To establish the therapeutic efficacy of 1041-D11-TNF α , the compound was tested on F9 teratoma (see Borsi et al., 2003 *Blood* 102, 4384-4392) in mouse models. The ED-B expression in mice is comparable to the human in vivo situation and is suitable for an evaluation of the therapeutic impact of 1041-D11-mTNF α on cancer, preferably in combination with a cytotoxic compound such as Melphalan. F9 teratoma is an aggressive tumor with high vascular density. Borsi et al described that targeting of mouse TNF α via EDB-antibodies improve the efficacy of Melphalan which is demonstrated by retardation in tumor growth. The experimental schedule for the efficacy study was adapted from Borsi, 2003.

20 Stage 1 defined the pharmacologic active and tolerable dose with endpoints relating to the ratio of tumor vs. body weight, weight loss and survival. The inventors found that 1041D11-TNF α is tolerated at highest dose (6.75 pmol/g) but has no suppressing effect on tumor growth (> 10 % body weight after 3, 4 and 8 days → animals were killed), whereas 1041D11-TNF α at lowest dose (0.25 pmol/g) seems to retard tumor growth. Dosing groups further used were descending from 2.25 pmol/g 1041D11-TNF α .

Stage 2 of the study defined the dose-dependent efficacy with Melphalan having as endpoint the retardation of tumor growth (animal weight loss > 10 %, tumor > 10 % body weight, ulceration of tumor). In the study, 1041D11/mTNF α , murine TNF α , in combination with melphalan were tested. 168 animals were used, 14 Dosing groups (8 mice per group recruited when bearing F9 tumors of 300 – 400 mm³); Administration of test sample i. v. followed by i. p. injection of Melphalan 24 h later Table 1 shows the dosing schedule:

Group	Test item	Dose		Route	Appl. vol	Animals (n)*
		Melphalan** (mg/kg)	TNF- α proteins (pmol/g)			
1	PBS	0	0	iv	10 ml/kg	8
2	mouse TNF- α fusion protein	0	2.25	iv	10 ml/kg	8
3	mouse TNF- α fusion protein	0	0.75	iv	10 ml/kg	8
4	mouse TNF- α fusion protein	0	0.25	iv	10 ml/kg	8
5	mouse TNF- α fusion protein	0	0.025	iv	10 ml/kg	8
6	mouse TNF- α fusion protein	0	0.0025	iv	10 ml/kg	8
7	Melphalan	4.5	0	ip	10 ml/kg	8
8	Melphalan/ mouse TNF- α fusion protein *	4.5	2.25	ip/iv	10/10 ml/kg	8
9	Melphalan/ mouse TNF- α fusion protein *	4.5	0.75	ip/iv	10/10 ml/kg	8
10	Melphalan/ mouse TNF- α fusion protein *	4.5	0.25	ip/iv	10/10 ml/kg	8
11	Melphalan/ mouse TNF- α fusion protein *	4.5	0.025	ip/iv	10/10 ml/kg	8
12	Melphalan/ mouse TNF- α fusion protein *	4.5	0.0025	ip/iv	10/10 ml/kg	8
13	mouse TNF- α	0	0.25	iv	10ml/kg	8
14	Melphalan/ mouse TNF- α	4.5	0.25	ip/iv	10/10 ml/kg	8

*animals with subcutaneous tumors of 300 –400 mm³
** Melphalan is applied 24 hours after mouse TNF- α protein injection
the MTD will be determined in study P10.0164

Figure 18 shows the relative tumor growth during the time of treatment (7 days). Fig. 18a clearly shows that our compound 1041-D11-TNF α in combination with Melphalan reduces the relative tumor growth more efficiently than mTNF α in combination with Melphalan or Melphalan alone. The tumor growth kinetic 7 days after treatment shows the significant reduction of tumors by 1041-D11-mTNF α . This is a clear evidence for efficacy in combination with Melphalan.

PUBLICATIONS

- 5 1. **Birchler, M., F. Viti, L. Zardi, B. Spiess, and D. Neri.** 1999. Selective targeting and photocoagulation of ocular angiogenesis mediated by a phage-derived human antibody fragment. *Nat Biotechnol* **17**:984-8.
2. **Brenmoehl, J., M. Lang, M. Hausmann, S. N. Leeb, W. Falk, J. Scholmerich, M. Goke, and G. Rogler.** 2007. Evidence for a differential expression of fibronectin splice forms ED-A and ED-B in Crohn's disease (CD) mucosa. *Int J Colorectal Dis* **22**:611-23.
- 10 3. **Dubin, D., J. H. Peters, L. F. Brown, B. Logan, K. C. Kent, B. Berse, S. Berven, B. Cercek, B. G. Sharifi, R. E. Pratt, and et al.** 1995. Balloon catheterization induced arterial expression of embryonic fibronectins. *Arterioscler Thromb Vasc Biol* **15**:1958-67.
- 15 4. **Goodsell, D. S.** 2001. FUNDAMENTALS OF CANCER MEDICINE: The Molecular Perspective: Antibodies. *The Oncologist* **6**:547-548.
5. **Kaczmarek, J., P. Castellani, G. Nicolo, B. Spina, G. Allemanni, and L. Zardi.** 1994. Distribution of oncofetal fibronectin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int J Cancer* **59**:11-6.
- 20 6. **Menrad, A., and H. D. Menssen.** 2005. ED-B fibronectin as a target for antibody-based cancer treatments. *Expert Opin Ther Targets* **9**:491-500.
7. **Pujuguet, P., A. Hammann, M. Moutet, J. L. Samuel, F. Martin, and M. Martin.** 1996. Expression of fibronectin ED-A+ and ED-B+ isoforms by human and experimental colorectal cancer. Contribution of cancer cells and tumor-associated myofibroblasts. *Am J Pathol* **148**:579-92.
- 25 8. **Trachsel, E., M. Kaspar, F. Bootz, M. Detmar, and D. Neri.** 2007. A human mAb specific to oncofetal fibronectin selectively targets chronic skin inflammation in vivo. *J Invest Dermatol* **127**:881-6.
- 30 9. **Van Vliet, A., H. J. Baelde, L. J. Vleming, E. de Heer, and J. A. Bruijn.** 2001. Distribution of fibronectin isoforms in human renal disease. *J Pathol* **193**:256-62.
10. **Lipovsek, D., and Pluckthun, A.** (2004). In-vitro protein evolution by ribosome display and mRNA display. *J. Immunol. Methods* **290**, 51-67.

11. **Ohashi, H., Shimizu, Y., Ying, B.W., and Ueda, T.** (2007). Efficient protein selection based on ribosome display system with purified components. *Biochem Biophys. Res. Commun.* **352**, 270-276.
12. **Studier, F.W.** (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41**, 207-234.
13. **Zahnd, C., Amstutz, P., and Plückthun, A.** (2007). Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target. *Nat. Methods* **4**, 269-279.

CLAIMS

1. A protein capable of binding the ED-B domain of fibronectin, comprising at least one modified ubiquitin protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 60%, wherein at least 3 amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66 and 68 of SEQ ID NO: 1 are modified by substitution, deletion or addition in order to obtain a modified ubiquitin protein with a detectable binding to said ED-B domain of fibronectin with a specific binding affinity of $K_d = 10^{-5} - 10^{-12}$ M.
2. The protein of claim 1, wherein said modifications form a contiguous stretch of amino acids located on the surface of said protein wherein at least 4 of said replaced amino acids are located within a beta sheet or within positions up to 3 amino acids adjacent to a beta sheet strand.
3. The protein according to anyone of the previous claims wherein said modifications are within the beta sheet strands of said ubiquitin ranging from 2 - 7, 12 - 16, 41 - 45 and 65 - 71, and/or within positions up to 3 amino acids adjacent to a beta sheet strand ranging from 62 - 64 or 8-11, said protein is having an amino acid sequence identity after modification of at least 70% with the corresponding sequence of SEQ ID NO: 1, and preferably wherein said modifications comprise at least 10% and at most 40% of those amino acids located in the beta sheet strands of the ubiquitin protein.
4. The protein according to anyone of the previous claims wherein at least 4 amino acids located in positions 2, 4, 6, 8, 62, 63, 64, 65, 66 and/or 68 are modified and wherein further 1 to 7 additional amino acids are modified, which are optionally selected from one or more of the amino acids in positions 36, 44, 70, 71, 72 and 73.
5. The protein according to anyone of the previous claims wherein at least two differently modified ubiquitin proteins are linked together in a head-to-tail arrangement or wherein at least one modified and at least one unmodified ubiquitin protein is linked together in a head-to-tail arrangement to obtain hetero-multimeric modified ubiquitin proteins, wherein said multimeric proteins are optionally dimeric or trimeric.

6. A protein capable of binding the extradomain B (ED-B) of fibronectin, comprising a modified multimeric, optionally dimeric or trimeric ubiquitin protein wherein at least two monomeric ubiquitin units are linked together in a head-to-tail arrangement, wherein each monomer of said multimeric protein is modified by substitutions of at least 3 or 4 amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66, and 68 of SEQ ID NO: 1, said modified monomeric ubiquitin unit having an amino acid identity to SEQ ID NO: 1 of at least one of the group of at least 60%, at least 85% and at least 90%, said protein having a specific binding affinity to said ED-B domain of fibronectin of $K_d = 10^{-5} - 10^{-12}$ M and exhibits a monovalent binding activity with respect to said extradomain B (ED-B) of fibronectin wherein in each monomeric ubiquitin at least 3 or 4 amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66 and 68 of SEQ ID NO: 1 are modified by substitution, deletion or addition in order to obtain a modified ubiquitin protein with a detectable binding to said ED-B domain of fibronectin with a specific binding affinity of $K_d = 10^{-5} - 10^{-12}$ M.
7. The protein of claim 6, wherein the multimer is formed of the same or different modified ubiquitin protein
8. The protein of claim 7, wherein the modified ubiquitin proteins are genetically or post-translationally fused.
9. The protein of one or more of claims 6-8, which is a hetero-dimer of said ubiquitin protein having modifications at least in positions 6, 8, 63-66 of the first ubiquitin monomer and in positions 6, 8, 62-66, and optionally in position 2 of the second ubiquitin monomer, preferably K6W, L8W, K63R, E64K, S65F, T66P; and K6T, L8Q, Q62W, K63S, E64N, S65W, T66E; optionally Q2R.
10. The protein of one or more of claims 5-8, which is a hetero-dimer of said ubiquitin protein comprising the sequence SEQ ID NO: 47.
11. The protein of one or more of claims 7-10, wherein both ubiquitin monomers are linked by a linker, preferably the linker of SEQ ID NO: 32 or by the sequence GIG.

12. The protein of one or more of claims 10 or 11, which comprises the ubiquitin hetero-dimer of SEQ ID NO: 33 or 34.
13. A fusion protein comprising a protein according to anyone of the previous claims fused to a pharmaceutically and/or diagnostically active component, wherein said pharmaceutically active component is optionally a cytokine, preferably TNF-alpha, a chemokine, a cytotoxic compound, or an enzyme, and wherein said diagnostically active component is selected from a fluorescent compound, a photosensitizer, or a radionuclide.
14. The fusion protein according to claim 13 wherein said fusion protein comprises a multimeric modified ubiquitin protein, which is optionally fused with a pharmaceutically or diagnostically active component, optionally a cytokine or wherein said protein comprises a multimeric modified ubiquitin protein wherein said multimeris formed via said pharmaceutically active component which is optionally TNF-alpha, or via said diagnostic component.
15. The fusion protein of one or more of the preceding claims, which is a trimer of a fusion protein of a ubiquitin hetero-dimer fused to TNF-alpha, wherein the fusion protein preferably has the sequence of SEQ ID NO: 35 or 36 or SEQ ID NO 47 or has an identity of at least 90% with the sequence of SEQ ID NO: 35 or 36 or SEQ ID NO 47.
16. The protein or fusion protein of one or more of the preceding claims, which is recombinant.
17. A pharmaceutical composition containing a recombinant protein or a recombinant fusion protein or a combination thereof in accordance with anyone of the preceding claims and a pharmaceutically acceptable carrier.
18. The pharmaceutical composition of claim 17, further comprising one or more chemotherapeutic agents, preferably selected from melphalan, doxorubicin,

cyclophosphamide, dactinomycin, fluorodesoxyuracil, cisplatin, paclitaxel, and gemcitabine; or from the group of kinase inhibitors or radiopharmaceuticals.

19. The pharmaceutical composition of claim 18, which is in the form of a combined preparation, preferably of a kit of parts.
20. A polynucleotide coding for a recombinant protein or fusion protein according to anyone of claims 1 - 16.
21. A vector comprising a polynucleotide according to claim 20.
22. A host cell comprising a protein according to anyone of claims 1 - 4,, a protein according to anyone of claims 5 - 12, a fusion protein according to anyone of claims 13 - 16, a vector according to claim 21 and/or a polynucleotide according to claim 19.
23. A diagnostic agent comprising a recombinant protein according to anyone of claims 1 - 4, a multimeric protein according to anyone of claims 5 - 12, or a fusion protein according to anyone of claims 13 - 16, with a diagnostically acceptable carrier.
24. A method for the generation of a recombinant protein according to anyone of claims 1 - 4 comprising the following steps:
 - a) providing a ubiquitin protein;
 - b) providing the ED-B domain of fibronectin;
 - c) modifying said ubiquitin protein in order to obtain a protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 60% wherein at least 3 or 4 amino acids are modified by substitution, deletion or addition of amino acids in positions 2, 4, 6, 8, 62, 63, 64, 65, 66, and/or 68 ;
 - d) contacting said modified ubiquitin protein with said ED-B domain of fibronectin;
 - e) screening for modified ubiquitin proteins which bind to said ED-B domain of fibronectin with a specific binding affinity of 10^{-5} - 10^{-12} M,
 - f) and isolating said modified ubiquitin proteins.

