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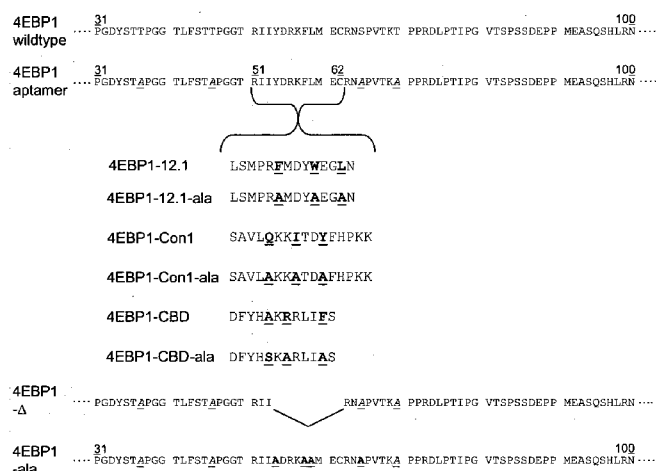
(54) Title: PROTEIN APTAMERS BASED ON UNSTRUCTURED SCAFFOLD PROTEINS

Figure 10

(57) Abstract: The present invention relates to protein aptamers based on unstructured scaffold proteins, in particular human 4EBP1 scaffolds, wherein at least one of amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 is mutated. The invention also relates to nucleic acid encoding these protein aptamers as well as methods for their production and use.

Protein Aptamers based on unstructured scaffold proteins

Field of the invention

The present invention relates to protein aptamers based on human 4EBP1 scaffolds, nucleic acid encoding them, as well as methods for their production and use.

Background

Target validation is a crucial step in the development of therapies for diseases. Validation of a target protein can be carried out by removing it or by inhibiting its interaction with other proteins, and observing the resulting downstream effects. Methods to deplete proteins include using small interfering RNAs (siRNA) for mRNA removal and animal knockout models for gene deletion. Alternatively, protein and nucleic acid aptamers can be used to inhibit a particular function of the target protein by specifically disrupting the interaction between the target protein and its binding partner or substrate, or by blocking its active site. The ability to examine function without inhibiting target protein expression is a distinct advantage, as complete or even partial removal of the target protein may result in multiple downstream effects and possibly compensatory mechanisms that will complicate analysis and obstruct the study of a specific function.

Protein aptamers are small recognition molecules that consist of an inhibitory peptide constrained within a scaffold protein. They are created by inserting binding peptides or peptide libraries, varying in length from 10 to 20 amino acids, into a small, compact and soluble protein scaffold.

One of the best characterized and commonly used scaffold proteins for aptamer construction is thioredoxin A (Trx), a small cytosolic protein (108 amino acids) from *Escherichia coli*. It has a rigid structure with a solvent accessible loop that is amenable to the insertion of peptides. There are many aptamers that use Trx as their scaffold. E2F-binding Trx aptamers isolated from a yeast two hybrid screen inhibited the activity of E2F and induced cell cycle arrest (Fabrizio E et al., *Oncogene*. 1999, 18, 4357-63). Trx aptamers that bind to the E6 protein of human papillomavirus specifically inhibited proliferation of E6 expressing mammalian cells by enabling accumulation of p53, resulting in apoptosis (Butz Ket al., *Proc Natl Acad Sci U S A*. 2000, 97, 6693-7). Mutant p53-binding Trx aptamers were able to inhibit the transcriptional activity of mutant p53 and specifically induce apoptosis in mutant p53 containing cells (Guida E

et al., Cancer Res. 2008, 68, 6550-8), and Mdm2 binding Trx aptamers were able to activate and stabilize the p53 protein (Bottger A et al., Curr Biol. 1997, 7, 860-9).

The role of a scaffold protein in an aptamer is to constrain and present the bioactive peptides for binding and to confer rapid, stable and detectable expression in both prokaryotic and eukaryotic systems, to facilitate the characterization of target protein function both *in vitro* and *in vivo*. Commonly it is believed that the scaffold protein should have enough flexibility to tolerate insertion of peptides that vary in length and sequence but on the other hand should be relatively rigid to enhance the presentation of the peptide for binding to its target protein (Hosse RJ et al. Protein Sci. 2006, 15, 14-27; Binz HK et al., Nat Biotechnol. 2005, 23, 1257-68).

It was found out that efficient target binding may also depend on the peptide scaffold interactions (Woodman R et al., J Mol Biol. 2005, 352, 1118-33) and thus the universal applicability of a particular scaffold is limited. For example, the insertion of peptides into the Trx scaffold destabilizes the structure of Trx (Brown CJ et al., J Mol Biol. 2010, 395, 871-83) and, in the case of an eIF4E binding peptide, the free peptide binds the target protein with higher affinity than the Trx aptamer encoding the same peptide. On the other hand, a Trx aptamer to Mdm2 showed very tight binding compared to the corresponding free peptide due to an interaction between the scaffold and the target. As a result thereof, to date no protein has been identified that is versatile enough to serve as an effective scaffold for aptamers against a variety of targets.

Summary of the Invention

The inventors of the present invention have now found that unstructured protein scaffolds, in particular a novel unstructured scaffold based on human 4EBP1, enable the functional display of a wide range of bioactive peptides. Surprisingly, this approach bypasses the conventional requirement for scaffolds to have a relatively rigid structure and yet serves to successfully present several peptides that need to adopt a wide range of conformations for binding to their target protein.

In a first aspect the present invention thus relates to a protein aptamer that includes an unstructured scaffold polypeptide, wherein at least one of the native amino acid sequence positions of the polypeptide is mutated such that it binds a non-natural ligand with detectable affinity. In one embodiment, the protein aptamer includes a human 4EBP1 scaffold, wherein at least one of amino acid sequence positions 51-62 of the linear polypeptide sequence of human

4EBP1 as set forth in SEQ ID NO:2 is mutated and wherein the protein aptamer binds a given non-natural ligand of 4EBP1 with detectable binding affinity.

In another aspect, the present invention relates to a method for the generation of a protein aptamer comprising a human 4EBP1 scaffold, wherein at least one of amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 as set forth in SEQ ID NO:2 is mutated and wherein the protein aptamer binds a given non-natural ligand of 4EBP1 with detectable binding affinity, comprising:

(a) subjecting a nucleic acid molecule encoding 4EBP1 to mutagenesis at at least one codon of any of the amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1, thereby obtaining a plurality of nucleic acids encoding 4EBP1 protein aptamers,

(b) expressing the one or more nucleic acid molecule(s) obtained in (a) in an expression system, thereby obtaining one or more protein aptamer(s) and

(c) enriching the one or more protein aptamer(s) obtained in (b) and having detectable binding affinity for a given non-natural ligand of 4EBP1 by means of selection and/or isolation.

The present invention also features a protein aptamer obtainable by this method.

In a further aspect, the present invention is directed to a nucleic acid molecule comprising a nucleotide sequence encoding a protein aptamer according to the invention.

In a still further aspect, the present invention also features a host cell containing a nucleic acid molecule according to the invention.

In another aspect, the present invention encompasses methods for the detection of a given non-natural ligand of human 4EBP1, comprising the steps of

(a) contacting a protein aptamer according to claim 1 with a sample suspected of containing the given ligand under suitable conditions, thereby allowing formation of a complex between the protein aptamer and the given ligand, and

(b) detecting the complexed protein aptamer by a suitable signal.

In still another aspect, the invention also relates to a method for the separation of a given non-natural ligand of human 4EBP1, comprising the steps of

(a) contacting protein aptamer according to claim 1 with a sample suspected to contain said ligand under suitable conditions, thereby allowing formation of a complex between the protein aptamer and the given ligand, and

(b) separating the protein aptamer/ligand complex from the sample.

In a further aspect, the invention features a method for the targeting of a compound to a preselected site in an organism or tissue, comprising the steps of

(a) conjugating a protein aptamer according to claim 1 with said compound, and

(b) delivering the protein aptamer/compound complex to the preselected site.

Brief Description of the Drawings

Figure 1 shows results of a binding assay of Thioredoxin (Trx)-based aptamers for eIF4E. (A) Western blot analysis of the precipitation of eIF4E using thioredoxin-based eIF4E binding aptamers. (B) Western blot analysis of the expression levels of streptavidin binding peptide (SBP)-tagged thioredoxin (Trx) aptamers in 293FT cells. The membrane was probed with anti-actin to check for equal loading. (C) Accumulation of the binding Trx aptamers after treatment of transfected cells with MG132, detected by western blot using a streptavidin peroxidase conjugate. Lane 1, Trx-eIF4G; lane 2, Trx-eIF4G-ala; lane 3, Trx-SG-eIF4G; lane 4, Trx-SG-eIF4G-ala; lane 5, SBP-GFP; lane 6, untransfected 293FT cells.

Figure 2 shows an analysis of streptavidin binding peptide (SBP)-tagged 4EBP1 aptamers expression in 293FT cells and their interaction with eIF4E. The cells were transfected and harvested for Western blot analysis and precipitation assays. Membrane was probed with (A) anti-actin to check for equal loading and with (B) streptavidin-peroxidase conjugate polymer to detect SBP-tagged 4EBP1 expression. (C) Precipitation of eIF4E by SBP-tagged 4EBP1 aptamers. The eluent was analyzed by western blot, using anti-eIF4E to check for interacting protein and (D) streptavidin-peroxidase conjugate to detect aptamer. Lane 1, WT 4EBP1; lane 2, non-phosphorylable 4EBP1; lane 3, non-phosphorylable 4EBP1 alanine control; lane 4, non-phosphorylable 4EBP1 deletion control.

Figure 3 shows the results of a bicistronic reporter assay measuring effects of eIF4E binding aptamers on cap-dependent translation. MCF7 cells were co-transfected with a bicistronic reporter vector and an aptamer encoding vector. The bicistronic vector consists of 2 luciferase genes: the *Renilla* luciferase at the 5' end and the firefly luciferase behind an internal ribosomal entry site (IRES). The ratio of cap-dependent translation (*Renilla* luciferase) to cap-independent

translation (firefly luciferase) was tabulated. The amount of *Renilla* luciferase measured is proportional to the activity of cap-dependent translation initiation factor eIF4E.