25. The method of claim 24 wherein chemical synthesis steps are included in said modification step.
26. The method of claim 24 or 25 wherein said modification is performed by genetic engineering on the DNA level, and expression of said protein is performed in prokaryotic or eukaryotic organisms or *in vitro*.
27. The method according to anyone of claims 24 - 26 wherein said recombinant protein is fused with a pharmaceutically active component, optionally a cytokine, preferably TNF-alpha, or a diagnostic component,
- or wherein said recombinant protein is fused with at least one second recombinant ubiquitin protein to obtain a multimer, optionally a dimer or trimer of said recombinant ubiquitin protein which is optionally fused with a pharmaceutically active component, optionally a cytokine, or a diagnostic component, or
wherein said recombinant protein is fused with at least one second recombinant ubiquitin protein to obtain a multimer, optionally a dimer or trimer of said recombinant ubiquitin protein wherein said multimer, dimer or trimer is formed via said pharmaceutically active component which is optionally TNF-alpha, or via said diagnostic component.
28. A method for generating a hetero-multimeric fusion protein according to anyone of claims 5-14, comprising the following steps:
 - a) providing a multimeric ubiquitin protein comprising two or more modified ubiquitin monomers linked either directly or by a suitable linker, wherein each monomer of said multimeric ubiquitin protein was modified in order to obtain a protein having an amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of at least 60% wherein at least 3 or 4 amino acids in each monomer are modified by substitution, deletion or addition of amino acids in positions 2, 4, 6, 62, 63, 64, 65, 66, and/or 68;
 - b) providing the ED-B of fibronectin;
 - c) contacting said hetero-multimeric modified ubiquitin protein with said ED-B of fibronectin;
 - d) screening for modified ubiquitin proteins which bind to said ED-B of fibronectin with a specific binding affinity of 10^{-5} - 10^{-12} M, and optionally

- e) isolating said modified hetero-multimeric ubiquitin proteins.
29. The method of claim 28, wherein the hetero-multimeric ubiquitin protein is a hetero-dimeric ubiquitin protein.
30. A recombinant protein according to anyone of claims 1 - , a multimeric protein according to anyone of claims 5 - 12, a fusion protein according to anyone of claims 13 - 16, for use in a method of medical treatment.
31. A library containing linear chains of modified multimeric ubiquitin proteins or ubiquitin fusion proteins as defined in one or more of claims 5 - 16 and being randomised in at least two interacting binding determining regions (BDR).

Occurrence of ED-B in Tumors

Tumor	Stroma/ Endothelium	Detection method	Literature
Glioblastoma multiforme (brain)		IH with L19	Pini 1998
Breast			
Lungs		I-Scinti with L19, mRNA	Oyama 1990
Adenocarcinoma of the lungs	Stroma+Endoth.	IH with C6, mRNA	Balza 2009, Oyama 1990, Pedretti 2009
Colorectal	Stroma	I-Scinti with L19	Pujuguet 1996
Mesothelioma	Stroma+Endoth.	IH with C6, IH with L19	Balza 2009, Pedretti 2009
Melanoma	Stroma+Endoth.	IH mit C6	Balza 2009
Squamous cell carcinoma	Stroma	mRNA	Oyama 1990, Pedretti 2009
Liver		mRNA	Oyama 1990
Small cell carcinoma		mRNA	Oyama 1990
Large cell carcinoma		mRNA	Oyama 1990
Non-small cell lung cancer		IH with L19	Pedretti 2009
Pancreas			Menrad & Menssen, 2005
Hodgkin lymphoma		¹³¹ I-L19SIP	Sauer et al 2009

Figure 1

FIGURE 3: Tetramerization leads to an increase in affinity

Variant	Target ED_B	Target c-FN
	K_D Monomer	K_D Tetramer
5E1	4,51 μ M	212 nM
1H4	9,98 μ M	6,78 nM

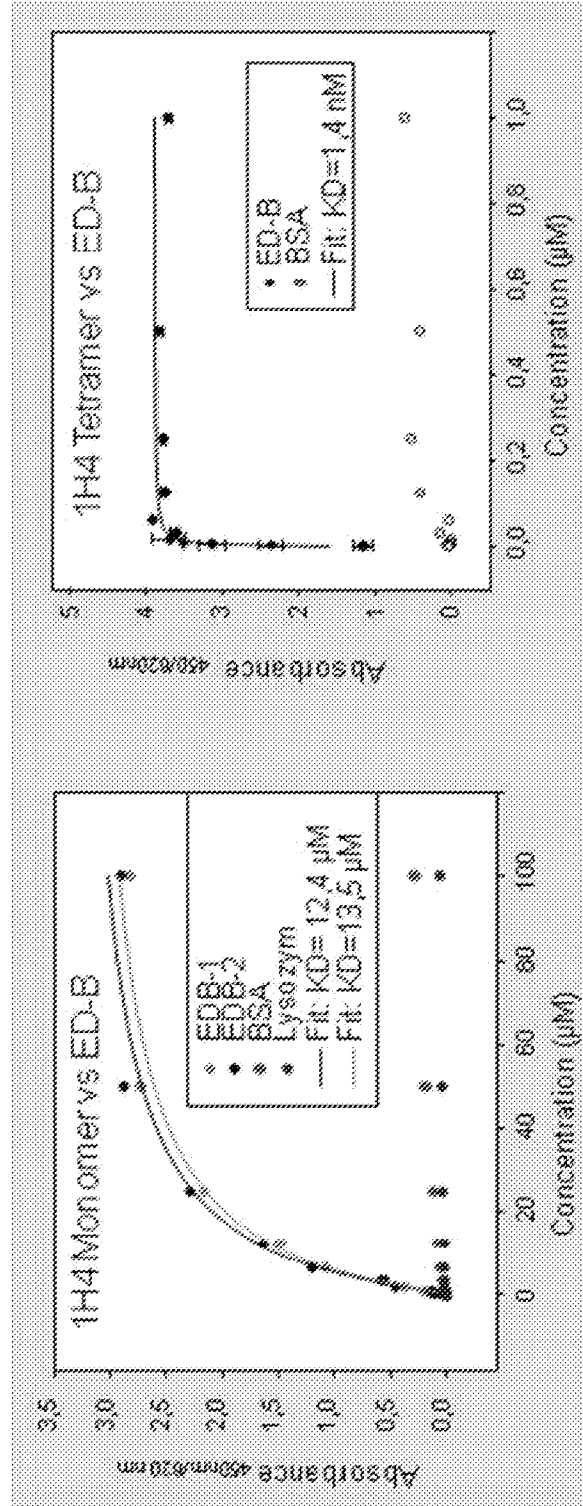


Figure 4: The recombination of the front monomer of clone 41B10 with a different rear monomer led to an increase of affinity as well as specificity

Figure 4A: Primary selection of SPW28-41B10

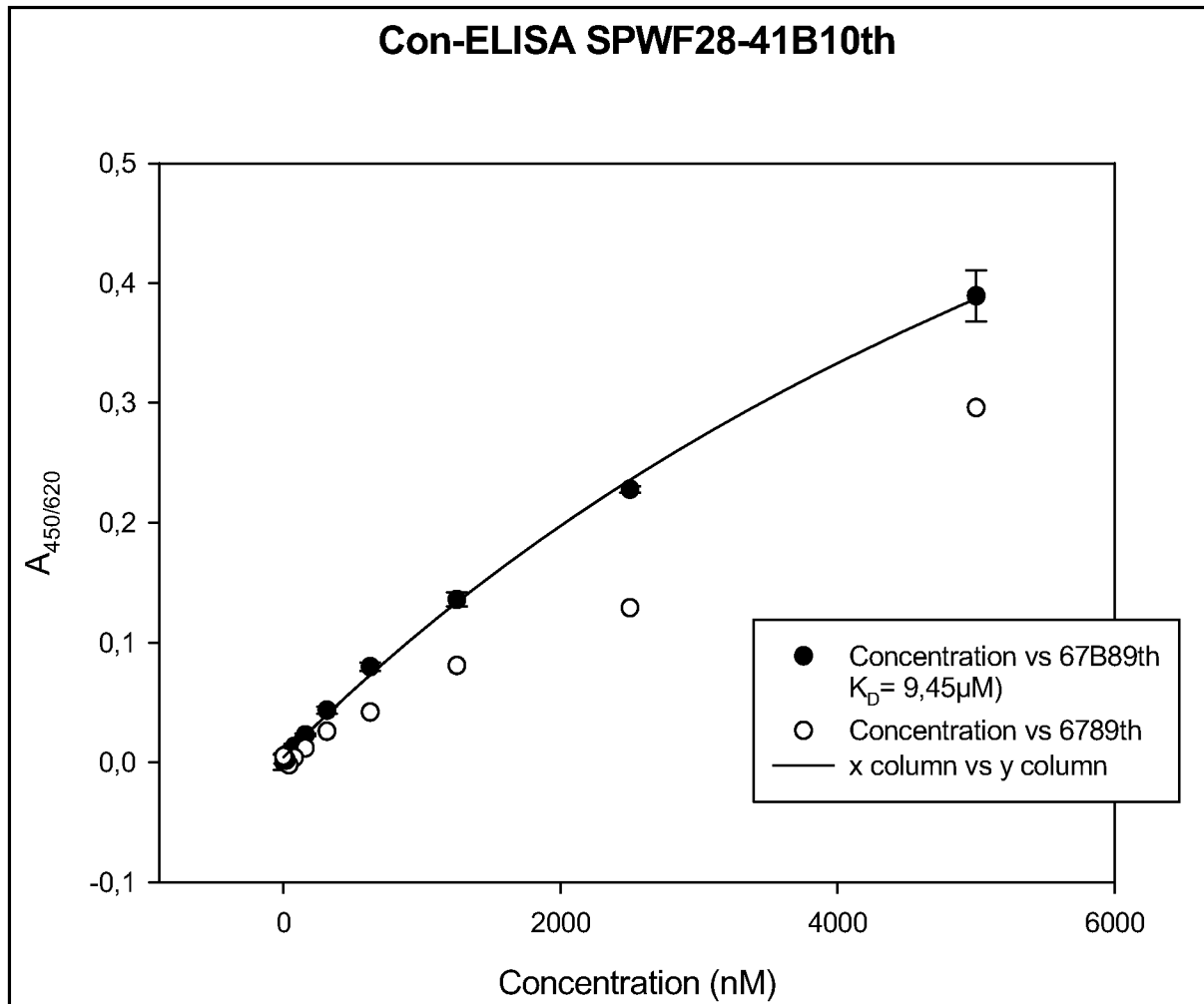
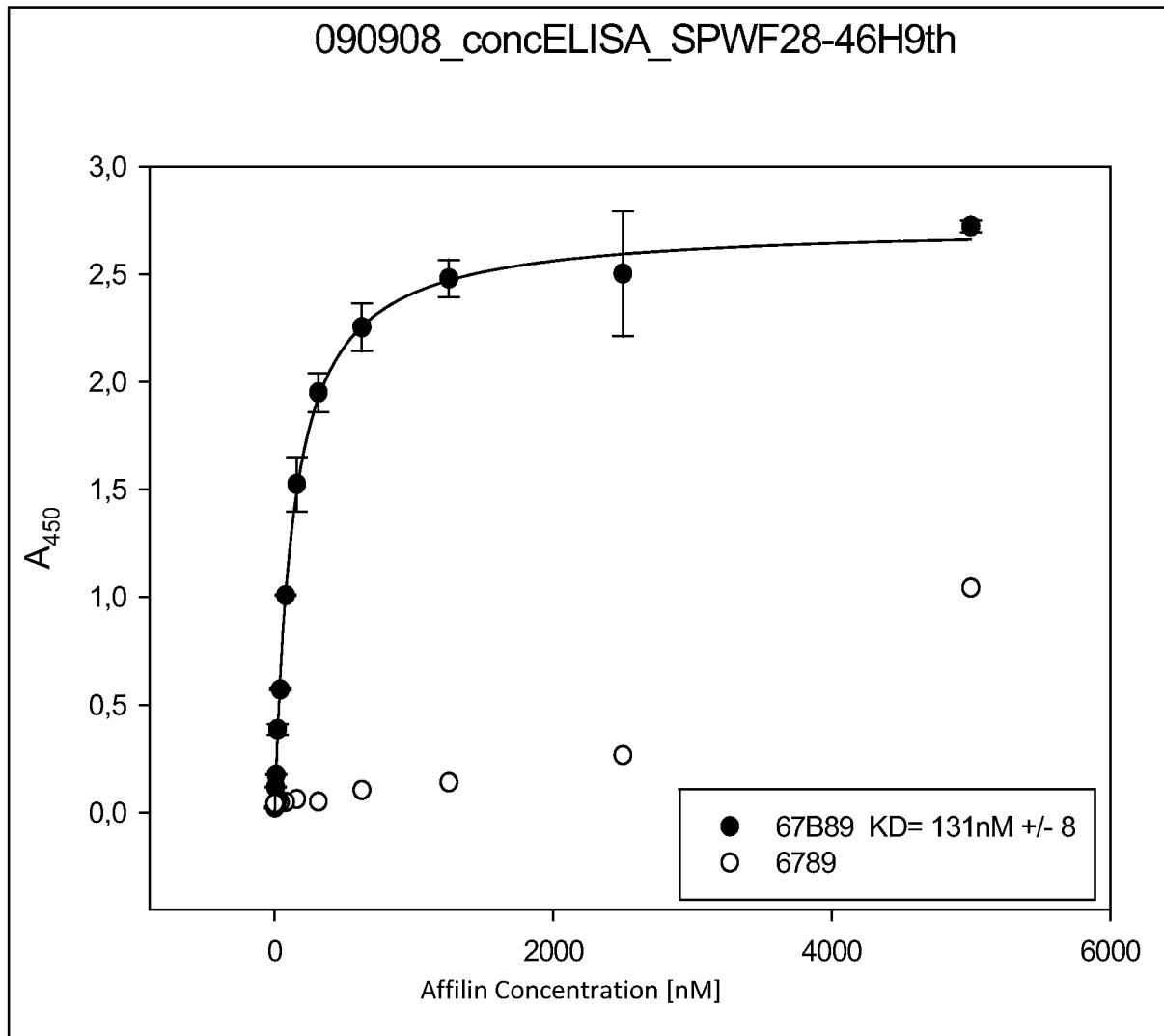


Figure 4B: After recombination with a different rear monomer (SPWF28-46H9)



Affilin = modified ubiquitin-based ED-B binding protein concentration

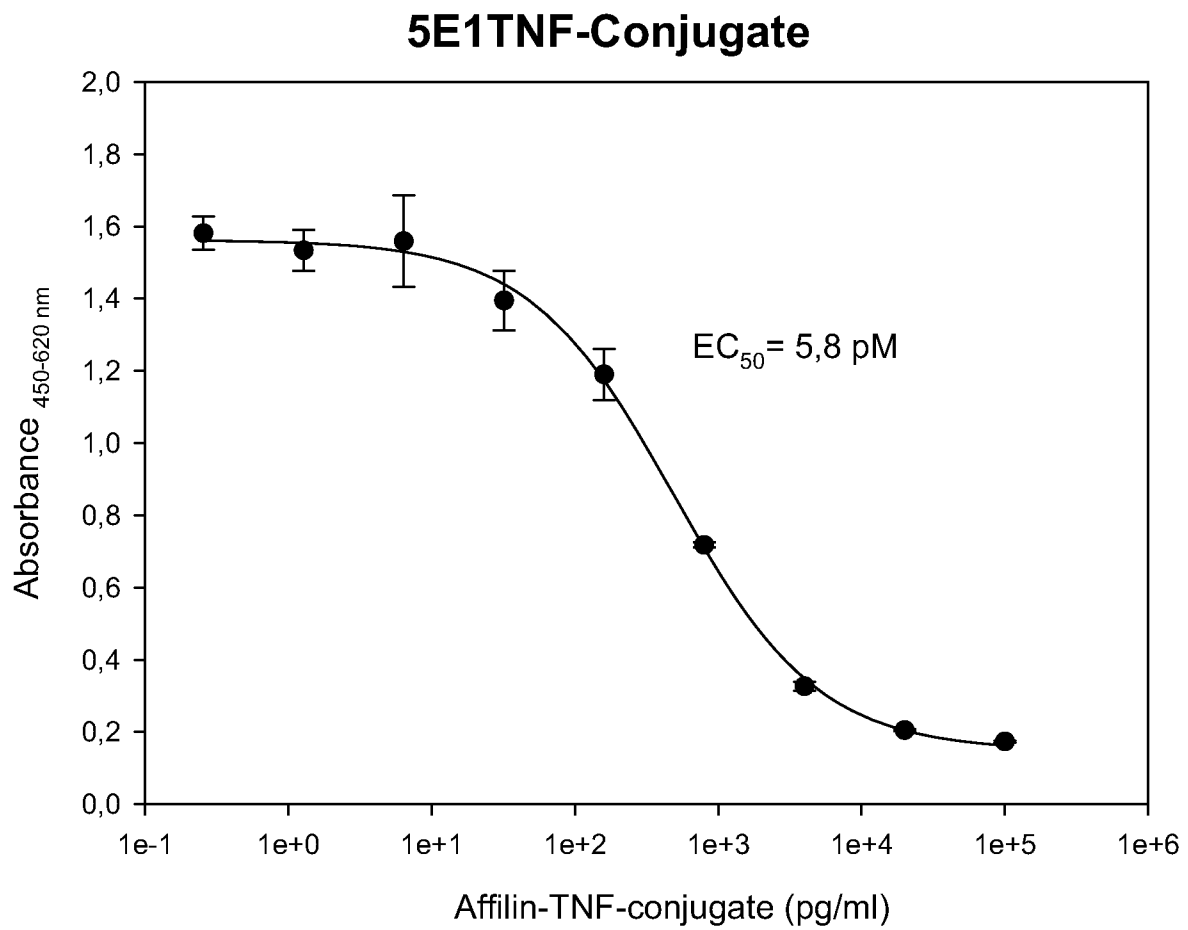
Figure 5: Modified ubiquitin-based ED-B binding -TNFalpha-Fusion protein trimerize and are active

Figure 5 A: drawing of modified ubiquitin-based ED-B based-effector-fusion protein; in green – effector, e.g. a cytokine, preferably TNF-alpha: red regions on effector;; brown: light brown: affilin structure; blue:

Effector

Affilin®



Figure 5B: The effector molecule is active (measured in L929 apoptosis assay)

Affilin = modified ubiquitin-based ED-B binding protein concentration

Figure 5C - Modified ubiquitin-based ED-B binding-TNFalpha-Fusion protein: High affinity binding to ED-B; 1H4-TNFalpha-Fusion

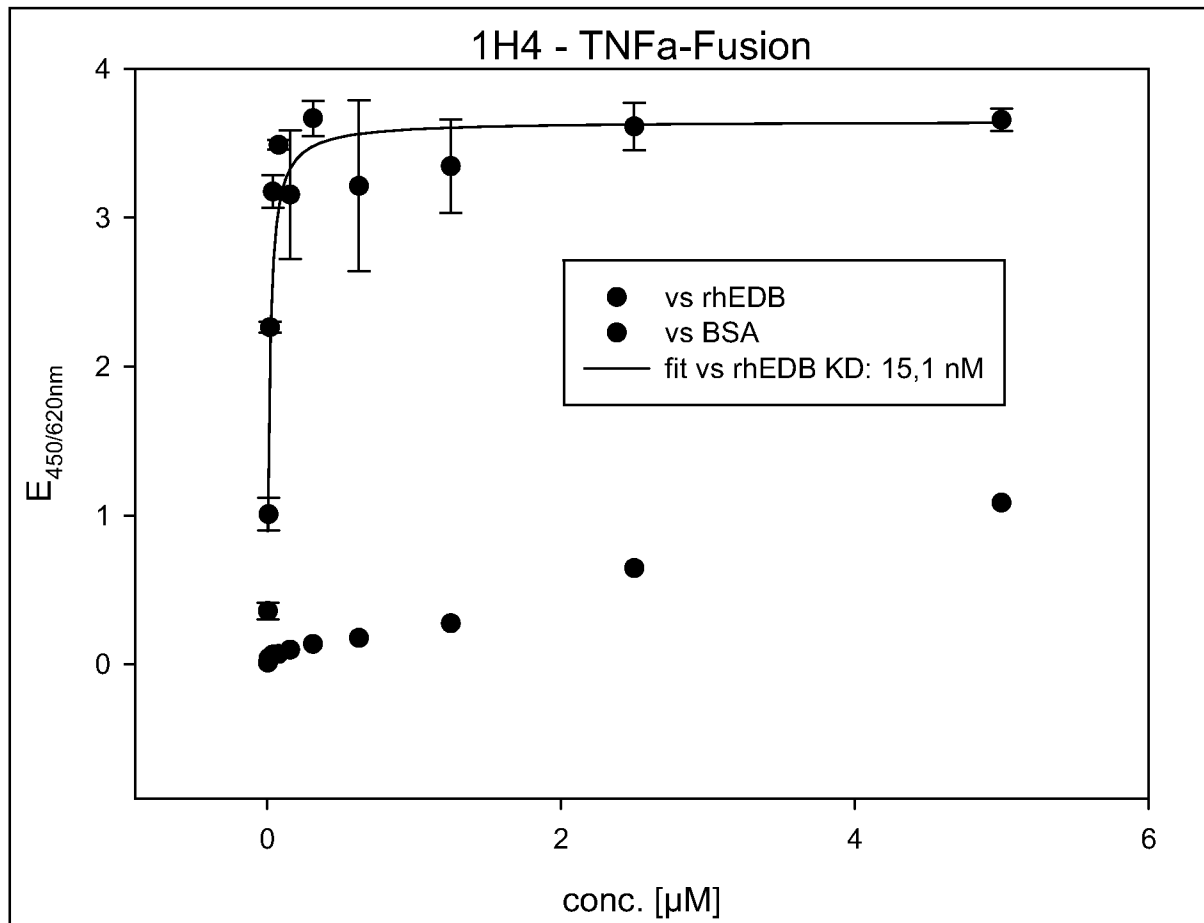


Figure 6 ~ Affinity and Activity of a Modified ubiquitin-based ED-B binding protein Dimer
Molecule fused to a Cytokine

5

- Apoptosis inducing activity of Affilin[®] cytokine fusion: EC₅₀ 0,78 ± 0,24 pM
- Apoptosis inducing activity of free cytokine: EC₅₀ 3,14 ± 3,59 pM

Figure 6 A shows the high affinity of modified ubiquitin based ED-B binding hetero-dimer 24H12 ($K_d = 50.7 \times 10^{-9} \text{ M}$).

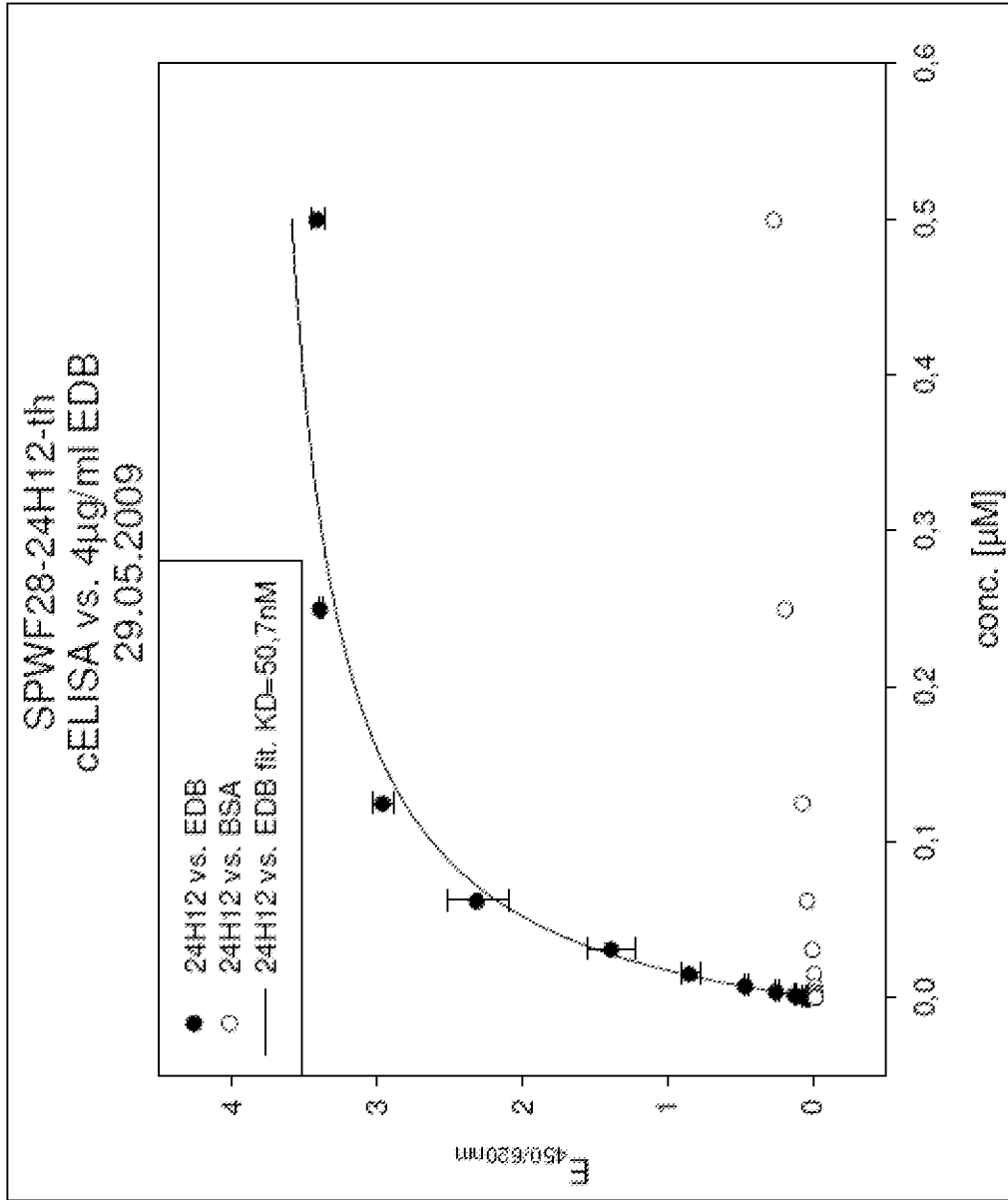


Figure 6 B shows the increased affinity of modified ubiquitin based ED-B binding heterodimer 24H12 fused to cytokine TNFalpha to result in a multimerisation of the heterodimer ($K_d = 5.6 \text{ nM} = 5.6 \times 10^{-9} \text{ M}$).