Figure 4 shows the results of the precipitation of Mdm2 from cell lysates using streptavidin binding peptide (SBP)-tagged aptamers. U2OS cells transfected with Mdm2 binding aptamers were harvested for precipitation assays. Equal amounts of streptavidin beads were used. The bound proteins were eluted and equal fractions of the elutant were analyzed by western blot. Lane 1, Trx-12.1; lane 2, Trx-12.1-ala; lane 3, SBP-4EBP1-12.1; lane 4, SBP-4EBP1-12.1-ala; lane 5, SBP-4EBP1 deletion control (SBP-4EBP1-delta); lane 6, SBP-GFP.

Figure 5 shows the results of a beta-galactosidase (beta-gal) reporter assay for studying the effect of the aptamers on p53 activity in cells. The Mdm2 binding aptamers were transfected into T22 cells which are stably transfected with a p53-responsive beta-galactosidase vector. The beta-gal readings measured are indicative of p53 activity. T22 cells were treated with 10nM of actinomycin D as a positive control. Results shown are representative of three independent experiments.

Figure 6 shows T22 cell staining pictures for beta-galactosidase (beta-gal) activity. T22 cells were transfected with the streptavidin binding peptide (SBP)-tagged 4EBP1 aptamers. The transfected cells were then fixed and stained for beta-gal expression to assess p53 activity. Cells were treated respectively with actinomycin D and DMSO as positive and negative controls for p53 activation.

Figure 7 shows the results of a streptavidin binding peptide (SBP)-tag precipitation experiment using transfected U2OS cells. (A) Western blot analysis of the proteins isolated by the 4EBP1-cyclin binding domain (CBD) aptamer precipitations. Lane 1, 4EBP1-CBD; lane 2, 4EBP1-CBD-ala. (B) Western blot analysis of the proteins isolated by the PCNA binding 4EBP1-Con1 aptamer precipitations. (C) The input and supernatant (Supt) of the 4EBP1-Con1 aptamer precipitations were analyzed for the presence of PCNA protein. Lane 3, 4EBP1-Con1; lane 4, 4EBP1-Con1-ala; lane 5, 4EBP1-del control; lane 6, SBP-GFP.

Figure 8 shows the percentage of transfected cells in G1 phase determined by flow cytometry analysis. U2OS cells were co-transfected with DNA encoding the PCNA binding aptamers and GFP, harvested and stained for cell cycle analysis. The cell cycle profiles of transfected fluorescent cells were analyzed. (A) Representative histograms of the cell cycle profiles

obtained for cells transfected with PCNA binding aptamers. (B) Tabulation of the percentages of transfected cells in G1 phase, obtained from 3 independent experiments.

Figure 9 shows the optimized structures of the 12.1 peptide (MPRFMDYWEGLN, SEQ ID NO:19), Con1 peptide (SAVLQKKTTITDYFHPKK, SEQ ID NO:21) and CBD peptide (DFYHAKRRLIFS, SEQ ID NO:23) in magenta ribbons bound to the surfaces of Mdm2, PCNA and Cyclin A respectively in A, B and C.

Figure 10 schematically illustrates sequence details of the polypeptide chains employed. The numbers indicate the relative position of the respective amino acid in the linear polypeptide chain of human 4EBP1 set forth in SEQ ID NO. 2. “....” indicates that the polypeptide chain comprises one or more additional amino acids on the respective terminus, for example amino acids corresponding to the respective amino acids of wildtype protein. Bold and underlined amino acids indicate amino acid positions that are involved in target binding. Italic and underlined amino acids indicate amino acid positions that can be phosphorylated in wt 4EBP1.

Detailed Description

In target validation the ability to examine function of a target protein without inhibiting its expression is highly significant, as complete or even partial removal of the target protein may result in undesired downstream effects and compensatory mechanisms that complicate and obstruct the study of a specific function. Hence, often the objective is to develop a method for the characterization of a specific protein function under physiological conditions with minimal disruption to other roles of the protein within the cell. For this purpose, for example protein aptamers have been developed. However, it was found that the use of protein aptamers that are based on a relatively rigid scaffold protein in an attempt to preserve the binding peptide's secondary structure is hampered by the fact that for the peptide to adopt the correct binding conformation the scaffold has to be destabilized, creating an entropic cost that reduces the effectiveness of the aptamer. Furthermore, it was found that the aptamers efficacy may depend on the compatibility of the scaffold and the target. In fact, due to the high variability of the binding peptides that are coupled to the scaffold, it was believed that a suitable scaffold may have to be selected depending on the conformation of the peptide binding loop to be inserted and that it may be impossible to use a general scaffold for essentially all bioactive peptides.

The inventors have now found by examination of the structure of a variety of protein peptide interactions that, since the bound peptides vary in conformation from highly helical to linear

extended strands, a given rigid aptamer scaffold may not be optimal for the presentation of such a wide variety of peptide conformations, but that, contrary to earlier beliefs, intrinsically unstructured proteins may be of use to solve the problem of providing a scaffold that is applicable for a wide variety of distinct binding peptides. Surprisingly, it was found that 4EBP1, an intrinsically unstructured protein, accommodates a variety of inserted peptides that significantly differ in their conformation and allows these peptides to adopt a number of conformations, one of which should be optimal for binding. Furthermore, insertion of peptides in this unstructured scaffold does not cause additional entropic penalty. It is thus expected that the discovery of such a generic unstructured scaffold will facilitate aptamer creation and expedite the process of target validation and drug discovery.

The present invention, in a first aspect, thus relates to a protein aptamer based on an unstructured scaffold protein that has been mutated such that it binds a given ligand with detectable affinity. In various embodiments, the scaffold is a naturally occurring protein or protein domain. Exemplary proteins and protein domains include, but are not limited to Arf (for example Homo sapiens Arf1, 3, 4, 5 or 6 with UniProtKB Accession Nos. P84077, P62330, P18085, P84085, P61204), p21^{Waf1/Cip1/Sdi1}, (for example Homo sapiens p21 with UniProtKB Accession No. P38936) p27 (for example Homo sapiens p27 with UniProtKB Accession No. P46527), 4EBP1 (for example with UniProtKB Accession No. Q13541), 4EBP2 (for example with UniProtKB Accession No. Q13542), 4EBP3 (for example with UniProtKB Accession No. O60516), non-A β component of AD amyloid precursor (NACP) (for example with UniProtKB Accession No. P37840), anti-sigma-28 factor (FlgM), calpastatin (for example with UniProtKB Accession No. P20810), microtubule-associated protein tau (for example with UniProtKB Accession No. P10636), microtubule-associated protein 2 (MAP2) (for example with UniProtKB Accession No. P11137) projection domain, titin (for example with UniProtKB Accession No. Q8WZ42) PEVK domain, SNAP25 (for example with UniProtKB Accession No. P60880) linker region, securing (for example with UniProtKB Accession No. O95997), stathmin (for example with UniProtKB Accession Nos. P16949, Q93045, Q9NZ72, Q9H169), thymosins (proT α) (for example with UniProtKB Accession Nos. P63313, P63328, POCG34, POCG35), caseins (for example with UniProtKB Accession No. P47710), salivary proline-rich protein (Prp) (for example with UniProtKB Accession Nos. P02812, P04286, P10163), desiccation stress protein (Dsp) 16, MAP2 microtubule-binding domain, caldesmon (for example with UniProtKB Accession No. Q05682), Bob1 (for example with UniProtKB Accession No. Q16633), λ phage N protein, sibling proteins, fibronectin receptor (MSCRAMM) D₁-D₄, CREB (for example with UniProtKB Accession No. P16220)

transactivator domain (TAD), Bcl-2 antiapoptotic protein (for example with UniProtKB Accession No. P10415) (aa 24-93), cAMP-dependent protein kinase inhibitor (PKI) (for example with UniProtKB Accession Nos. Q9Y2B9, Q9C010, P61925), cFOS (for example with UniProtKB Accession No. P01100) transactivator domain (TAD), the proline-rich C-terminal domain of RNA polymerase II, amino acids 1-80 of p53 (for example with UniProtKB Accession No. P04637), amino acids 1-80 of p63 (for example with UniProtKB Accession No. Q9H3D4), amino acids 1-80 of p73 (for example with UniProtKB Accession No. O15350), and the transactivator domain (TAD) of transcription factors. Unstructured proteins and protein domains that may be used as scaffolds according to the invention are reviewed in Tompa (Trends in biochemical sciences, 2002, 27(10):537-533), which is hereby incorporated by reference in its entirety.

In one embodiment, the protein aptamers of the invention are created by inserting binding peptides or single peptides from a peptide library into the unstructured scaffold protein. These peptides can be 6 to 35 amino acids in length, e.g. 8 to 30, 10 to 30, 10 to 25 or 20 to 30 amino acids. In various embodiments, the unstructured scaffold protein is a small, compact and soluble protein scaffold, e.g. comprises about 50 to 200 amino acids, is a globular protein and is highly soluble in water.

"Unstructured" as used herein relates to the secondary structure of the protein and indicates that under natural conditions more than 50 %, preferably more than 60, more than 70 %, or more than 80 % of the protein is neither in alpha-helical, beta-sheet or beta-loop conformation. This usually means that the unstructured regions are in a random coil conformation.

The fact that the scaffold is unstructured does not exclude that the inserted peptide sequences have a secondary structure, such as alpha-helical, beta-sheet or beta-loop structures or combinations thereof.

In a preferred embodiment, the protein aptamer of the invention is based on human 4EBP1, wherein at least one of amino acids in the binding loop of 4EBP1, comprising amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 as set forth in SEQ ID NO:2, is mutated. In one embodiment, this protein aptamer binds a given non-natural ligand of 4EBP1 with detectable binding affinity.

“Protein aptamer”, “peptide aptamer” or “aptamer” as used interchangeably herein, relate to scaffold proteins into which a linear peptide sequence with binding affinity for a given ligand has been inserted. The inserted sequence can replace one or more amino acids of the scaffold or can be inserted between two amino acids of the scaffold. Commonly, a loop sequence of the scaffold is replaced by a linear amino acid stretch of the desired sequence. The inserted sequence is usually about 2 to 50 amino acids in length, commonly 10 to 30, for example 20 to 30 amino acids. The inserted sequence can be a bioactive peptide, including peptides comprising known ligand-binding motifs of larger proteins. The scaffold can be any protein, but usually proteins that are advantageous with respect to size, solubility, stability, immunogenicity etc. are used. The present invention is based on the inventors’ surprising discovery that an essentially unstructured small human protein of 118 amino acids length, termed 4EBP1, has advantageous properties as a scaffold protein in that it can accommodate a variety of different peptide sequences that differ significantly in their conformation and have binding activity for a given non-natural ligand.

“Mutation”, as used herein, relates to an alteration of the nucleotide or amino acid sequence of a nucleic acid, peptide or protein molecule. “Mutation” thus includes substitutions of one or more nucleotides or amino acids as well as deletions, insertions and inversions of one or more nucleotides or amino acids. Included are also substitutions of one or more amino acids by one or more other amino acids, for example 2-5 or 2-10 amino acids and the respective modifications on the nucleotide level required for such an alteration.

The term “non-natural ligand” refers to a compound, which does not bind to native 4EBP1 under physiological conditions. “Not binding” means that native 4EBP1 binds this ligand only with an affinity in the millimolar range or worse. The target (ligand) may be any chemical compound in free or conjugated form which exhibits features of an immunological hapten, a hormone such as steroid hormones or any biopolymer or fragment thereof, for example, a protein or protein domain, a peptide, an oligodeoxynucleotide, a nucleic acid, an oligo- or polysaccharide or conjugates thereof, a lipid or another macromolecule.

The protein aptamers of the invention are able to bind the desired target with detectable affinity, e.g. with a dissociation constant of at least about 1 μ M. Preferred in some embodiments are aptamers, which bind the desired target with a dissociation constant for a given target of at least 500, 200, 100, 20, 1 nM or even less. The binding affinity of an aptamer to the desired target

can be measured by a multitude of methods such as fluorescence titration, competition ELISA or surface plasmon resonance (Biacore).

“4EBP1” (UniprotKB/Swiss-Prot databank entry Q13541; NCBI Reference Sequence: NP_004086.1; SEQ ID NO:2; encoded by nucleotides 73-429 of NCBI Reference Sequence NM_004095 ; SEQ ID NO:1) is a known binding partner of eIF4E. It is a small protein of 118 amino acids length with no other known interaction partners besides eIF4E. The activity of 4EBP1 is tightly regulated by mTOR phosphorylation. 4EBP1 has 4 phosphorylation sites which regulate binding to eIF4E. Phosphorylation of threonines 37, 46, 70 and of serine 65 by mTOR results in the dissociation of 4EBP1 from eIF4E, and subsequently the degradation of 4EBP1. Mature 4EBP1 is obtained by processing and cleavage of the N-terminal methionine. Although the present invention generally makes reference to human 4EBP1, it is understood that homologs of 4EBP1 are also contemplated to fall within the scope of the present invention.

In various embodiments of the protein aptamers of the invention, at least 2 amino acids of the indicated amino acid sequence positions are mutated. In other embodiments, at least 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 amino acids of the amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 are mutated.. Various embodiments relate to protein aptamers wherein the amino acids in sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 are deleted and replaced by a variable loop of 6 to 35 amino acids. In various embodiments, this loop is not identical to or has no significant sequence homology to the native amino acids 51-62 of 4EBP1. In certain embodiments, the loop may comprise 8 to 30, 10 to 30, 10 to 20, 10 to 15, or 20 to 30 amino acids. The inserted loop may have any secondary structure, i.e. it may have an alpha-helical or beta-sheet structure or both or may be unstructured. The insertion of such a loop means that natural amino acids 51-62 of the linear polypeptide sequence of human 4EBP1 are replaced by a linear oligo- or polypeptide comprising 6 to 35, 8 to 30, 10 to 30, 10 to 20, 10 to 15, or 20 to 30 amino acids such that the N-terminus of the inserted peptide is coupled to the C-terminus of the amino acid in position 50 of the linear polypeptide sequence of human 4EBP1 and the C-terminus of the inserted peptide is coupled to the N-terminus of the amino acid in position 63 of the linear polypeptide sequence of human 4EBP1. The coupling is usually covalent, preferably by a peptide bond. Although the deletion and insertion may be done on the protein level by using specific proteases, the deletion and insertion are preferably made on the nucleic acid level by generating a nucleic acid molecule that encodes the desired protein.

If a linear peptide stretch is inserted to replace amino acid positions 51-62 of 4EBP1, this peptide sequence may have no or little homology to the native amino acid sequence that is replaced.

5 In various embodiments, the sequence identity between the inserted sequence and the replaced sequence is 90 % or lower, preferably 80 % or lower, more preferably 70% or lower, most preferably 60 or lower. In further embodiments, the sequence homology between the inserted sequence and the replaced sequence is 90 % or lower, preferably 80 % or lower, more preferably 70 or lower, most preferably 60 % or lower.

10 The variable loop, i.e. the linear peptide sequence that is inserted, may originate from a library of random peptides comprising a variety of bioactive peptides of different lengths, conformations and binding affinities. This includes a library of nucleic acid molecules that encode this peptide library and which are used for production of recombinant nucleic acid
15 molecules that encode the protein aptamers.

In various embodiments, the variable loop that is inserted into the 4EBP1 Scaffold to replace amino acids 51-62 is selected from the group consisting of the amino acid sequences set forth in any one of SEQ ID Nos. 19, 21, 23.

20 The protein aptamers of the invention may, in addition to the mutation or variation in positions 51-62 of the linear polypeptide sequence of human 4EBP1, comprise additional mutations in amino acid sequence positions positions 1-50 and 63-118. Such mutations can be accomplished very easily on DNA level using established standard methods (Sambrook, J. et al. (1989)
25 *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Possible alterations of the amino acid sequence are insertions or deletions as well as amino acid substitutions. Such substitutions may be conservative, i.e. an amino acid residue is replaced with a chemically similar amino acid residue. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine,
30 serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan. One the other hand, it is also possible to introduce non-conservative alterations in the amino acid sequence.

Such modifications of the amino acid sequence include directed mutagenesis of single amino acid positions in order to simplify sub-cloning of the 4EBP1 aptamer gene or its parts by incorporating cleavage sites for certain restriction enzymes. In addition, these mutations can also be incorporated to further improve the affinity of an aptamer for a given target.

5 Furthermore, mutations can be introduced in order to modulate certain characteristics of the aptamer such as to influence stability, bioavailability, solubility, *in vivo* half-life, coupling sites for modifications and labels and the like. These additional mutations may serve to remove phosphorylation sites in the 4EBP1 protein. In one embodiment of the invention, at least one of amino acid sequence positions 37, 46, 65 and 70 of human 4EBP1 is substituted by any amino
10 acid other than the naturally occurring amino acid or deleted. In other embodiments at least 2, 3 or all 4 of these positions are substituted or deleted. If substituted, the substituting amino acid is preferably not serine or threonine. In further embodiments, the substituting amino acid is not glutamic acid. A preferred replacement amino acid in these positions is alanine.

15 It has been reported that these four positions are phosphorylated by mTOR *in vivo* and result in dissociation from eIF4E and subsequent degradation. Thus, in order to construct a stable and persistent aptamer, it is advantageous to modify these positions by mutation to render them insensitive to mTOR phosphorylation. It was found that the thus modified protein is significantly more stable than the wildtype.

20 “Wildtype” as used in connection with the 4EBP1 nucleotide or amino acid sequence relates to the naturally occurring sequence. The respective sequences are set forth in SEQ ID Nos. 1 and 2.

25 Other positions that may be subject to phosphorylation and thus be mutated include, but are not limited to, amino acid positions 10, 34-36, 41, 44, 45, 50, 54, 68, 70, 77, 82, 83, 94, 101 and 112 of the linear polypeptide sequence of human 4EBP1.

The protein aptamers of the invention may be truncated, i.e. lack one or more amino acid at their
30 N-terminus, C-terminus or both. The truncation may be of 1 to 20 amino acids, for example 1 to 10 amino acid. In one specific embodiment of the invention, the aptamer of the invention lacks the N-terminal methionine. This residue has been reported to be removed during processing *in vivo*. Thus in certain embodiments, the N-terminus of the aptamer starts with amino acid position 2 of the linear polypeptide sequence of human 4EBP1, i.e. a serine residue. This serine
35 residue may be modified, for example by acetylation, thus forming N-acetylserine.

In other embodiments, the C-terminus may be fused to another protein or peptide, such as for example a tag, such as a His-tag (6-His-tag) or Strep-Tag. In such embodiments, the C-terminus may lack one or more of the naturally occurring C-terminal amino acids of human 4EBP1.

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In various other embodiments, the cysteine residues in positions 7 and 62 of the linear polypeptide sequence of human 4EBP1 are mutated to other amino acids or deleted, for example to prevent disulphide bridge formation. In other embodiments, the protein aptamers of the invention may also include variants wherein one or more amino acids are mutated to cysteine residues in order to generate coupling sites for modifications, for example for the conjugation to other compounds, such as polyethylene glycol (PEG), biotin, peptides or proteins, or for the formation of non-naturally occurring disulphide linkages.

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As already noted above, the invention also encompasses protein aptamers of the invention, wherein the protein aptamer is at its N-terminus or its C-terminus operably fused to an enzyme, a protein or a protein domain, a peptide, a signal sequence and/or an affinity tag. Affinity tags such as the Strep-tag[®] or Strep-tag[®] II, the *myc*-tag, the FLAG-tag, the His₆-tag or the HA-tag or proteins such as Streptavidin binding protein (SBP) and glutathione-S-transferase also allow easy detection and/or purification of recombinant proteins and are examples of fusion partners. Furthermore, proteins with chromogenic or fluorescent properties such as the green fluorescent protein (GFP) or the yellow fluorescent protein (YFP) are suitable fusion partners for a protein aptamer of the invention as well.

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The term "fusion protein" as used herein also comprises aptamers according to the invention containing a signal sequence. The signal sequence may be fused to the C- or, preferably, the N-terminus. Signal sequences at the N-terminus of a polypeptide may direct this polypeptide to a specific cellular compartment, for example the periplasm of *E. coli* or the endoplasmatic reticulum of eukaryotic cells. A large number of signal sequences are known in the art. A preferred signal sequence for secretion a polypeptide into the periplasm of *E. coli* is the OmpA-signal sequence. In other embodiments, the signal sequence may be a nuclear localization signal (NLS). The NLS may be fused to the N-terminus of the aptamer. A variety of NLS sequences are known in the art and include, but are not limited to the NLS of SV40 large T antigen (PKKKRKV) and the NLS of nucleoplasmin (KR[PAATKKAGQA]KKKK).