SPWF28-24H12-10a ELISA 15.07.2009

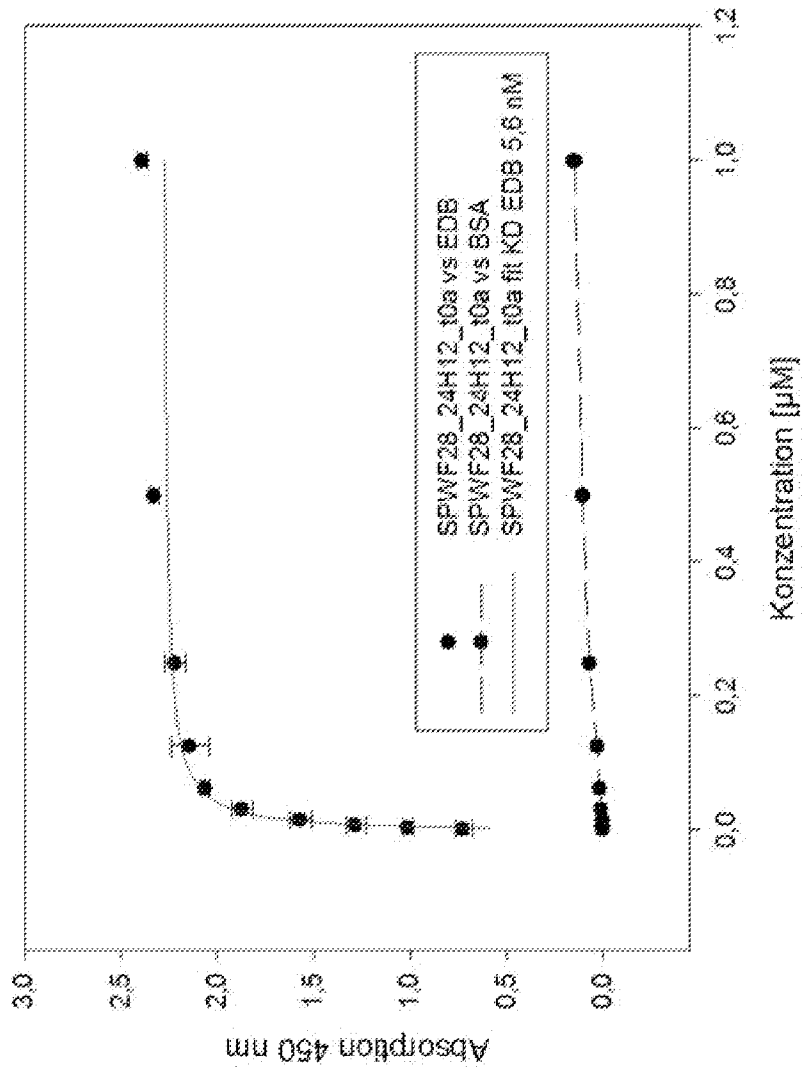
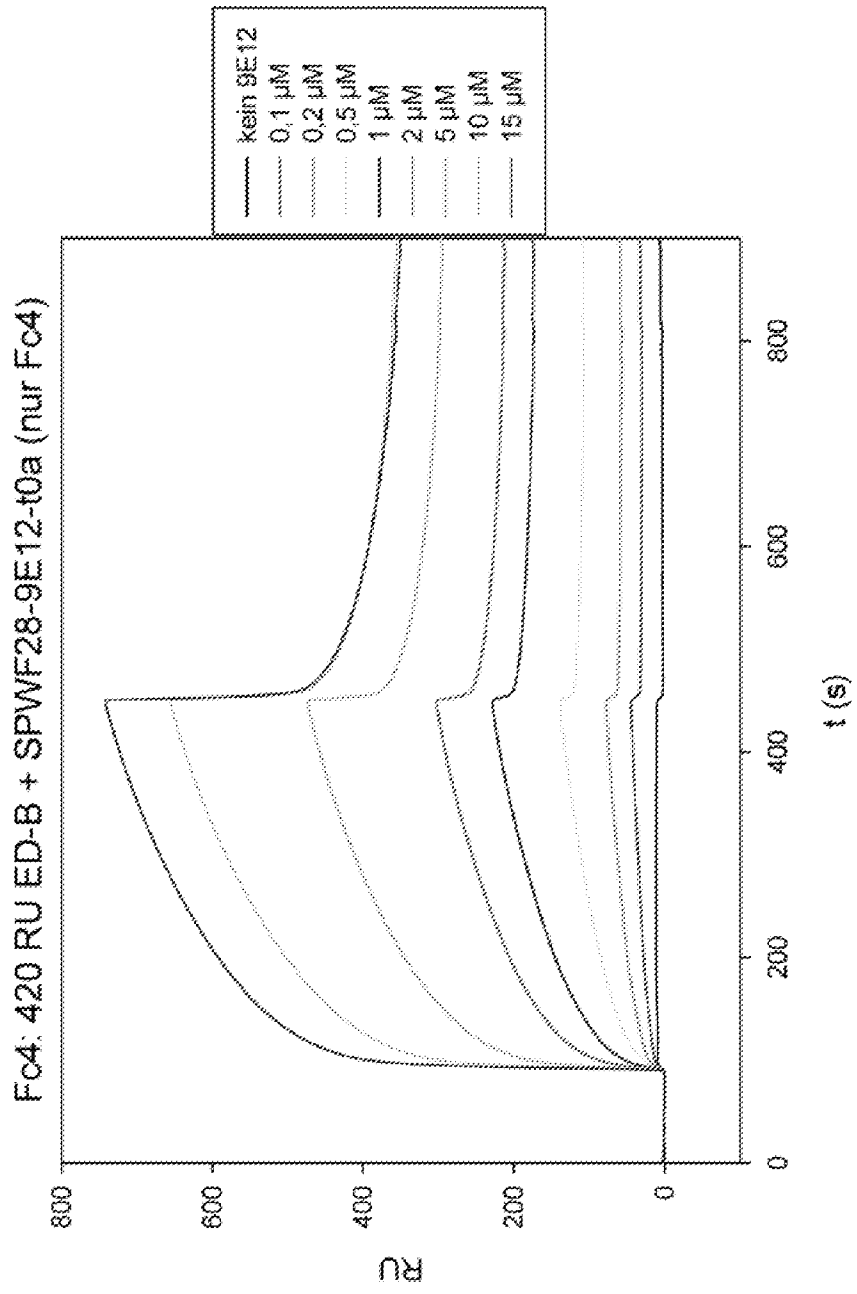


Figure 6 C Analysis of candidates from dimer library selection, for example 9E12 22D1 24H12 41B10

Variant	Target ED_B				Target c-FN
	K _D ELISA	Biacore			
		K _{on} (M ⁻¹ s ⁻¹)	K _{off} (s ⁻¹)	K _D (nM)	
9E12	9,5 nM	Not determinable	Not determinable	Not determinable	61,2 nM
22D1	594 nM	-	-	-	711 nM
24H12	50,7 nM	-	-	-	286 nM
41B10	310 nM	293	1,82·10 ⁻⁴	623 nM	280 nM

Figure 6D



5

10

15

20

Figure 6E

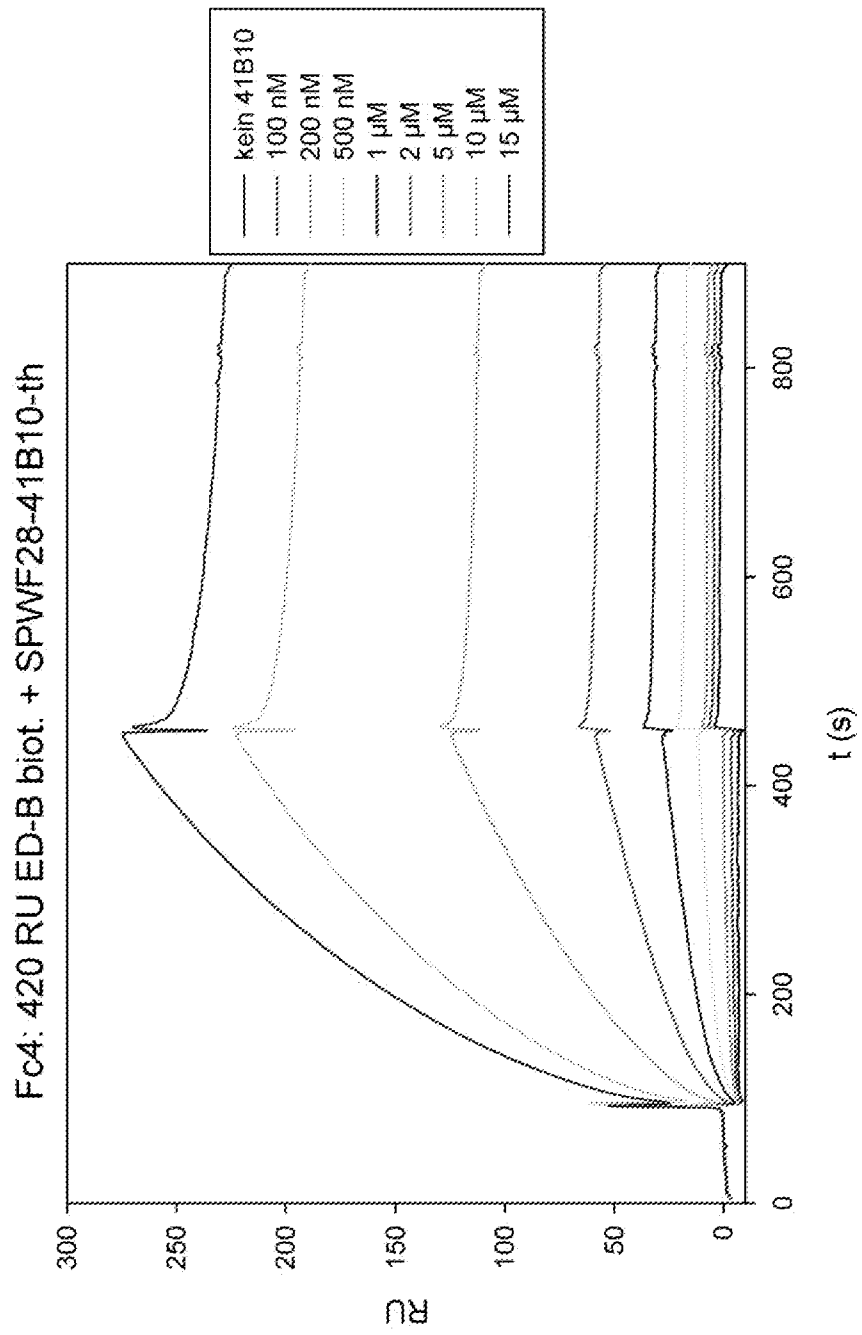
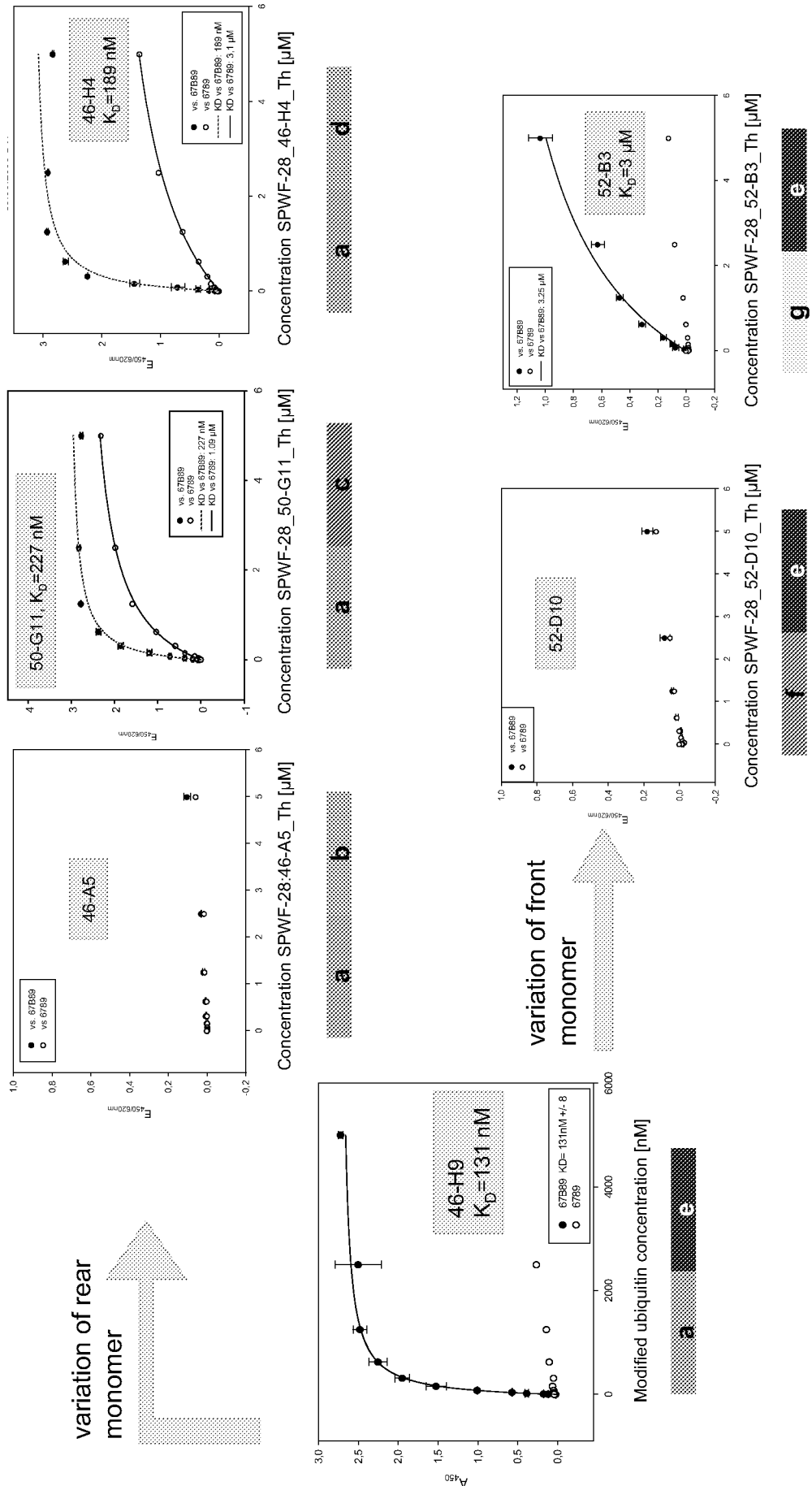


FIGURE 7: Contribution of different monomers to binding affinity and specificity



Multi-way Protein alignment

Sequences: 2 Scoring matrix BL O SUM 62

Sequence View: Similarity Format, Color behind non-matches

	10	20	30	40	50	60	70	80	90
WubiHub-Protein	•	•	•	•	•	•	•	•	•
pPR-IBAi-46H9_Ts	•	•	•	•	•	•	•	•	•
WubiHub-Protein	MQIFV	KTLTGK	TITLEVE	PSD	TIENVK	AKIQD	KEGIP	PDQQR	LIWAGKQ
pPR-IBAi-46H9_Ts	MGI	V	R	T	L	T	G	K	T
WubiHub-Protein	IFV	K	T	E	T	G	K	T	I
pPR-IBAi-46H9_Ts	IFV	H	T	M	T	G	K	T	I

FIGURE 8

FIGURE 9. Sequence comparison of variant 1041-D11 with ubiquitin

1041-D11_TsX9	1	MQIFVTTTGTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN
Ub2_TsX9	1	MQIFVKLTGTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN
Ubi-Dimer wt (Pr	1	MQIFVKLTGTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN
Ubi-Monomer wt	1	MQIFVKLTGTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN
1041-D11_TsX9	61	IQRKFLHLVLRLEGGGIGMRIFVTTQTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQ
Ub2_TsX9	61	IQKESTLHLVLRLEGGGIGMQIFVKLTGTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQ
Ubi-Dimer wt (Pr	61	IQKESTLHLVLRLEGG---MQIFVKLTGTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQ
Ubi-Monomer wt	61	IQKESTLHLVLRLEGG-----
1041-D11_TsX9	121	RLIFAGKQLEDGRTLSDYNIWSNWEIHLVLRRAA
Ub2_TsX9	121	RLIFAGKQLEDGRTLSDYNIQKESTLHLVLRRAA
Ubi-Dimer wt (Pr	118	RLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLEGG
Ubi-Monomer wt		-----