Also contemplated are protein aptamers fused to a moiety that extends the serum half-life of the protein aptamer. Such moieties are well-known in the art and those skilled in the art may resort to routine practice to identify suitable moieties. Exemplary moieties include, but are not limited to an Fc part of an immunoglobulin, a CH3 domain of an immunoglobulin, a CH4 domain of an immunoglobulin, albumin or an albumin fragment, an albumin binding peptide, an albumin binding protein, transferrin, a polyalkylene glycol molecule, hydroxyethyl starch, palmitic acid and other fatty acid molecules. The fusion may be to the N- or C-terminus, but also may be to an amino acid side chain that is amenable to such modification, including cysteine, lysine, serine, threonine, glutamic acid and aspartic acid side chains.

For some applications, it is also useful to employ the aptamers of the invention in a labeled form. Accordingly, the invention is also directed to protein aptamers which are conjugated to a label selected from the group consisting of enzyme labels, radioactive labels, fluorescent labels, chromogenic labels, luminescent labels, haptens, digoxigenin, biotin, metal complexes, metals, and colloidal gold. The aptamer may also be conjugated to an organic molecule. The term "organic molecule" as used herein preferably denotes an organic molecule comprising at least two carbon atoms, but preferably not more than 7 or 12 rotatable carbon bonds, having a molecular weight in the range between 100 and 2000 Dalton, preferably between 100 and 1000 Dalton, and optionally including one or two metal atoms.

In general, it is possible to label the protein aptamer with any appropriate chemical substance or enzyme, which directly or indirectly generates a detectable compound or signal in a chemical, physical or enzymatic reaction. An example for a physical reaction is the emission of fluorescence upon irradiation or the emission of X-rays when using a radioactive label. Alkaline phosphatase, horseradish peroxidase or β -galactosidase are examples of enzyme labels which catalyze the formation of chromogenic reaction products. In general, all labels commonly used for antibodies (except those exclusively used with the sugar moiety in the Fc part of immunoglobulins) can also be used for conjugation to the aptamers of the present invention. The aptamers of the invention may also be conjugated with any suitable therapeutically active agent, e.g., for the targeted delivery of such agents to a given cell, tissue or organ or for the selective targeting of cells, e.g., of tumor cells without affecting the surrounding normal cells. Examples of such therapeutically active agents include radionuclides, toxins, small organic molecules, and therapeutic peptides (such as peptides acting as agonists/antagonists of a cell surface receptor or peptides competing for a protein binding site on a given cellular target). The aptamers of the invention may, however, also be conjugated with therapeutically active nucleic

acids such as antisense nucleic acid molecules, small interfering RNAs, micro RNAs or ribozymes. Such conjugates can be produced by methods well known in the art.

The non-natural ligand of 4EBP1 that is bound by the protein aptamers of the invention with detectable affinity may be a protein or a fragment thereof. By way of example, such targets include, but are not limited to Mdm2, PCNA and Cyclin A. Generally, targets can include proteins involved in signal transduction, such as receptors, for example growth factor receptors, including receptor tyrosine kinases (RTKs), kinases in general, including serine/threonine kinases, tyrosine kinases and dual specificity kinases, translation factors, growth factors and the like. Targets also include the expression products of known oncogenes or tumor suppressor genes. Further targets include proteins of pathogens, such as viruses, bacteria, fungi and parasites, in particular cell surface proteins of these pathogens.

The protein aptamers of the invention can inhibit or block the binding of their target to its natural binding partner. Also contemplated is however the formation of a ternary or higher molecular complex, where binding of the aptamer stabilizes an existing interaction. The interruption of target binding to the natural binding partner may be achieved by blocking the natural binding site or by induction of a conformational change upon binding that changes conformation of the natural binding site.

In specific embodiments of the invention, the protein aptamers of the invention target Mdm2, PCNA or Cyclin A and act as antagonists on these proteins, i.e. inhibit or block their natural activity.

In certain embodiments, the binding affinity of the protein aptamer for its target is at least 1 μ M or less, 500 nM or less, 200 nM or less, 100 nM or less or 50 nM or less. Accordingly in one embodiment of the invention, the protein aptamer binds Mdm2, PCNA or Cyclin A with a K_D of 1 μ M or less.

Specific embodiments of the inventive aptamers include those having the amino acid sequence set forth in any one of SEQ ID Nos. 10, 12, 14 or of a fragment or variant thereof.

The term "fragment", as used herein with respect to aptamers of the invention, relates to N-terminally and/or C-terminally shortened protein aptamers, which retain the capability of the full length protein to bind the desired ligand. Similarly, the term with respect to a ligand relates

to N-terminally and/or C-terminally shortened proteins or peptides, which retain the capability of the full length protein or peptide to be recognized and/or bound by an aptamer according to the invention.

- 5 The term "variant" as used in the present invention relates to derivatives of a protein or peptide that comprise modifications of the amino acid sequence, for example by substitution, deletion, insertion or chemical modification. Preferably, such modifications do not reduce the functionality of the protein or peptide. Such variants include proteins, wherein one or more amino acids have been replaced by their respective D-stereoisomers or by amino acids other than the naturally occurring 20 amino acids, such as, for example, ornithine, hydroxyproline, citrulline, homoserine, hydroxylysine, norvaline. However, such substitutions may also be conservative, i.e. an amino acid residue is replaced with a chemically similar amino acid residue. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) 10 asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, and valine; 15 and 6) phenylalanine, tyrosine, and tryptophan.

In another aspect, the invention provides for a method for the generation of the protein aptamers of the invention. This method comprises the steps of:

- 20 (a) subjecting a nucleic acid molecule encoding 4EBP1 to mutagenesis at at least one codon of any of the amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1, thereby obtaining a plurality of nucleic acids encoding protein aptamers, (b) expressing the one or more nucleic acid molecule(s) obtained in (a) in an expression system, thereby obtaining one or more protein aptamer(s) and 25 (c) enriching the one or more protein aptamer(s) obtained in (b) and having detectable binding affinity for a given non-natural ligand of 4EBP1 by means of selection and/or isolation.

In various embodiments, the mutagenesis can be random mutagenesis.

- 30 The term "mutagenesis" as used herein means that the experimental conditions are chosen such that the amino acid naturally occurring at a given sequence position of human 4EBP1 can be substituted by at least one amino acid that is not present at this specific position in the respective natural polypeptide sequence. The term "mutagenesis" also includes the (additional) modification of the length of sequence segments by deletion or insertion of one or more amino acids. Thus, it is within the scope of the invention that, for example, one amino acid at a chosen 35

sequence position is replaced by a stretch of three random mutations, leading to an insertion of two amino acid residues compared to the length of the respective segment of the wild type protein. Such an insertion or deletion may be introduced independently from each other in any of the peptide segments that can be subjected to mutagenesis in the invention. The term "random mutagenesis" means that no predetermined single amino acid (mutation) is present at a certain sequence position but that at least two amino acids can be incorporated with a certain probability at a predefined sequence position during mutagenesis.

The coding sequence of human 4EBP1 (nucleotides 73-429 of NCBI Reference sequence NM_004095, SEQ ID NO:1) is used as a starting point for the mutagenesis of the peptide segment selected in the present invention. For the mutagenesis of the recited amino acid positions, the person skilled in the art has at his disposal the various established standard methods for site-directed mutagenesis (Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

A commonly used technique is the introduction of mutations by means of PCR (polymerase chain reaction) using mixtures of synthetic oligonucleotides, which bear a degenerate base composition at the desired sequence positions. For example, use of the codon NNK or NNS (wherein N = adenine, guanine or cytosine or thymine; K = guanine or thymine; S = adenine or cytosine) allows incorporation of all 20 amino acids plus the amber stop codon during mutagenesis, whereas the codon VVS limits the number of possibly incorporated amino acids to 12, since it excludes the amino acids Cys, Ile, Leu, Met, Phe, Trp, Tyr, Val from being incorporated into the selected position of the polypeptide sequence; use of the codon NMS (wherein M = adenine or cytosine), for example, restricts the number of possible amino acids to 11 at a selected sequence position since it excludes the amino acids Arg, Cys, Gly, Ile, Leu, Met, Phe, Trp, Val from being incorporated at a selected sequence position. In this respect it is noted that codons for other amino acids (than the regular 20 naturally occurring amino acids) such as selenocystein or pyrrolysine can also be incorporated into a nucleic acid of an aptamer. It is also possible, as described by Wang, L., et al. (2001) *Science* **292**, 498-500 to use "artificial" codons such as UAG which are usually recognized as stop codons in order to insert other unusual amino acids, for example o-methyl-L-tyrosine or p-aminophenylalanine.

The use of nucleotide building blocks with reduced base pair specificity, as for example inosine, 8-oxo-2'-deoxyguanosine or 6(2-deoxy- β -D-ribofuranosyl)-3,4-dihydro-8H-pyrimindo-1,2-oxazine-7-one (Zaccolo et al. (1996) *J. Mol. Biol.* **255**, 589-603), is another option for the introduction of mutations into a chosen sequence segment.

A further possibility is the so-called triplet-mutagenesis. This method uses mixtures of different nucleotide triplets, each of which codes for one amino acid, for incorporation into the coding sequence (Virnekäs et al. 1994 *Nucleic Acids Res* **22**, 5600-5607).

One possible strategy for introducing mutations in the selected regions of the respective polypeptides is based on the use of four oligonucleotides, each of which is partially derived from one of the corresponding sequence segments to be mutated. When synthesizing these oligonucleotides, a person skilled in the art can employ mixtures of nucleic acid building blocks for the synthesis of those nucleotide triplets which correspond to the amino acid positions to be mutated so that codons encoding all natural amino acids randomly arise, which at last results in the generation of a protein aptamer library. For example, the first oligonucleotide corresponds in its sequence - apart from the mutated positions - to the coding strand for the peptide segment to be mutated at the most N-terminal position of the protein aptamer. Accordingly, the second oligonucleotide corresponds to the non-coding strand for the second sequence segment following in the polypeptide sequence. The third oligonucleotide corresponds in turn to the coding strand for the corresponding third sequence segment. Finally, the fourth oligonucleotide corresponds to the non-coding strand for the fourth sequence segment. A polymerase chain reaction can be performed with the respective first and second oligonucleotide and separately, if necessary, with the respective third and fourth oligonucleotide.

The amplification products of both of these reactions can be combined by various known methods into a single nucleic acid comprising the sequence from the first to the fourth sequence segments, in which mutations have been introduced at the selected positions. To this end, both of the products can for example be subjected to a new polymerase chain reaction using flanking oligonucleotides as well as one or more mediator nucleic acid molecules, which contribute the sequence between the second and the third sequence segment. In the choice of the number and arrangement within the sequence of the oligonucleotides used for the mutagenesis, the person skilled in the art has numerous alternatives at his disposal.