Figure 10

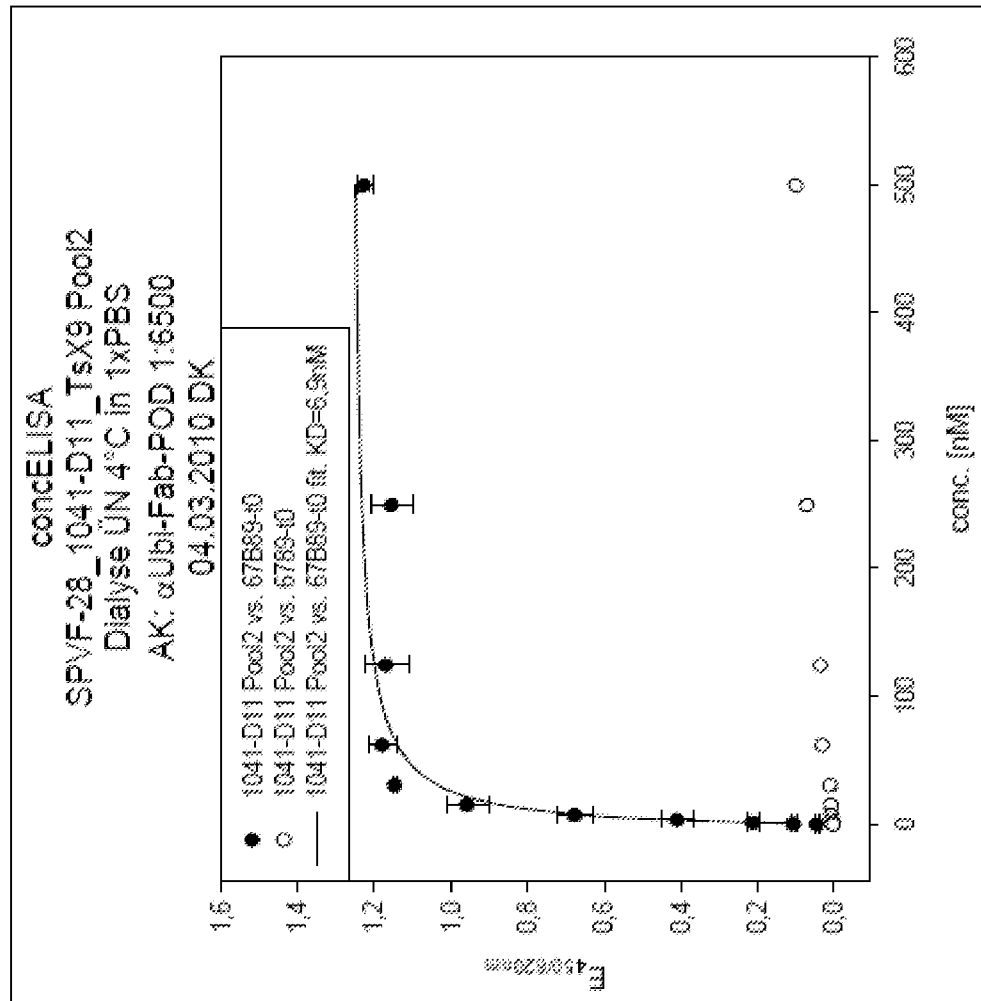


Figure 11

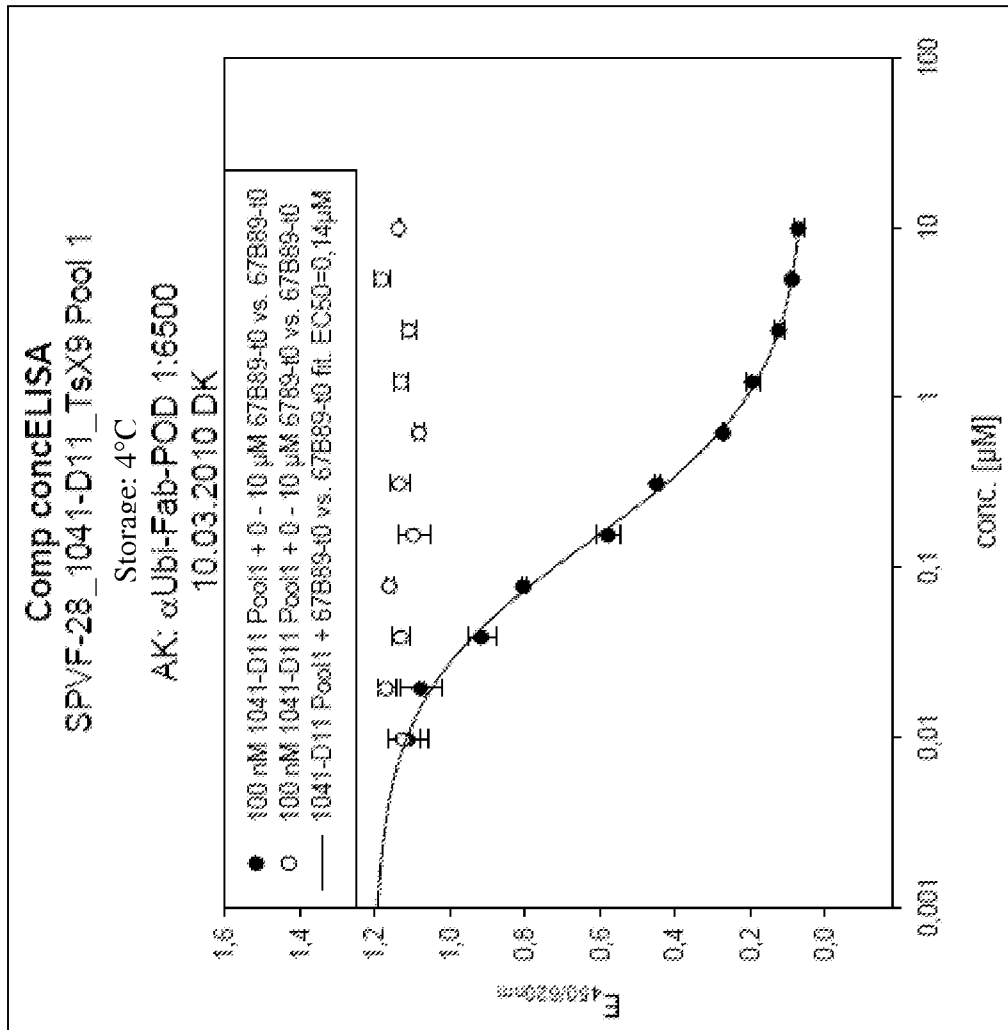


Figure 12

Fc2: 460 RU 67B89_T0L1 (SA-Chip)
+ SPVF-28_1041-D11_TsX9 Pool 2 (TG 23.3.10)
in 1x PEST 0,005%

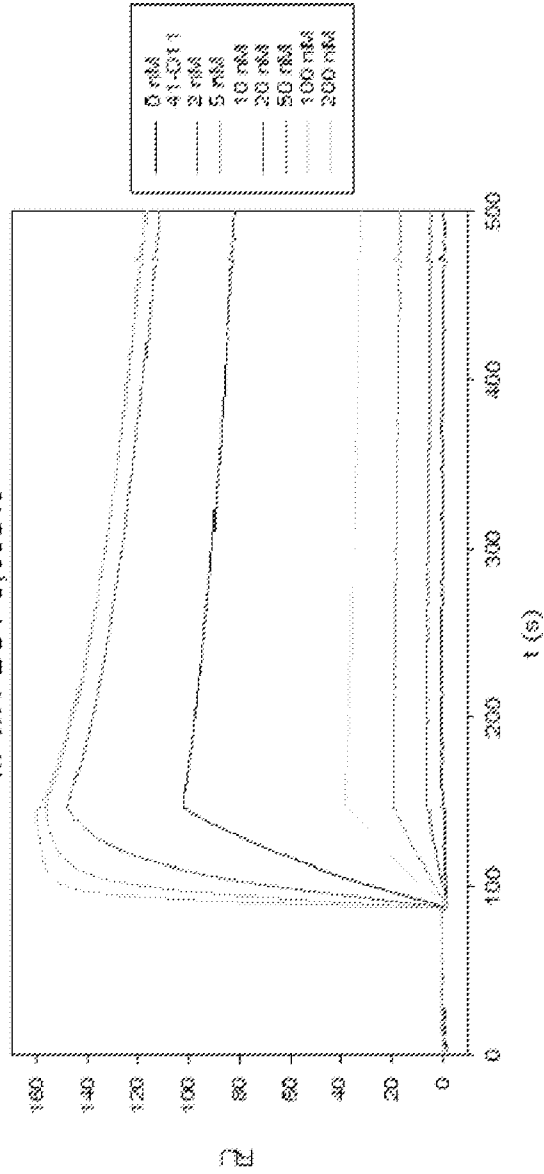


Figure 13

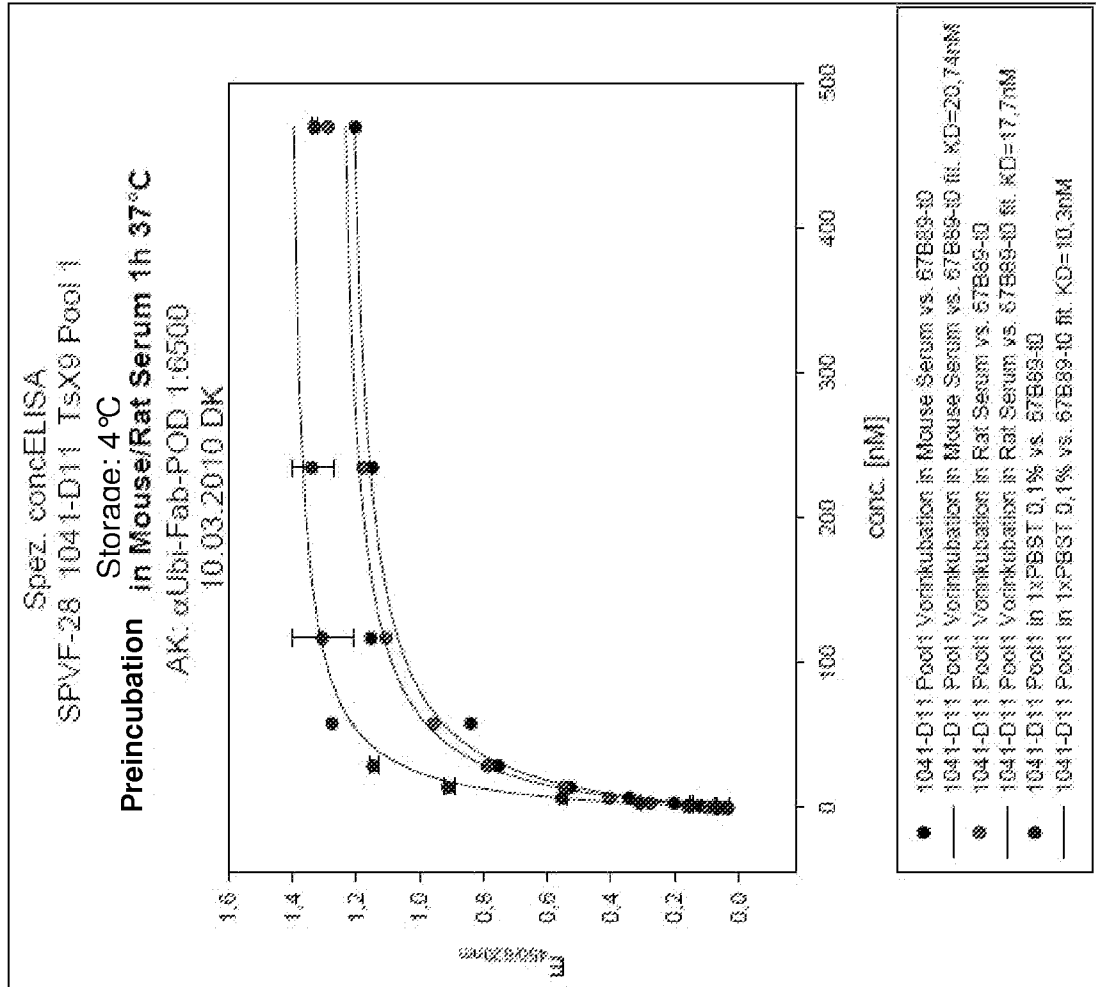


FIGURE 14. SE-HPLC.
Fig. 14 A. Complex with ED-B (67B89)

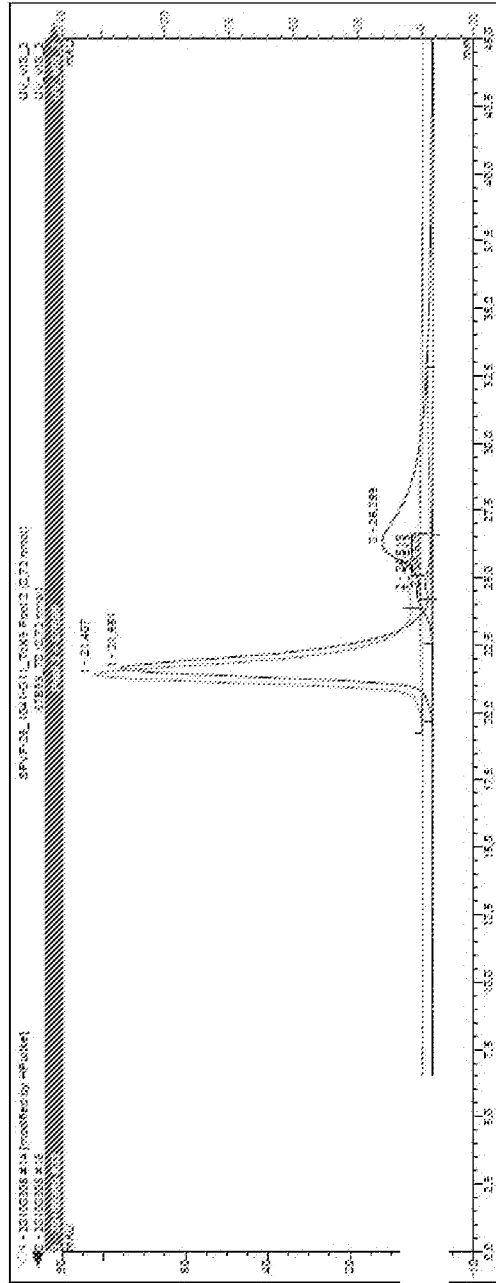


Fig. 14 B. No complex with control (6789)

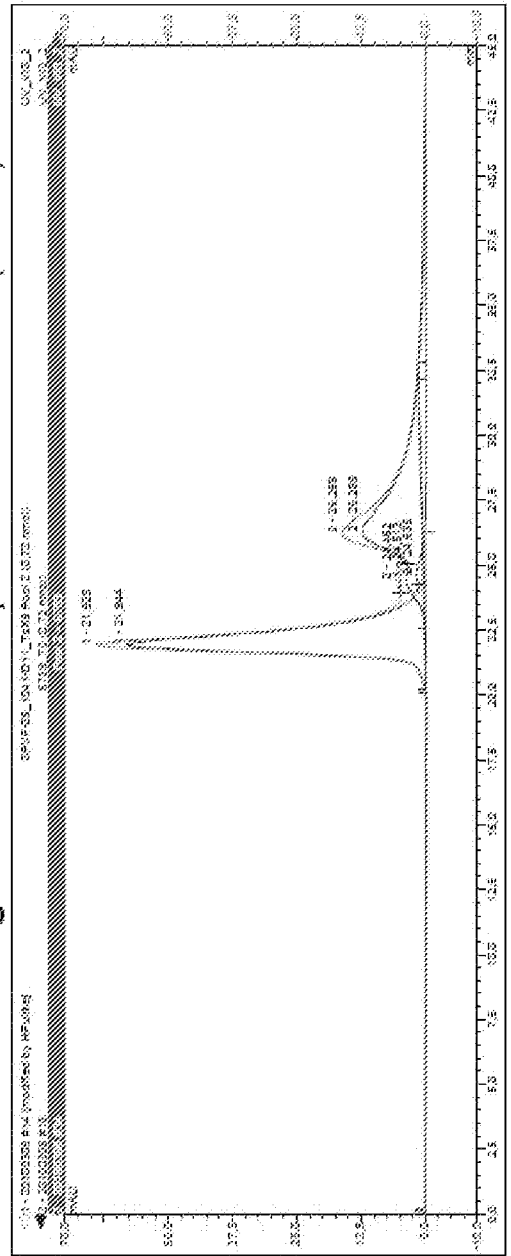


FIGURE 15 A.

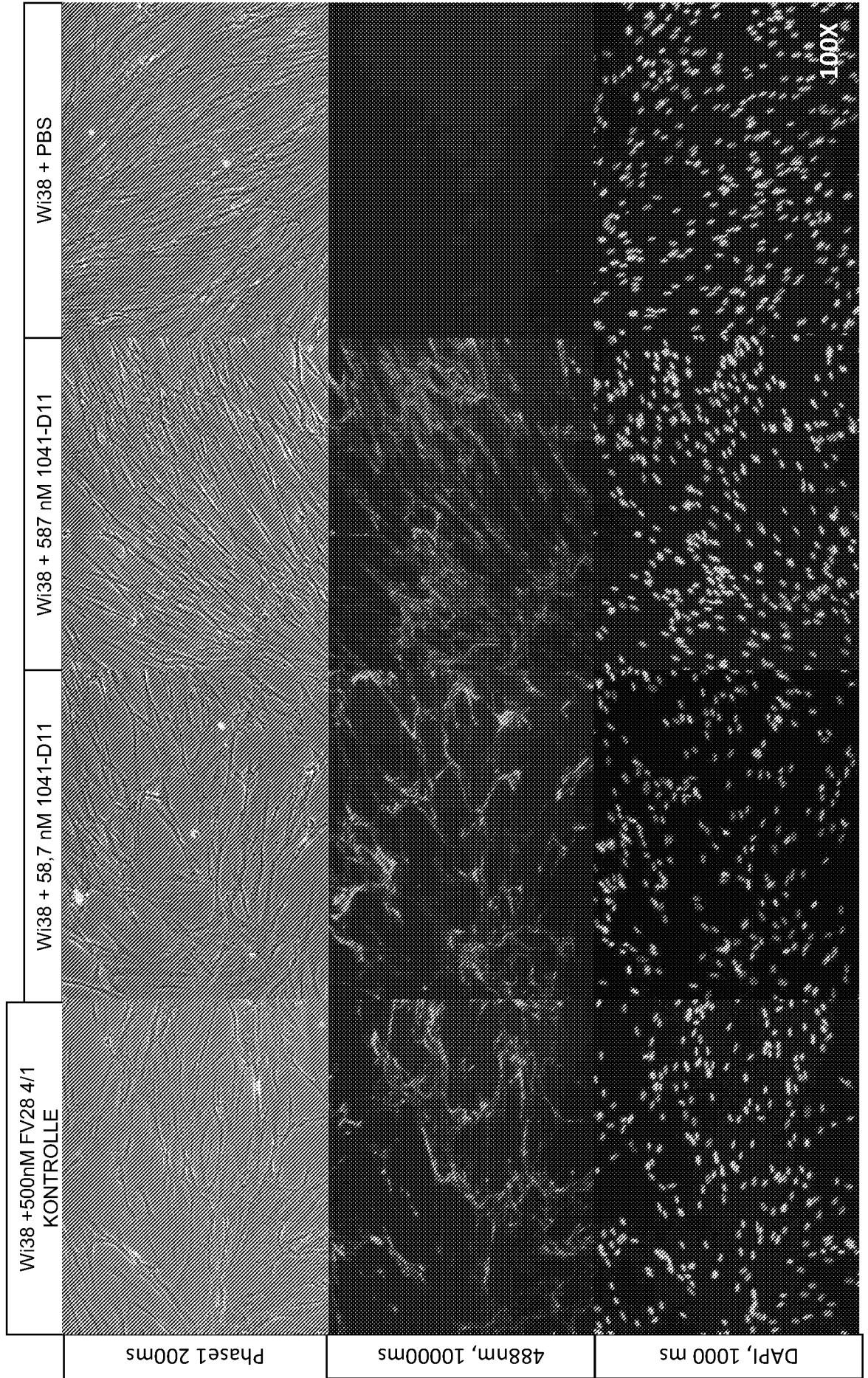


Figure 15C. 1041-D11 on fixed murine Balb 3T3-cells

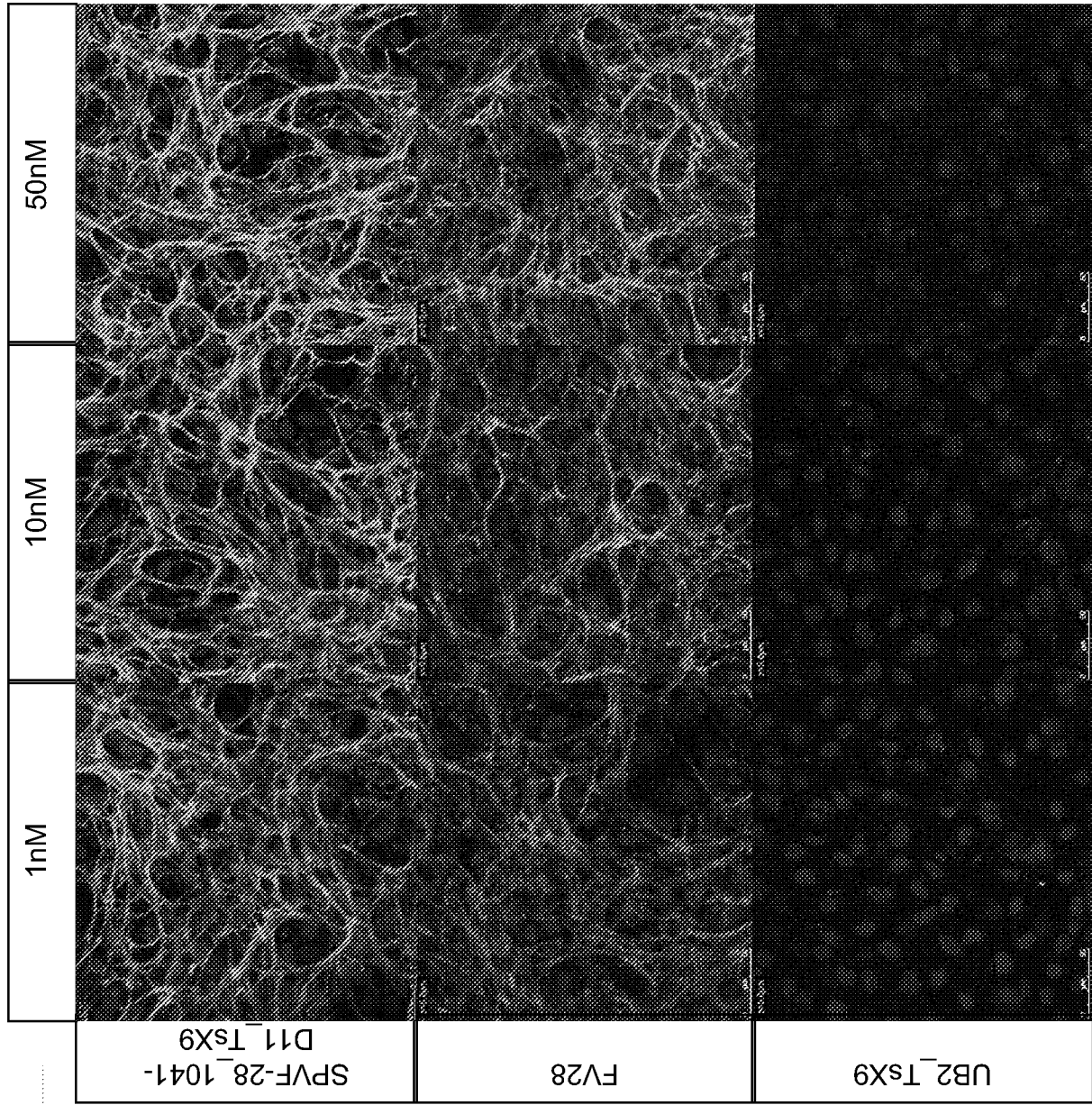


Figure 15D. 1041-D11 on fixed murine ST-2 cells (konfovoales mikroskop)

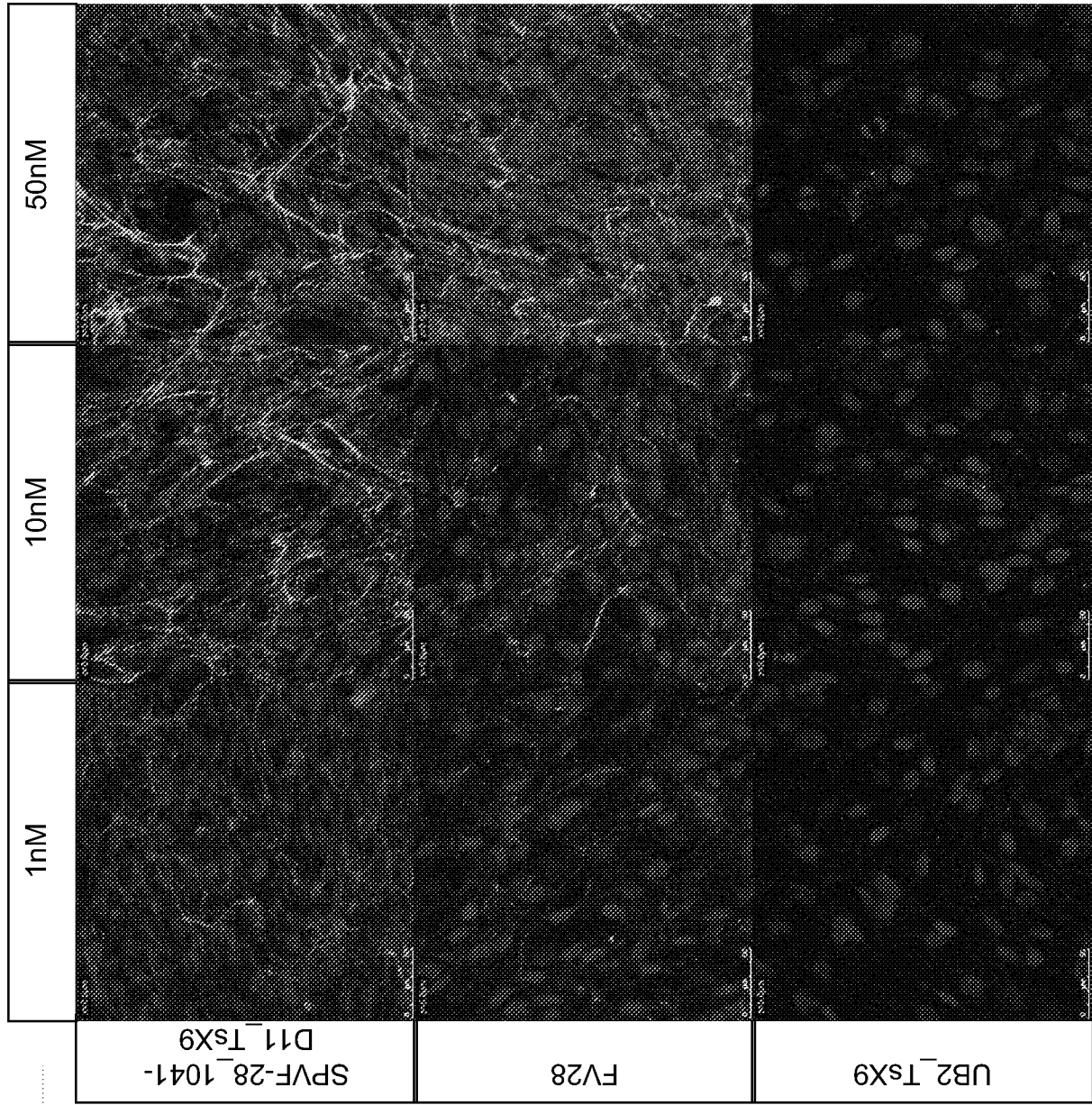


FIGURE 16 A Specificity of 1041-D11 in Tissue Sections

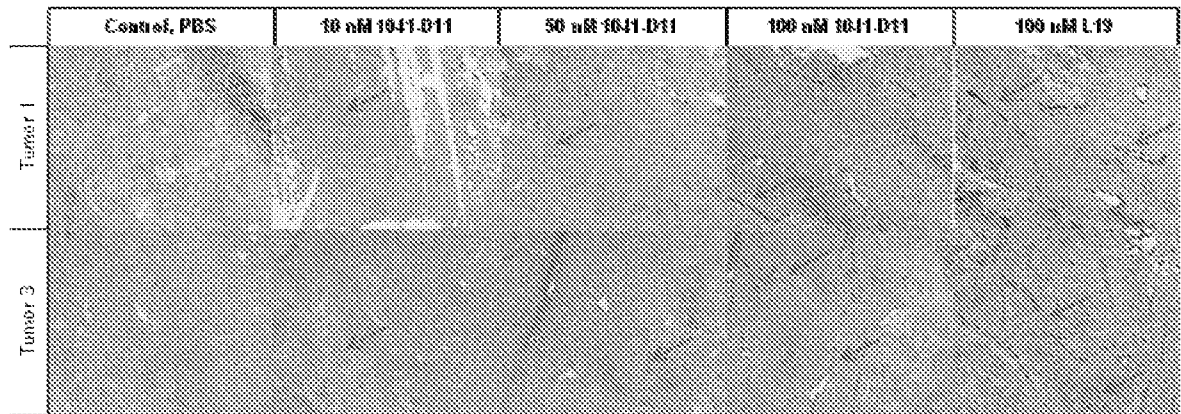


FIGURE 16 B – Accumulation in tumor cells

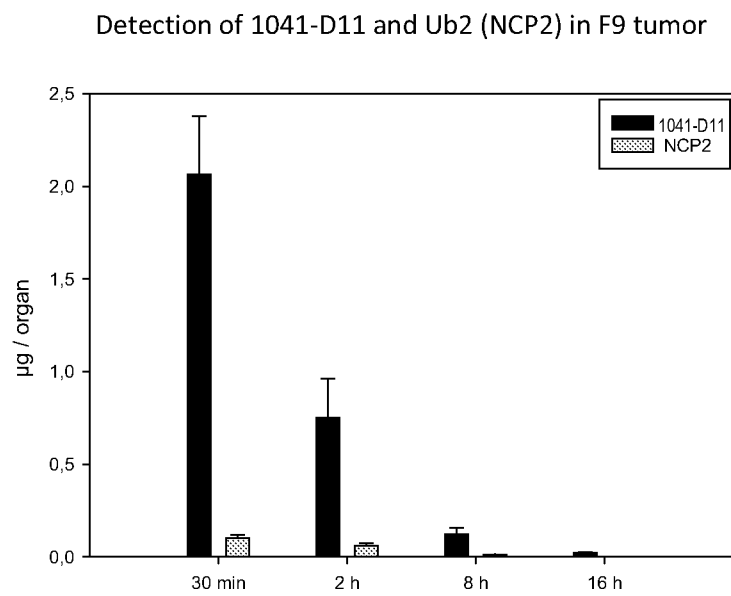


FIGURE 17.