The nucleic acid molecules defined above can be connected by ligation with the missing 5'- and 3'-sequences of a nucleic acid encoding an aptamer polypeptide and/or the vector, and can be cloned in a known host organism. A multitude of established procedures are available for ligation and cloning (Sambrook, J. et al. (1989), *supra*). For example, recognition sequences for restriction endonucleases also present in the sequence of the cloning vector can be engineered

into the sequence of the synthetic oligonucleotides. Thus, after amplification of the respective PCR product and enzymatic cleavage the resulting fragment can be easily cloned using the corresponding recognition sequences.

- 5 Longer sequence segments within the gene coding for the protein selected for mutagenesis can also be subjected to random mutagenesis via known methods, for example by use of the polymerase chain reaction under conditions of increased error rate, by chemical mutagenesis or by using bacterial mutator strains. Such methods can also be used for further optimization of the target affinity or specificity of a protein aptamer.

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- According to the method of the invention a protein aptamer is obtained starting from a nucleic acid encoding 4EBP1. Such a nucleic acid is subjected to mutagenesis and introduced into a suitable bacterial or eukaryotic host organism by means of recombinant DNA technology. Obtaining a nucleic acid library of 4EBP1 can be carried out using any suitable technique that is
15 known in the art for generating muteins with antibody-like properties, i.e. muteins that have affinity towards a given target. Examples of such combinatorial methods are described with respect to lipocalin proteins in detail in the international patent applications WO 99/16873, WO 00/75308, WO 03/029471, WO 03/029462, WO 03/029463, WO 2005/019254, WO 2005/019255, WO 2005/019256, or WO 2006/56464 for instance. The content of each of these
20 patent applications is incorporated by reference herein in its entirety. After expression of the nucleic acid sequences that were subjected to mutagenesis in an appropriate host, the clones carrying the genetic information for the plurality of respective protein aptamers, which bind a given target can be selected from the library obtained. Well known techniques can be employed for the selection of these clones, such as phage display, colony screening, ribosome display or
25 mRNA display.

- In this method, in step (a) at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 codons of any of the amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 may be mutated. This includes embodiments, wherein in step (a) the codons encoding amino acid
30 sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 are deleted and replaced by codons encoding a variable loop of 6 to 35, 8 to 30, 10 to 30, 10 to 20, 10 to 15 or 20 to 30 amino acids. In one embodiment, the thus inserted loop is 10 to 25 or 20 to 30 amino acids long.

The thus inserted loop may be defined as described in connection with the invented protein aptamers above.

In order to generate protein aptamers having the phosphorylation sites at amino acid positions 37, 46, 65 and 70 removed, in step (a) of the invented method 1, 2, 3 or all 4 codons encoding amino acid sequence positions 37, 46, 65 and 70 of human 4EBP1 can be substituted by codons encoding any other amino acid, for example an amino acid other than serine, threonine or glutamic acid, such as, e.g. alanine, or can be deleted.

Similarly, in step (a) the nucleic acid molecule encoding the 4EBP1 scaffold can be mutated to include additional mutations, such as substitutions or deletions, or truncations at the N-terminus, C-terminus or both. In certain embodiments, the nucleic acid is mutated such that the expressed protein aptamer lacks one or more, such as for example 1 to 20 amino acids, at the N- or C-terminus or both compared to the wildtype human 4EBP1 amino acid sequence as set forth in SEQ ID NO:2. One embodiment provides for an aptamer lacking the N-terminal methionine compared to the human 4EBP1 sequences of SEQ ID NO:2.

In various embodiments, step (c) of the methods of the invention further comprises:

- i. providing as a given ligand a compound selected from the group consisting of a chemical compound in free or conjugated form that exhibits features of an immunological hapten, a peptide, a protein or another macromolecule,
- ii. contacting the plurality of protein aptamers with said ligand in order to allow formation of complexes between said ligand and protein aptamers having binding affinity for said ligand, and
- iii. removing protein aptamers having no or no substantial binding affinity.

In these methods, the ligand may be a protein or a fragment thereof.

It is readily apparent to the skilled person that complex formation is dependent on many factors such as concentration of the binding partners, the presence of competitors, ionic strength of the buffer system etc. Selection and enrichment can be generally performed under conditions allowing the isolation of aptamers having, in complex with the desired target, a dissociation constant of at least 1 μ M. However, the washing and elution steps can be carried out under varying stringency. A selection with respect to the kinetic characteristics is possible as well. For example, the selection can be performed under conditions, which favor complex formation of

the target with aptamers that show a slow dissociation from the target, or in other words a low k_{off} rate. Alternatively, selection can be performed under conditions, which favor fast formation of the complex between the aptamer and the target, or in other words a high k_{on} rate.

5 In this context it is also noted that the complex formation between the respective aptamer and its ligand is influenced by many different factors such as the concentrations of the respective binding partners, the presence of competitors, pH and the ionic strength of the buffer system used, and the experimental method used for determination of the dissociation constant K_D (for example fluorescence titration, competition ELISA or surface plasmon resonance, just to name
10 a few) or even the mathematical algorithm which is used for evaluation of the experimental data.

Therefore, it is also clear to the skilled person that the K_D values (dissociation constant of the complex formed between the respective aptamer and its ligand) given here may vary within a
15 certain experimental range, depending on the method and experimental setup that is used for determining the affinity of a particular protein aptamer for a given ligand. This means, there may be a slight deviation in the measured K_D values or a tolerance range depending, for example, on whether the K_D value was determined by surface plasmon resonance (Biacore) or by competition ELISA.

20 In one embodiment of these methods, the selection in step (c) is carried out under competitive conditions. Competitive conditions as used herein means that selection of aptamers encompasses at least one step in which the aptamers and the given non-natural ligand of human 4EBP1 (target) are brought in contact in the presence of an additional ligand, which competes
25 with binding of the aptamers to the target. This additional ligand may be a physiological ligand of the target, an excess of the target itself or any other non-physiological ligand of the target that binds at least an overlapping epitope to the epitope recognized by the aptamers of the invention and thus interferes with target binding of the aptamers. Alternatively, the additional ligand competes with binding of the aptamers by complexing an epitope distinct from the binding site
30 of the aptamers to the target by allosteric effects.

The invention is also directed to protein aptamers obtainable or obtained by the methods of invention.

In another aspect, the invention relates to a nucleic acid molecule comprising a nucleotide sequence encoding a protein aptamer according to the invention. Since the degeneracy of the genetic code permits substitutions of certain codons by other codons specifying the same amino acid, the invention is not limited to a specific nucleic acid molecule encoding a protein aptamer of the invention but includes all nucleic acid molecules comprising nucleotide sequences encoding a functional aptamer.

A nucleic acid molecule disclosed in this application may be "operably linked" to a regulatory sequence (or regulatory sequences) to allow expression of this nucleic acid molecule.

A nucleic acid molecule, such as DNA, is referred to as "capable of expressing a nucleic acid molecule" or capable "to allow expression of a nucleotide sequence" if it comprises sequence elements which contain information regarding to transcriptional and/or translational regulation, and such sequences are "operably linked" to the nucleotide sequence encoding the polypeptide.

An operable linkage is a linkage in which the regulatory sequence elements and the sequence to be expressed are connected in a way that enables gene expression. The precise nature of the regulatory regions necessary for gene expression may vary among species, but in general these regions comprise a promoter which, in prokaryotes, contains both the promoter *per se*, i.e. DNA elements directing the initiation of transcription, as well as DNA elements which, when transcribed into RNA, will signal the initiation of translation. Such promoter regions normally include 5' non-coding sequences involved in initiation of transcription and translation, such as the -35/-10 boxes and the Shine-Dalgarno element in prokaryotes or the TATA box, CAAT sequences, and 5'-capping elements in eukaryotes. These regions can also include enhancer or repressor elements as well as translated signal and leader sequences for targeting the native polypeptide to a specific compartment of a host cell.

In addition, the 3' non-coding sequences may contain regulatory elements involved in transcriptional termination, polyadenylation or the like. If, however, these termination sequences are not satisfactory functional in a particular host cell, then they may be substituted with signals functional in that cell.

Therefore, a nucleic acid molecule of the invention can include a regulatory sequence, preferably a promoter sequence. In another preferred embodiment, a nucleic acid molecule of the invention comprises a promoter sequence and a transcriptional termination sequence.

Suitable prokaryotic promoters are, for example, the *tet* promoter, the *lacUV5* promoter or the

T7 promoter. Examples of promoters useful for expression in eukaryotic cells are the SV40 promoter or the CMV promoter.

The nucleic acid molecules of the invention can also be part of a vector or any other kind of cloning vehicle, such as a plasmid, a phagemid, a phage, a baculovirus, a cosmid or an artificial chromosome.

Such cloning vehicles can include, aside from the regulatory sequences described above and a nucleic acid sequence encoding a protein aptamer of the invention, replication and control sequences derived from a species compatible with the host cell that is used for expression as well as selection markers conferring a selectable phenotype on transformed or transfected cells. Large numbers of suitable cloning vectors are known in the art, and are commercially available.

The DNA molecule encoding protein aptamers of the invention, and in particular a cloning vector containing the coding sequence of such a protein aptamer can be transformed into a host cell capable of expressing the gene. Transformation can be performed using standard techniques (Sambrook, J. et al. (1989), *supra*). Thus, the invention is also directed to a host cell containing a nucleic acid molecule as disclosed herein.

The transformed host cells are cultured under conditions suitable for expression of the nucleotide sequence encoding a protein aptamer or protein aptamer fusion protein of the invention. Suitable host cells can be prokaryotic, such as *Escherichia coli* (*E. coli*) or *Bacillus subtilis*, or eukaryotic, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, SF9 or High5 insect cells, immortalized mammalian cell lines (e.g. HeLa cells or CHO cells) or primary mammalian cells.

The invention also relates to a method for the production of a protein aptamer of the invention, wherein the aptamer, a fragment of the aptamer or a fusion protein of the aptamer and another polypeptide is produced starting from the nucleic acid coding for the aptamer by means of genetic engineering methods. The method can be carried out *in vivo*, the aptamer can for example be produced in a bacterial or eucaryotic host organism and then isolated from this host organism or its culture. In another embodiment, the aptamers of the invention may be produced by *in vitro* transcription/translation employing well-established methods known to those skilled in the art.