FIGURE 17A:

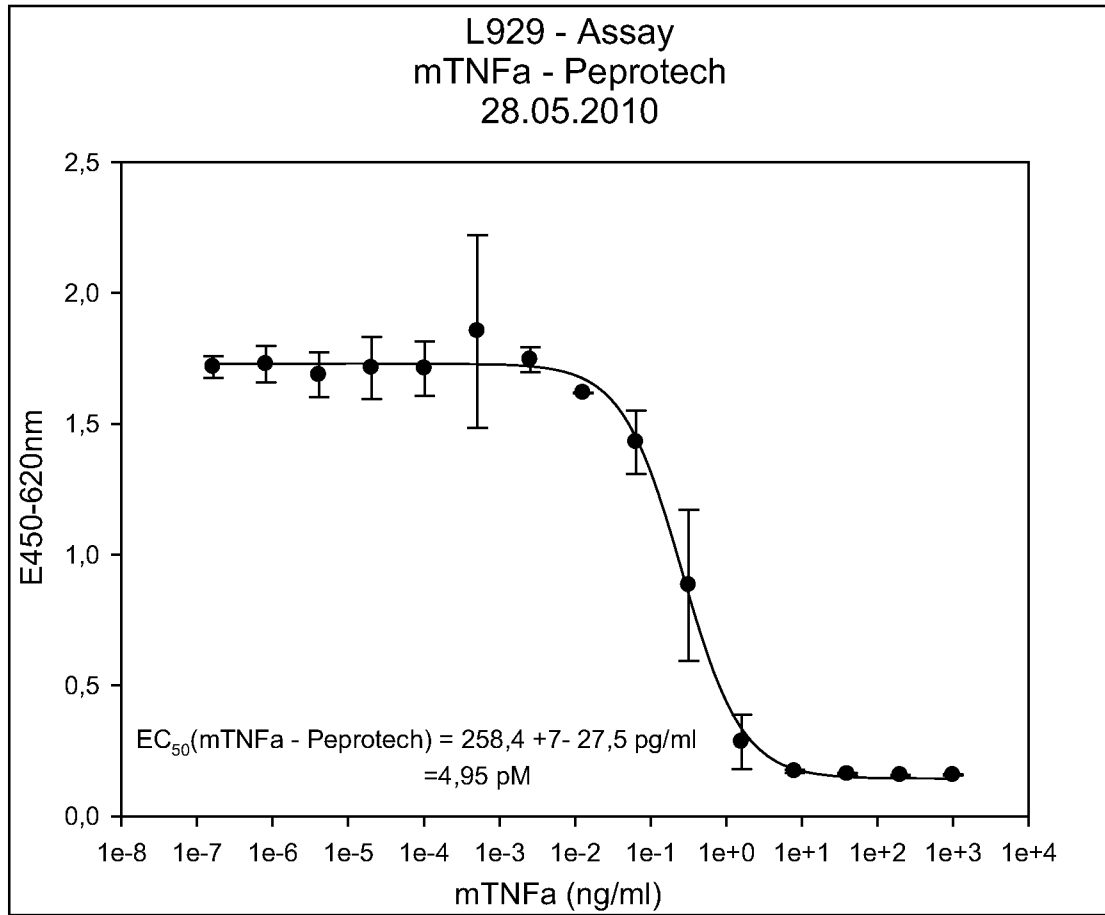


FIGURE 17B:

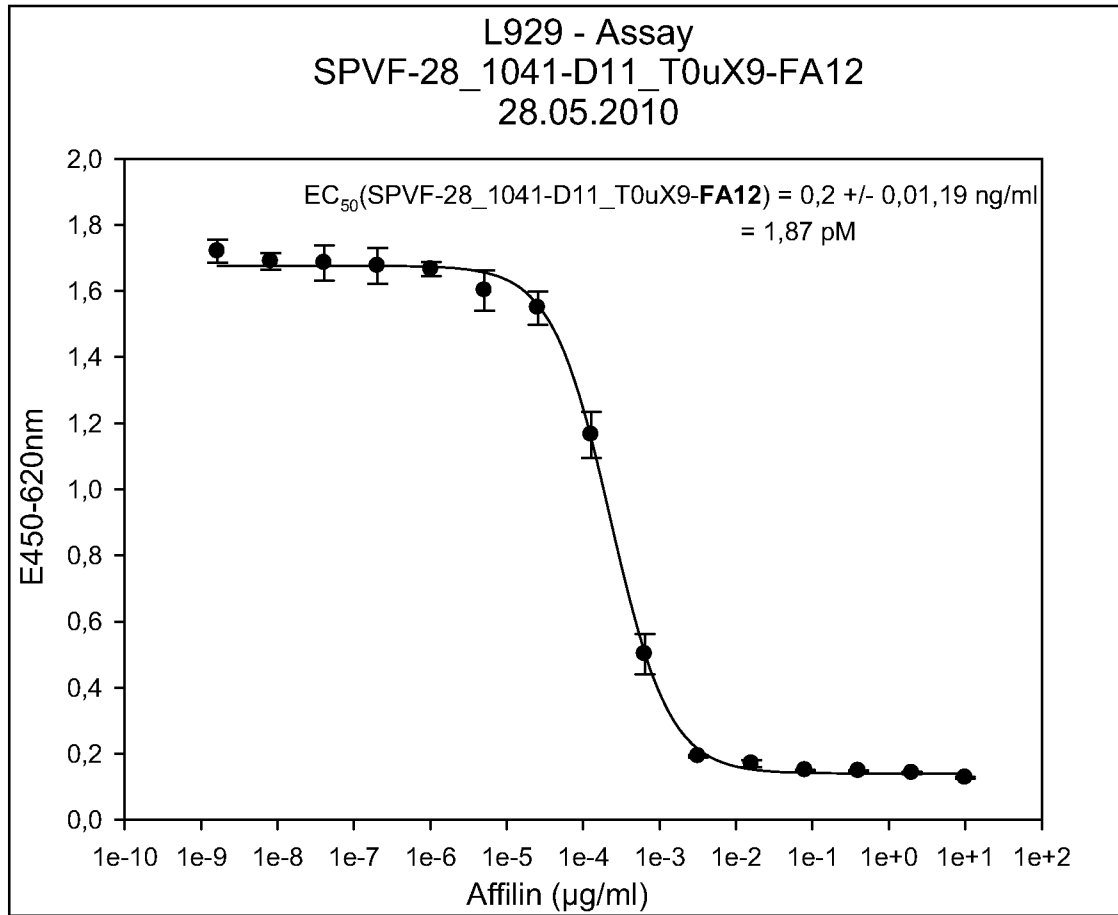


FIGURE 17C:

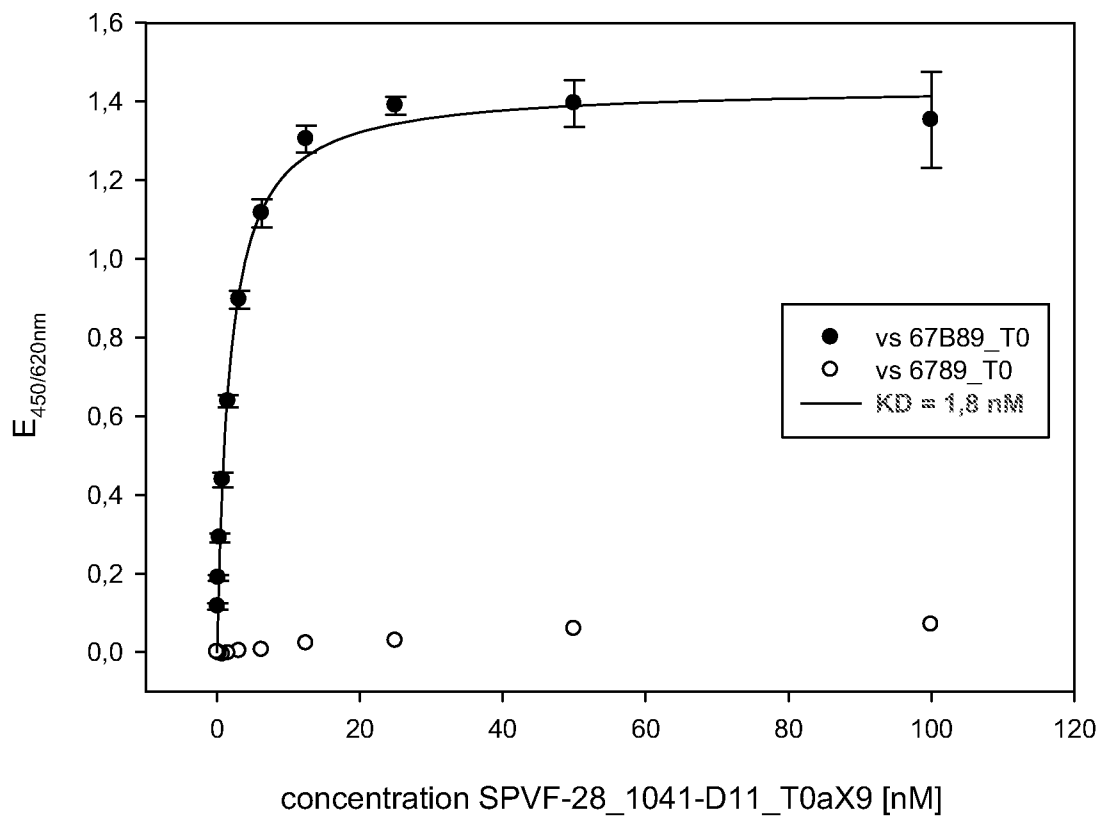
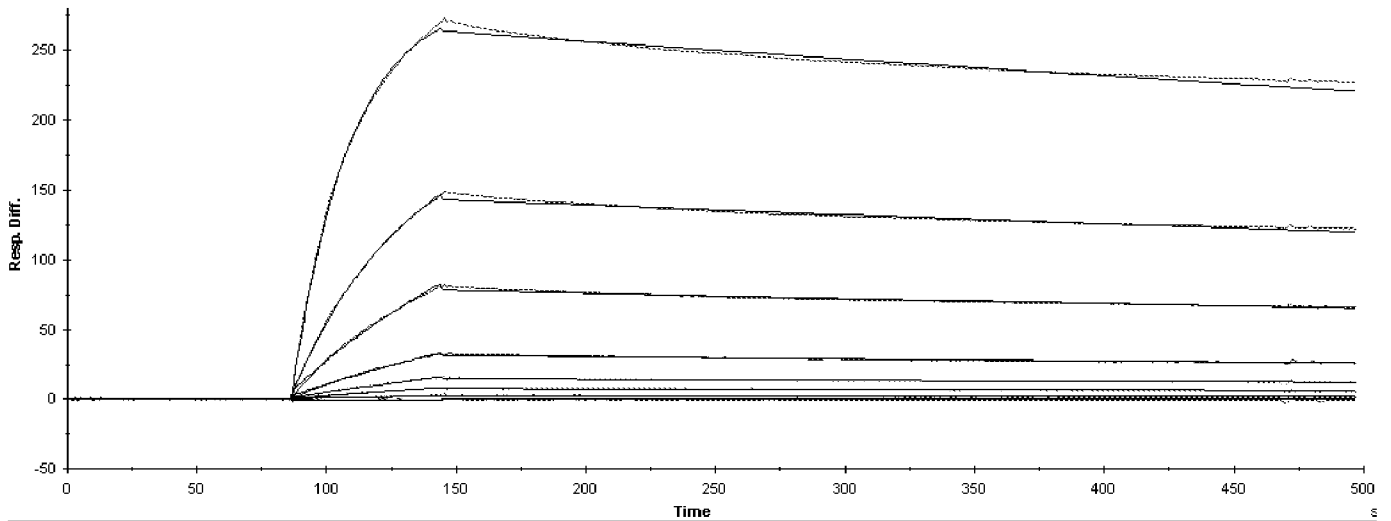


FIGURE 17D:



	ka (1/Ms)	kd (1/s)	Rmax (RU)	RI (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
	4.46e5	5.05e-4				8.84e8	1.13e-9			0.916
20100706 Lauf TG Fce=2.1 .1			0.443	-0.462	0			0	5.05e-4	
20100706 Lauf TG Fce=2.1 .2			90.8	0.345	1n			42.6	9.52e-4	
20100706 Lauf TG Fce=2.1 .3			152	0.773	2n			97.2	1.4e-3	
20100706 Lauf TG Fce=2.1 .4			122	1.46	5n			99.6	2.74e-3	
20100706 Lauf TG Fce=2.1 .5			139	2.52	10n			125	4.97e-3	
20100706 Lauf TG Fce=2.1 .6			196	5.4	20n			185	9.43e-3	
20100706 Lauf TG Fce=2.1 .7			200	3.42	50n			195	0.0228	
20100706 Lauf TG Fce=2.1 .8			288	2.37	100n			285	0.0451	

FIGURE 17E:

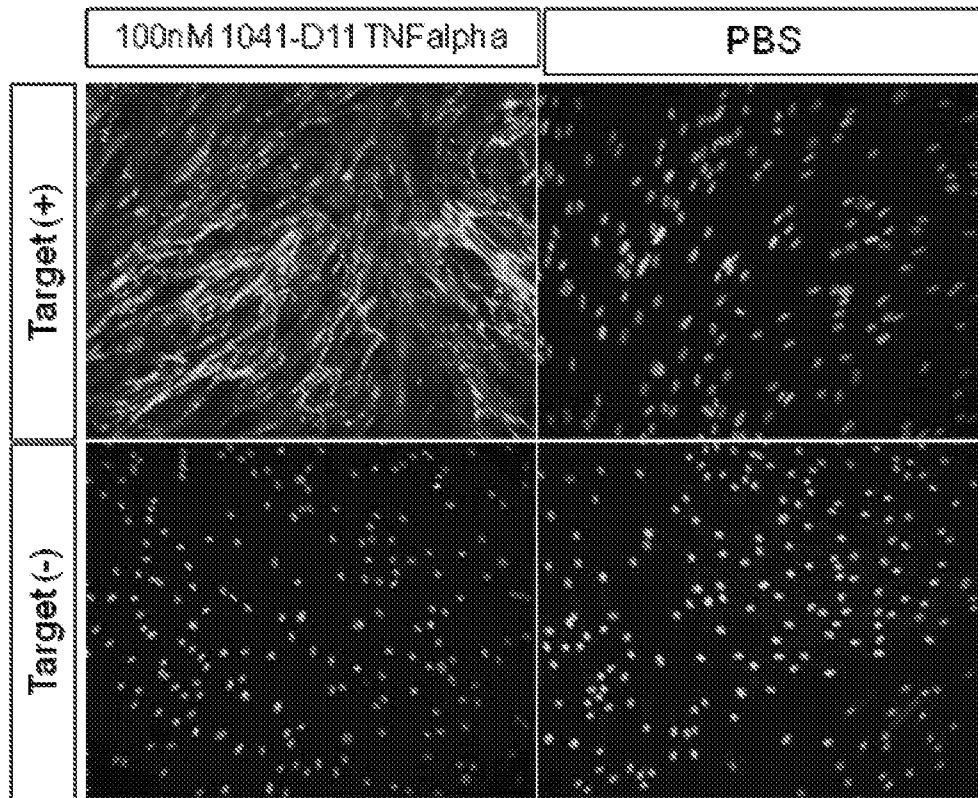
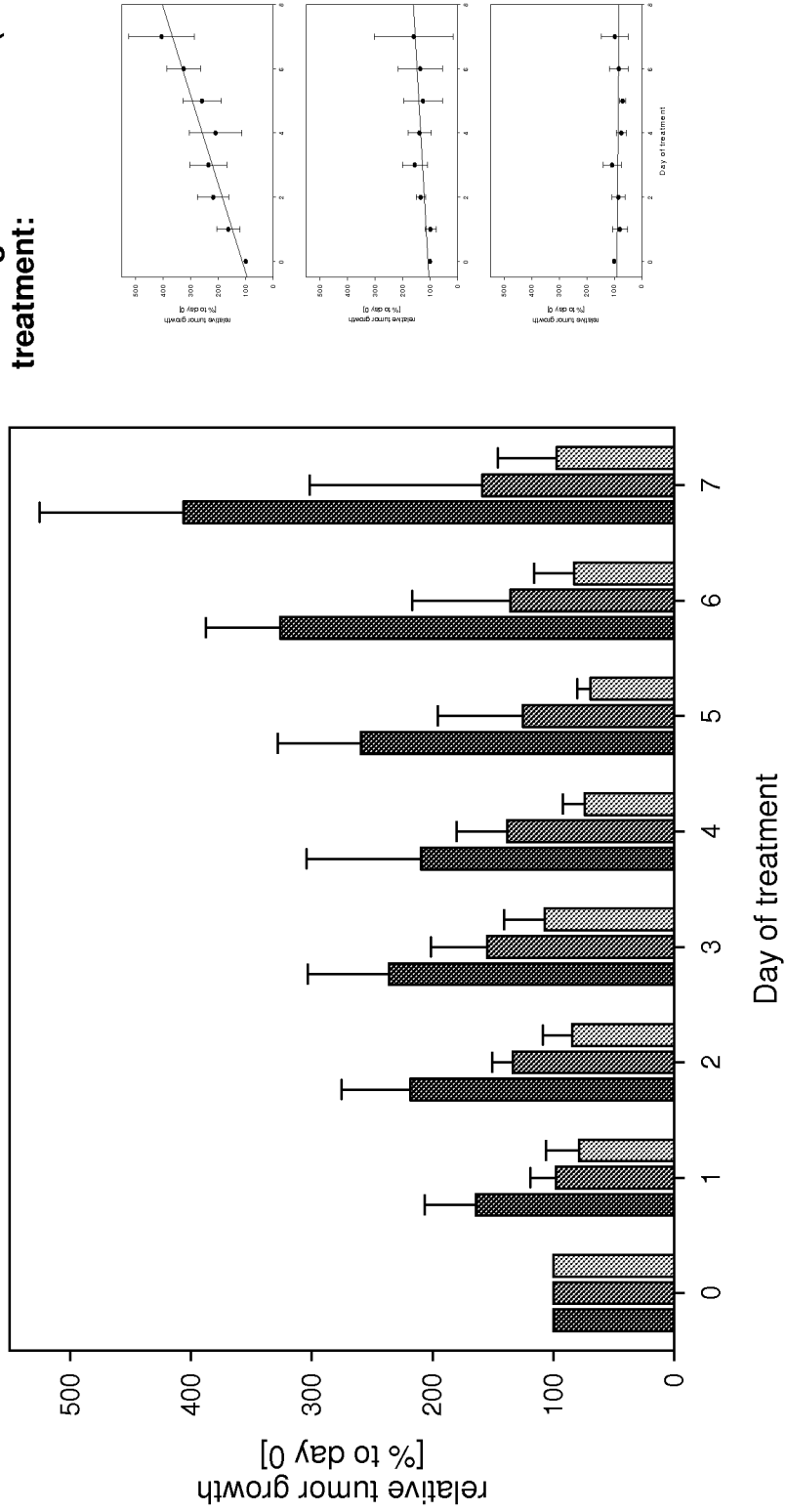


Figure 18: Efficacy of 1041-D11-TNFAlpha in combination with Melphalan

Tumor growth kinetic (linear regression) 7 days after treatment:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/069666

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/00 C12N15/10 C40B40/02 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K C12N C40B		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2004/106368 A1 (SCIL PROTEINS GMBH [DE]; FIEDLER MARKUS [DE]; FIEDLER ULRIKE [DE]; RUD) 9 December 2004 (2004-12-09) cited in the application * abstract page 6, line 1 - page 9, line 18 page 22, line 21 - page 24, line 28 page 25, line 11 - line 16 page 31, line 26 - page 32, line 26 page 34, line 9 - line 24; examples 2,4; tables 1,2 <p style="text-align: center;">----- -/--</p>	1-5,13, 14, 16-27, 30,31
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.		
<input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family
Date of the actual completion of the international search <p style="text-align: center;">31 March 2011</p>		Date of mailing of the international search report <p style="text-align: center;">13/04/2011</p>
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer <p style="text-align: center;">Mossier, Birgit</p>

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/069666

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 2008/022759 A2 (EIDGENOESS TECH HOCHSCHULE [CH]; GRABULOVSKI DRAGAN [CH]; NERI DARIO []) 28 February 2008 (2008-02-28) cited in the application * abstract page 11, paragraph 4 - page 13, paragraph 2; claim 7; example 3 page 8, paragraph 2 - page 11, paragraph 2 - & GRABULOVSKI DRAGAN ET AL: "A novel, non-immunogenic Fyn SH3-derived binding protein with tumor vascular targeting properties." THE JOURNAL OF BIOLOGICAL CHEMISTRY 2 FEB 2007 LNKD- PUBMED:17130124, vol. 282, no. 5, 2 February 2007 (2007-02-02), pages 3196-3204, XP002630189, ISSN: 0021-9258 the whole document</p>	1-5, 13, 14, 16-27, 30, 31
A	<p>----- US 2003/045681 A1 (NERI DARIO [CH] ET AL) 6 March 2003 (2003-03-06) * abstract figure 1; examples 1-3, 5</p>	1-31
A	<p>----- WO 2008/096012 A2 (SCIL PROTEINS GMBH [DE]; CELARES GMBH [DE]; KRAEHMER RALF [DE]; LEENDE) 14 August 2008 (2008-08-14) * abstract page 13, paragraph 3 - page 14, paragraph 3 page 15, paragraph 6 - page 16, paragraph 1 page 26 - page 31; figures 1-4</p>	1-31
A	<p>----- WO 2006/040129 A2 (SCIL PROTEINS GMBH [DE]; FIEDLER ERIK [DE]; EBERSBACH HILMAR [CH]; HEY) 20 April 2006 (2006-04-20) page 11, paragraph 5 - page 13, paragraph 3; example 2 * abstract</p>	1-31
A	<p>----- WO 2008/059011 A1 (SCIL PROTEINS GMBH [DE]; SCHRAEML MICHAEL [DE]; FIEDLER ERIK [DE]) 22 May 2008 (2008-05-22) the whole document</p>	1-31
A	<p>----- WO 01/04144 A2 (FIEDLER ULRIKE [DE]; RUDOLPH RAINER [DE]) 18 January 2001 (2001-01-18) cited in the application * abstract example 2</p> <p>-----</p>	1-31
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/069666

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FIEDLER ET AL: "Affilin(TM) Molecules", FOOD AND BIOPRODUCTS PROCESSING, INSTITUTION OF CHEMICAL ENGINEERS, RUGBY, GB, vol. 84, no. 1, 1 March 2006 (2006-03-01), pages 3-8, XP022525357, ISSN: 0960-3085, DOI: DOI:10.1205/FBP.05222 * abstract page 4, column 1, paragraph 4 - column 2, paragraph 1</p>	1-31
A	<p>-----</p> <p>GEBAUER M ET AL: "Engineered protein scaffolds as next-generation antibody therapeutics", CURRENT OPINION IN CHEMICAL BIOLOGY, CURRENT BIOLOGY LTD, LONDON, GB, vol. 13, no. 3, 1 June 2009 (2009-06-01), pages 245-255, XP026285197, ISSN: 1367-5931, DOI: DOI:10.1016/J.CBPA.2009.04.627 [retrieved on 2009-06-06] * abstract</p>	1-31
A	<p>-----</p> <p>HEY T ET AL: "Artificial, non-antibody binding proteins for pharmaceutical and industrial applications", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 23, no. 10, 1 October 2005 (2005-10-01), pages 514-522, XP025290753, ISSN: 0167-7799, DOI: DOI:10.1016/J.TIBTECH.2005.07.007 [retrieved on 2005-10-01] page 518, column 1, paragraph 4 - page 519, column 1, paragraph 1; figures 1,2; tables 1-4</p>	1-31
A	<p>-----</p> <p>BOLTON D ET AL: "Structure and properties of a dimeric N-terminal fragment of human ubiquitin", JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 314, no. 4, 7 December 2001 (2001-12-07), pages 773-787, XP004466212, ISSN: 0022-2836, DOI: DOI:10.1006/JMBI.2001.5181 * abstract figures 2,5; table 1</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-31

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/069666

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KRANTZ B A ET AL: "Discerning the Structure and Energy of Multiple Transition States in Protein Folding using psi-Analysis", JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 337, no. 2, 19 March 2004 (2004-03-19), pages 463-475, XP004493197, ISSN: 0022-2836, DOI: DOI:10.1016/J.JMB.2004.01.018 * abstract figures 5-7</p> <p style="text-align: center;">-----</p>	1-31
A	<p>KIEL C ET AL: "The Ubiquitin Domain Superfold: Structure-based Sequence Alignments and Characterization of Binding Epitopes", JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 355, no. 4, 27 January 2006 (2006-01-27), pages 821-844, XP024950609, ISSN: 0022-2836, DOI: DOI:10.1016/J.JMB.2005.10.010 [retrieved on 2006-01-27] * abstract figures 2,6,7</p> <p style="text-align: center;">-----</p>	1-31

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2010/069666

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
 - on paper
 - in electronic form
 - b. (time)
 - in the international application as filed
 - together with the international application in electronic form
 - subsequently to this Authority for the purpose of search
2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/069666

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004106368	A1	09-12-2004	AT 499382 T 15-03-2011
			AU 2004242851 A1 09-12-2004
			CA 2524899 A1 09-12-2004
			CN 1956996 A 02-05-2007
			DE 10324447 A1 30-12-2004
			EP 1626985 A1 22-02-2006
			EP 2163559 A1 17-03-2010
			EP 2295446 A1 16-03-2011
			JP 2008500953 T 17-01-2008
			US 2008171851 A1 17-07-2008
US 2006099686 A1 11-05-2006			
WO 2008022759	A2	28-02-2008	AU 2007287807 A1 28-02-2008
			CA 2661160 A1 28-02-2008
			CN 101506230 A 12-08-2009
			EP 1892248 A1 27-02-2008
			EP 2054432 A2 06-05-2009
			JP 2010500875 T 14-01-2010
			KR 20090053926 A 28-05-2009
			RU 2009110180 A 27-09-2010
			US 2010119446 A1 13-05-2010
			US 2003045681
JP 2009280607 A 03-12-2009			
ZA 200007211 A 16-08-2002			
WO 2008096012	A2	14-08-2008	CA 2677041 A1 14-08-2008
			CN 101616691 A 30-12-2009
			EP 1955712 A1 13-08-2008
			EP 2109463 A2 21-10-2009
			JP 2010519182 T 03-06-2010
			US 2010143387 A1 10-06-2010
WO 2006040129	A2	20-04-2006	CA 2583009 A1 20-04-2006
			CN 101084237 A 05-12-2007
			DE 102004049479 A1 13-04-2006
			EP 1675623 A2 05-07-2006
			JP 2008516210 T 15-05-2008
			US 2007248536 A1 25-10-2007
WO 2008059011	A1	22-05-2008	AT 496999 T 15-02-2011
			CN 101563456 A 21-10-2009
			DK 2094845 T3 21-03-2011
			EP 1925664 A1 28-05-2008
			EP 2094845 A1 02-09-2009
			US 2010130720 A1 27-05-2010
			WO 0104144
AU 5827800 A 30-01-2001			
CA 2378871 A1 18-01-2001			
CN 1371415 A 25-09-2002			
CN 101693889 A 14-04-2010			
DE 19932688 A1 18-01-2001			
DK 1200583 T3 19-02-2007			
EP 1200583 A2 02-05-2002			
ES 2273705 T3 16-05-2007			
JP 4221174 B2 12-02-2009			
JP 2003504081 T 04-02-2003			

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/069666

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		JP 4597177 B2	15-12-2010
		JP 2008019276 A	31-01-2008
		PT 1200583 E	29-12-2006
		US 7601803 B1	13-10-2009
		US 2007111287 A1	17-05-2007
<hr style="border-top: 1px dashed black;"/>			