When producing the aptamer *in vivo*, a nucleic acid encoding an aptamer of the invention is introduced into a suitable bacterial or eukaryotic host organism by means of recombinant DNA technology (as already outlined above). For this purpose, the host cell is first transformed with a cloning vector comprising a nucleic acid molecule encoding an aptamer of the invention using established standard methods (Sambrook, J. et al. (1989), *supra*). The host cell is then cultured under conditions, which allow expression of the heterologous DNA and thus the synthesis of the corresponding polypeptide. Subsequently, the polypeptide is recovered either from the cell or from the cultivation medium.

10 The protein aptamers of the invention can be used in various applications, including therapeutic or diagnostic applications.

In one aspect, the invention thus relates to a method for the detection of a given non-natural ligand of human 4EBP1, comprising the steps of

- 15 (a) contacting a protein aptamer according to the invention with a sample suspected of containing the given ligand under suitable conditions, thereby allowing formation of a complex between the protein aptamer and the given ligand, and
(b) detecting the complexed protein aptamer by a suitable signal.

20 The detection may for example be achieved by a detectable label that is coupled to the protein aptamer, as defined above. Alternatively, the detectable signal can be caused by a change of physical properties due to the binding, i.e. the complex formation, itself. One example is plasmon surface resonance, the value of which is changed during binding of binding partners from which one is immobilized on a surface such as a gold foil.

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In another aspect, described is a method for the separation of a given non-natural ligand of human 4EBP1, comprising the steps of

- (a) contacting a protein aptamer according to the invention with a sample suspected to contain said ligand under suitable conditions, thereby allowing formation of a complex
30 between the protein aptamer and the given ligand, and
(b) separating the protein aptamer/ligand complex from the sample.

In such a method, the protein aptamer/ligand complex may be bound onto a solid support.

Also contemplated is a method for the targeting of a compound to a preselected site in an organism or tissue, comprising the steps of

- (a) conjugating a protein aptamer according to the invention with said compound, and
- (b) delivering the protein aptamer/compound complex to the preselected site.

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This use is in particular suitable, but not restricted to, for delivering a drug (selectively) to a preselected site in an organism, such as an infected body part, tissue or organ which is supposed to be treated with the drug. Such a conjugate of aptamer and compound may also include a linker that covalently links aptamer and compound to each other. Optionally, such a linker is stable in the bloodstream but is cleavable in a cellular environment.

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Another related and preferred use of a protein aptamer described herein is target validation, i.e. the analysis whether a polypeptide assumed to be involved in the development or progress of a disease or disorder is indeed somehow causative of that disease or disorder. This use for validating a protein as a pharmacological drug target takes advantage of the ability of a protein aptamer of the present invention to specifically recognize a surface area of a protein in its native conformation, i.e. to bind to a native epitope. However, the use of an inventive aptamer for validation of a drug target is not limited to the detection of proteins as targets, but also includes the detection of protein domains, peptides, nucleic acid molecules, organic molecules or metal complexes.

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The invention is further illustrated by the following non-limiting examples.

Examples

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Example 1: Reagents

AC-15, eIF4E(P-2) and Cyclin A(H-432) (all from Santa Cruz Biotechnology) were used for detection of beta-actin, eIF4E and Cyclin A respectively. "Anti-Thio" (Invitrogen, #R920-25) was used for detection of the thioredoxin scaffold. Antibodies against PCNA (Clone S83) and Mdm2 (Clone 2A9) were generous gifts from Dr. Borivoj Vojtesek (Department of Clinical and Experimental Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic). A streptavidin-peroxidase conjugate (Sigma, #S2438) was used to detect the SBP-tagged aptamers.

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Example 2: Construction of aptamers

pET32a (Novagen) encoding a His-tagged thioredoxin (Trx) was cleaved with the endonuclease restriction enzyme RsrII. The following oligonucleotides were annealed and inserted into Trx:

Trx-eIF4G:

5' GTCCGCCTAAAAACGCTATGATCGCGAATTTCTGCTGGGCTTTCAGTTTGAAAA

5 CG 3' (SEQ ID NO:55) and

5'GACCGTTTTCAAACGCTGAAAGCCCAGCAGAAATTCGCGATCATAGCGTTTTTTAG

GCG 3' (SEQ ID NO:56)

Trx-SG-eIF4G:

5'GTCCGCCTAGCGGCAAAAAACGCTATGATCGCGAATTTCTGCTGGGCTTTCAGTT

10 TGAAAACG 3' (SEQ ID NO:57) and

5' GACCGTTTTCAAACGCTGAAAGCCCAGCAGAAATTCGCGATCATAGCGTTTTTTGCC

GCTAGGCG 3' (SEQ ID NO:58)

Trx-eIF4G-ala:

5' GTCCGCCTAAAAACGCGCGGATCGCGAATTTGCG GCGGGCTTTCAGTTTGAAA

15 ACG 3' (SEQ ID NO:59) and

5'GACCGTTTTCAAACGCTGAAAGCCCGCCGCAAATTCGCGATCCGCGCGTTTTTTAGG

CG 3' (SEQ ID NO:60)

Trx-SG-eIF4G-ala:

5' GTCCGCCTAGCGGCAAAAAACGCGCGGATCGCGAATTTGCGGCGGGCTTTCAGT

20 TTGAAAACG 3' (SEQ ID NO:61) and

5'GACCGTTTTCAAACGCTGAAAGCCCGCCGCAAATTCGCGATCCGCGCGTTTTTTGCC

GCTAGGCG 3' (SEQ ID NO:62)

The method is described in more detail in Bottger A et al., Curr Biol. 1997, 7, 860-869 and
25 Brown CJ et al., J Mol Biol. 2010, 395, 871-883, both of which are hereby incorporated by
reference in their entirety.

DNAs encoding for 4EBP1 wildtype (SEQ ID NO: 1), 4EBP1 aptamer (SEQ ID NO:3), 4EBP1-
12.1 (SEQ ID NO: 9), 4EBP1-Con1 (SEQ ID NO: 11) and 4EBP1-CBP (SEQ ID NO: 13) were
30 ordered and synthesized by Midland, Molecular Biology Group, Texas.

Site directed mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis
Kit (Stratagene) to obtain the DNA sequences encoding for the respective non-binding controls
of the respective aptamers 4EBP1-ala (SEQ ID Nos. 5 and 6), 4EBP1-delta (SEQ ID Nos. 7

and 8), 4EBP1-12.1-ala (SEQ ID Nos. 49 and 15), 4EBP1-Con1-ala (SEQ ID Nos. 50 and 16) and 4EBP1-CBD-ala (SEQ ID Nos. 51 and 17).

Construct	Forward primer	Reverse primer	NT Sequence	AA Sequence
4EBP1-ala	4-p-4EBP1-ala, SEQ ID NO: 63	4-p-4EBP1-ala, SEQ ID NO: 64	SEQ ID NO: 5	SEQ ID NO: 6
4EBP1-delta	4-p-4EBP1-del, SEQ ID NO: 65	4-p-4EBP1-del, SEQ ID NO: 66	SEQ ID NO: 7	SEQ ID NO: 8
4EBP1-12.1-ala	4-p-EBP1-ST-ala SEQ ID NO: 71	4-p-EBP1-ST-ala SEQ ID NO: 72	SEQ ID NO: 49	SEQ ID NO: 15
4EBP1-Con1-ala	4EBP1-Con1-ala, SEQ ID NO: 67	4EBP1-Con1-ala, SEQ ID NO: 68	SEQ ID NO: 50	SEQ ID NO: 16
4EBP1-CBD-ala	4EBP1-CBD-ala, SEQ ID NO: 69	4EBP1-CBD-ala, SEQ ID NO: 70	SEQ ID NO: 51	SEQ ID NO: 17

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Using the Gateway[®] technology (Invitrogen) all aptamers to be expressed in mammalian cells were cloned into the pcDNA6.2 vector (Invitrogen) comprising an N-terminal tagged with a streptavidin binding peptide (SBP, nucleotide sequence SEQ ID No: 27, protein sequence SEQ ID NO: 28; Keefe AD et al., Protein Expr Purif. 2001, 23, 440-6) to facilitate detection and selective precipitation. Vectors comprising the following nucleotide sequences and encoding for the respective SBP-fusion protein were obtained: SBP-4EBP1-wildtype (SEQ ID Nos. 40 and 32), SBP-4EBP1 aptamer (SEQ ID Nos. 41 and 33), SBP-4EBP1-12.1 (SEQ ID Nos. 29 and 30), SBP-4EBP1-12.1-ala (SEQ ID Nos. 52 and 31), SBP-4EBP1-ala (SEQ ID Nos. 42 and 34), SBP-4EBP1-delta (SEQ ID Nos. 43 and 35), SBP-4EBP1-Con1 (SEQ ID Nos. 44 and 36), SBP-4EBP1-Con1-ala (SEQ ID Nos. 53 and 37), SBP-4EBP1-CBD (SEQ ID Nos. 45 and 38) and SBP-4EBP1-CBD-ala (SEQ ID Nos. 54 and 39).

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Example 3: Expression of aptamers and eIF4E precipitation assay

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His-tagged aptamers were incubated with Ni-NTA beads (Qiagen) in PBS for 1hr at 4°C on a rotator followed by 2 washes with PBS containing 0.05% Tween20 to remove the unbound protein. The soluble fraction of bacterial lysate, from BL21 cells induced to express eIF4E, were added to the beads and incubated for 1hr at 4°C. The beads were washed 5 times with PBS containing 0.05% Tween 20 and the bound proteins were eluted by heating at 95°C in 2X NuPAGE LDS sample buffer (Invitrogen).

Example 4: Cell based assays

All mammalian cell transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MCF7 cells were transfected with 0.5 μ g of bicistronic vector and 3.5 μ g of aptamer DNA in a 6 well plate and the luciferase measurements were carried out using the Dual luciferase reporter assay kit (Promega) 24hr post transfection. The bicistronic luciferase reporter construct was a generous gift from Dr Anne-Catherine Prats from INSERM, France (Delluc-Clavieres A et al., Gene Ther. 2008, 15, 1090-8; Conte Cet al., PLoS One. 2008, 3, e3078).

T22 cells stably transfected with a p53-responsive beta-galactosidase reporter (Bottger A et al., Curr Biol. 1997, 7, 860-9; Berkson RG et al., Int J Cancer. 2005, 115, 701-10) were transfected with 1.6 μ g of aptamer DNA in a 12 well plate for 30hrs. The measurements for betagal were carried out using the beta-Galactosidase Enzyme Assay System kit (Promega) and cell staining for beta-gal expression was performed using the *In Situ* beta-Galactosidase Staining Kit (Roche) according to manufacturer's instructions.

Example 5: Streptavidin binding peptide (SBP) tag precipitations

293FT cells transfected with 4 μ g of aptamer DNA in 6 well plates, were harvested by scraping 30hrs post transfection. The cells were lysed in 200 μ l of NP40 lysis buffer (50mM Hepes, pH7.5, 100mM NaCl, 5mM EDTA and 0.5% NP40) supplemented with protease inhibitors. The supernatant was incubated with 50 μ l of washed avidin agarose beads for 1 hour at 4 °C on a rotator. The precleared supernatant was then incubated with 50 μ l of washed agarose streptavidin beads or 50 μ l of streptavidin-coupled Dynabeads (Invitrogen) for 1 hour at 4 °C on a rotator. The beads were then washed 4X with 1ml of NP40 lysis buffer and the bound proteins were eluted with 1mM biotin.

Example 6: Flow cytometry analysis

Flow cytometry analysis was carried out on a BD LSRII flow cytometer. U2OS cells were transfected with 1 μ g of a transmembrane GFP construct and 1 μ g of aptamer DNA, and harvested after 48hrs. The cells were fixed overnight in 70% ethanol and stained with 0.025 μ g/ μ l of propidium iodide and 0.25 μ g/ μ l of RNaseA. The cell cycle profiles of the transfected cells were analyzed after gating for GFP fluorescent cells.

Example 7: Three dimensional (3D) structure modeling

The 3D structures used for the modeling were taken from the crystal structures of the complexes between Mdm2 and p53 (PDB ID: 1YCR, resolved at 2.6Å; (Kussie PH et al., Science. 1996, 274, 948-53), PCNA and p21 peptide (PDB ID: 1AXC resolved at 2.6Å; (Gulbis JM et al., Cell. 1996, 87, 297-306) and, between Cyclin A and peptide (PDB ID: 2CCH.pdb resolved at 1.7Å; (Cheng KY et al., J Biol Chem. 2006, 281, 23167-79). The peptides in all the 3 complexes were modified to correspond to the sequences used in the experiments: MPRFMDYWEGLN (SEQ ID NO: 19) for the peptide in the Mdm2-peptide complex, SAVLQKKTTITDYFHPKK (SEQ ID NO: 21) in the PCNA-Cyclin A complex and DFYHAKRRLIFS (SEQ ID NO: 23) in the Cyclin A-peptide complex. The structures were prepared as previously described (Dastidar SG et al., J Am Chem Soc. 2008, 130, 13514-5) and were subject to the charmm27 force field (Brooks BR et al., J Comput Chem. 2009, 30, 1545-614) with the GBSW implicit solvation continuum model (Im Wet al., J Comput Chem. 2003, 24, 1691-702). Cycles of optimizations of the structures and energies of the complexes using Steepest Descent, Conjugate Gradient and Adopted Basis Newton Raphson methods were carried out to generate the final models.

Example 8: Thioredoxin (Trx) based eIF4E binding aptamers

The peptide sequence (KKRYDREFLLGF) binding to eIF4G was selected and inserted into Trx for the construction of a bioactive aptamer – Trx-eIF4G. The critical binding residues were mutated to alanines (KKRADREFAAGF) to generate a non-binding control – Trx-eIF4G-ala. A modified Trx-SG-eIF4G aptamer with additional serine and glycine residues inserted (SGKKRYDREFLLGF) was found to exhibit enhanced binding to eIF4E in a precipitation assay performed using bacterially expressed proteins (Figure 1A).

The Trx aptamers were next transfected into mammalian cells and checked for expression efficiency. The protein levels of the binding aptamers Trx-eIF4G and Trx-SG-eIF4G were found to be undetectable in comparison to their respective non-binding controls as shown in Figure 1B.

Upon the addition of proteasome inhibitor MG132, 4 hours prior to harvesting, accumulation of the binding aptamer proteins was detected by western blot (Figure 1C). The Trx aptamers were found to be able to bind eIF4E but were dependent on the addition of MG132 for accumulation of binding aptamer protein (data not shown). The streptavidin-horseradish peroxidase (HRP) conjugate was able to bind the SBP-tag and facilitate detection of aptamer expression by western blot (Figure 1C).

These results illustrate the convenience of the SBP-tag for detection of aptamer expression and confirm the successful use of the Trx scaffold to construct aptamers that bind eIF4E. Addition of the serine and glycine residues was found to improve the binding of the aptamer (Trx-SG-eIF4G) to eIF4E. However, the binding aptamers constructed, using Trx as scaffold, were found to be unstable in cells when compared to their respective non-binding controls.

Example 9: Conversion of 4EBP1 into an eIF4E aptamer

A stable and persistent 4EBP1-based eIF4E binding aptamer was constructed by mutating the mTOR phosphorylation sites (threonines 37, 46, 70 and of serine 65) rendering them insensitive to phosphorylation by mTOR. The modified 4EBP1 (subsequently referred to as 4EBP1 aptamer, SEQ ID NO: 4) resembles hypophosphorylated 4EBP1 and functions as an eIF4E binding aptamer. For the generation of control aptamers, the critical contact residues in 4EBP1 essential for binding to eIF4E were mutated to alanine (Y54, F58, L59) to give 4EBP1-ala (SEQ ID NO: 6). In addition, 9 amino acids (aa 54-62) from the eIF4E binding sequence of 4EBP1 were deleted to create the additional non-binding control 4EBP1-delta (SEQ ID NO: 8) for comparison purposes (Haghighat A et al., EMBO J. 1995, 14, 5701-5709). The transfection of the aptamers into cells led to stable expression of the 4EBP1 aptamers.

The expression levels of the 4EBP1 aptamer were slightly reduced in comparison to WT 4EBP1 and the control aptamers as shown in lane 2 of Figure 2B. Nevertheless, the difference in expression levels was less pronounced in comparison to that of the Trx aptamers in the absence of MG132. A precipitation assay was performed using streptavidin beads and SBP-tagged 4EBP1 aptamers to determine the binding ability of the aptamers. The SBP-tagged aptamers were successfully precipitated by the streptavidin beads. The binding of the 4EBP1 aptamer to eIF4E was found to be equal to if not better than WT 4EBP1 (Figure 2C). The binding is specific as the alanine and deletion control aptamers did not pull down any eIF4E. These results demonstrate the utility of the SBP-tag in precipitations and show that the modifications of 4EBP1 did not affect the stability or the ability of the protein to bind to its target *in vitro*.

Example 10: Assessment of the bioactivity of the 4EBP1 aptamer

To assess the *in vivo* bioactivity of the 4EBP1 aptamer, the effects of the 4EBP1 aptamers on cap-dependent translation were analyzed. The 4EBP1 aptamer target eIF4E plays an essential role in translation. As host mammalian cells were chosen. A reporter construct that enables the measurement and comparison of cap-dependent and -independent translation was used to assess the effect of eIF4E binding aptamers in the cells. The construct consists of a *Renilla* luciferase

gene that is translated in a cap-dependent manner followed by a firefly luciferase gene that is translated in a cap-independent mode, on the same plasmid. The ratio of *Renilla* luciferase to firefly luciferase readings was used to assess the effects of the eIF4E binding aptamers on cap-dependent translation (Villalonga P et al., J Biol Chem. 2009, 284, 35287-96).

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As shown in Figure 3, normalized *Renilla* luciferase activity was substantially reduced for cells transfected with the non-phosphorylatable 4EBP1 aptamer. The reduction in *Renilla* luciferase activity was more pronounced for the 4EBP1 aptamer in comparison to wild type 4EBP1. Cells transfected with non-binding 4EBP1 aptamers controls had higher levels of *Renilla* luciferase activity. These results demonstrate that the non-phosphorylatable 4EBP1 aptamer exhibits a greater inhibitory effect on cap-dependent translation in comparison to WT 4EBP1.

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Example 11: Construction of bioactive Mdm2 binding aptamers using the 4EBP1 scaffold and comparison with thioredoxin (Trx) based Mdm2 binding aptamers

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In order to examine the potential of unstructured, non-phosphorylatable 4EBP1 aptamer as a scaffold protein, it was used to construct aptamers that bind Mdm2. Mdm2 is an E3 ubiquitin ligase that ubiquitinates tumour suppressor p53 and targets it for degradation. Inhibition of Mdm2 activity leads to the stabilization of p53 protein and its increased activity. A bioactive Mdm2 binding aptamer (SuperTIP) has been successfully created by Böttger A et al, using the well characterized scaffold protein thioredoxin (Bottger A et al., Curr Biol. 1997, 7, 860-9). The peptide used (12.1; LSMPRFMDYWEGLN, SEQ ID NO: 19, encoded by SEQ ID NO: 18) for the construction of SuperTIP (subsequently referred to as Trx-12.1) was grafted onto 4EBP1 (4EBP1-12.1, SEQ ID NO: 10) to assess its aptitude as a scaffold. Amino acids 51 to 62 of 4EBP1 that confer binding to eIF4E were deleted and replaced by the Mdm2-binding 12.1 peptide. The critical contact residues were mutated to alanines (LSMPRAMDYAEGAN, SEQ ID NO: 24, encoded by SEQ ID NO: 46) to create a non-binding control (4EBP1-12.1-ala, SEQ ID NO: 34).

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Precipitation assays were performed using the SBP-tagged 4EBP1-12.1 (SEQ ID NO: 30). The 4EBP1-12.1 aptamer bound specifically to Mdm2 as shown in Figure 4 (lane 3), and pulled down more Mdm2 in comparison to Trx-12.1 (Figure 4, lane 1). Also, the expression level of the binding Trx-12.1 aptamer was observed to be lower compared to its non-binding control (Trx-12.1-ala), as in the case for eIF4E binding Trx-eIF4G aptamers. The results demonstrate that the binding affinity of 4EBP1-12.1 aptamer for Mdm2 is higher than that of Trx-12.1 and further supports our prior observations that the binding aptamers constructed using Trx as

scaffold are unstable in cells. To further evaluate the bioactivity of the 4EBP1-12.1 aptamer, cell based assays were carried out in T22 cells. This mouse fibroblast cell line was stably transfected with a plasmid encoding betagalactosidase (beta-gal) gene under the control of a p53-dependent promoter (Berkson R G et al., Int J Cancer. 2005, 115, 701-10). Upon activation or accumulation of p53, the beta-gal gene is transcribed. The level of p53 activity can therefore be assessed by the amount of beta-gal activity measured.

The 4EBP1-12.1 and Trx-12.1 aptamers were transfected into T22 cells and the beta-gal activity of the cells was analyzed after 30 hours. Cells were treated with actinomycin D to serve as a positive control. The cells transfected with 4EBP1-12.1 and Trx-12.1 aptamers were found to have higher levels of beta-gal readings in comparison to their respective negative controls. The beta-gal activity induced by 4EBP1-12.1 was higher than that of Trx-12.1. In addition, there was higher background activity measured for Trx-12.1-ala compared to 4EBP1-12.1-ala. The beta-gal reading obtained for cells transfected with 4EBP1-12.1 was found to be similar to that of the positive control. The transfected cells were fixed and stained for beta-gal expression as shown in Figure 6. Similarly, the cells transfected with 4EBP1-12.1 stained more strongly for beta-gal expression compared to Trx-12.1 transfected cells, whereas the staining for cells transfected with Trx-12.1-ala was more intense compared to that for 4EBP1-12.1-ala. The beta-gal assay further demonstrates that 4EBP1-12.1 is a better Mdm2-binding aptamer than Trx-12.1 in terms of specificity and biological activity.

Example 12: Further validation of the versatility of 4EBP1 scaffold by inserting binding peptides to PCNA and Cyclin A

The potential of using 4EBP1 as an aptamer scaffold was further tested by constructing aptamers against targets such as proliferating cell nuclear antigen (PCNA) and Cyclin A. PCNA and Cyclin A are proteins that are essential for cell cycle progression. The peptide sequences for binding to PCNA and Cyclin A were derived from p21. The peptide sequences SAVLQKKITDYFHPKK, named Con1 (SEQ ID NO: 21, encoded by SEQ ID NO: 20; Zheleva Diet al., Biochemistry. 2000, 39, 7388-97; Warbrick E, Oncogene. 2006, 25, 2850-9), and DFYHAKRRLIFS, named Cyclin-binding domain or CBD (SEQ ID NO: 23, encoded by SEQ ID NO: 22; Zheleva Diet al., J Pept Res. 2002, 60, 257-70), were used to construct binding aptamers to PCNA and Cyclin A respectively. The critical binding residues were mutated (SAVLAKKATDAFHPKK, SEQ ID NO: 25, encoded by SEQ ID NO: 47, and DFYHAKRLIAS, SEQ ID NO: 26, encoded by SEQ ID NO: 48) to abrogate binding. The peptides were inserted into the 4EBP1 scaffold as before and precipitation assays were

performed using transfected cell lysates to examine interaction between the aptamers and their target proteins. The 4EBP1-CBD and 4EBP1-Con1 aptamers were found to specifically bind and precipitate Cyclin A (Figure 7A) and PCNA (Figure 7B) respectively. The endogenous level of Cyclin A in the cell lysate was too low for analysis of the total input and unbound fraction by western blot. But the binding of 4EBP1-Con1 was able to deplete PCNA from the cell lysate used for the precipitation (Figure 7C, lane 3).

After establishment of the physical interaction between the 4EBP1 aptamers and their respective targets, it was tested whether the binding of the aptamers had physiological significance. U2OS cells were transfected with the 4EBP1-CBD and -Con1 aptamers, and analyzed for cell cycle inhibition.

Cells transfected with a vector DNA encoding for the 4EBP1-CBD aptamer that successfully bound Cyclin A *in vitro* did not exhibit any significant change in cell cycle profile. This may be due to weaker or transient binding. Alternatively, it is also possible that blocking the substrate binding site on Cyclin A is not effective in blocking Cyclin dependent kinase (Cdk) function. On the other hand, cells transfected with 4EBP1-Con1 that binds PCNA were found to exhibit G1 arrest when compared to cells transfected with 4EBP1-Con1-ala (Figure 8). The cell cycle profile of cells transfected with 4EBP1-Con1-ala resembles that of 4EBP1-deletion control and SBP-GFP control (data not shown). These results describe the successful use of 4EBP1 as a scaffold for construction of aptamers against PCNA and Cyclin A, with the PCNA binding aptamer (4EBP1-Con1) demonstrating both physical interaction and bioactivity in cells.

All documents cited herein, are hereby incorporated by reference in their entirety.

The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those

skilled in the art, and that such modifications and variations are considered to be within the scope of this invention. The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further embodiments of the invention will become apparent from the following claims.

What is claimed is:

1. Protein aptamer comprising an unstructured scaffold polypeptide, wherein at least one of the amino acid sequence positions of the unstructured scaffold polypeptide is mutated such that the protein aptamer binds a given non-natural ligand of the unstructured scaffold polypeptide with detectable binding affinity.
2. The protein aptamer of claim 1, wherein the unstructured scaffold polypeptide is a naturally occurring protein or protein domain.
3. The protein aptamer of claim 1, wherein the unstructured scaffold polypeptide is selected from the group consisting of Arf, p21^{Waf1/Cip1/Sdi1}, p27, 4EBP1, 4EBP2, 4EBP3, non-A β component of AD amyloid precursor (NACP), p21, anti-sigma-28 factor (FlgM), calpastatin, microtubule-associated protein tau, microtubule-associated protein 2 (MAP2) projection domain, titin PEVK domain, SNAP25 linker region, securin, stathmin, thymosins (proT α), casein, salivary proline-rich protein (Prp), desiccation stress protein (Dsp) 16, MAP2 microtubule-binding domain, caldesmon, Bob1, λ phage N protein, sibling proteins, fibronectin receptor (MSCRAMM) D₁-D₄, CREB transactivator domain (TAD), Bcl-2 antiapoptotic protein (24-93), cAMP-dependent protein kinase inhibitor (PKI), cFOS transactivator domain (TAD), the proline-rich C-terminal domain of RNA polymerase II, p53 (aa 1-80), p63 (aa 1-80), p73 (aa 1-80), and the transactivator domain (TAD) of transcription factors.
4. The protein aptamer of claim 1, wherein the unstructured scaffold is a human 4EBP1 scaffold, wherein at least one of amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 as set forth in SEQ ID NO:2 is mutated and wherein the protein aptamer binds a given non-natural ligand of 4EBP1 with detectable binding affinity.
5. The protein aptamer of claim 3, wherein at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12, amino acids of the amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 are mutated.

6. The protein aptamer of claim 3, wherein amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 are deleted and replaced by a variable loop of 6 to 35 amino acids.
- 5 7. The protein aptamer of claim 6, wherein the variable loop is of 10 to 25 amino acids in length.
8. The protein aptamer of claim 6, wherein the variable loop originates from a peptide library.
- 10 9. The protein aptamer of claim 6, wherein the variable loop has the amino acid sequence set forth in any one of SEQ ID Nos. 19, 21, 23.
- 15 10. The protein aptamer of claim 3, wherein 1, 2, 3 or all 4 of amino acid sequence positions 37, 46, 65 and 70 of human 4EBP1 is substituted by any amino acid other than serine or threonine or deleted.
11. The protein aptamer of claim 10, wherein 1, 2, 3 or all 4 of amino acid sequence positions 37, 46, 65 and 70 of human 4EBP1 are substituted by alanine.
- 20 12. The protein aptamer of claim 3, wherein the 4EBP1 scaffold is truncated by 1 to 20 amino acids at the N-terminus, C-terminus or both.
13. The protein aptamer of claim 3, wherein the protein aptamer is at its N-terminus or
25 its C-terminus operably fused to an enzyme, a protein or a protein domain, a peptide, a signal sequence and/or an affinity tag.
14. The protein aptamer of claim 3, wherein the protein aptamer is fused to a moiety that extends the serum half-life of the protein aptamer.
- 30 15. The protein aptamer of claim 14, wherein the moiety that extends the serum half-life is selected from the group consisting of an Fc part of an immunoglobulin, a CH3 domain of an immunoglobulin, a CH4 domain of an immunoglobulin, albumin or an albumin fragment, an albumin binding peptide, an albumin binding protein, transferrin,

a polyalkylene glycol molecule, hydroxyethyl starch, palmitic acid and other fatty acid molecules.

16. The protein aptamer of claim 3, wherein the aptamer is conjugated to a label selected from the group consisting of organic molecules, enzyme labels, radioactive labels, fluorescent labels, chromogenic labels, luminescent labels, haptens, digoxigenin, biotin, metal complexes, metals, and colloidal gold.

17. The protein aptamer of claim 3, wherein said non-natural ligand of 4EBP1 is a protein or a fragment thereof.

18. The protein aptamer of claim 17, wherein the protein or fragment thereof is selected the group of Mdm2, PCNA and Cyclin A.

19. The protein aptamer of claim 18, wherein the protein aptamer acts as an Mdm2, PCNA or Cyclin A antagonist.

20. The protein aptamer of claim 18, wherein the protein aptamer binds Mdm2, PCNA or Cyclin A with a K_D of 1 μ M or less.

21. The protein aptamer according to claim 3, wherein the protein aptamer has an amino acid sequence as set forth in any one of SEQ ID Nos.: 10, 12 or 14 or of a fragment or variant thereof.

22. A method for the generation of a protein aptamer comprising a human 4EBP1 scaffold, wherein at least one of amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 as set forth in SEQ ID NO:2 is mutated and wherein the protein aptamer binds a given non-natural ligand of 4EBP1 with detectable binding affinity, comprising:

(a) subjecting a nucleic acid molecule encoding 4EBP1 to mutagenesis at at least one codon of any of the amino acid sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1, thereby obtaining a plurality of nucleic acids encoding 4EBP1 protein aptamers,

(b) expressing the one or more nucleic acid molecule(s) obtained in (a) in an expression system, thereby obtaining one or more protein aptamer(s) and

(c) enriching the one or more protein aptamer(s) obtained in (b) and having detectable binding affinity for a given non-natural ligand of 4EBP1 by means of selection and/or isolation.

- 5 23. The method of claim 22, wherein in step (a) at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12
 codons of any of the amino acid sequence positions 51-62 of the linear polypeptide
 sequence of human 4EBP1 are mutated.
- 10 24. The method of claim 22, wherein in step (a) the codons encoding amino acid
 sequence positions 51-62 of the linear polypeptide sequence of human 4EBP1 are
 deleted and replaced by codons encoding a variable loop of 6 to 35 amino acids.
25. The method of claim 24, wherein the variable loop is of 10 to 25 amino acids in
 length.
- 15 26. The method of claim 24, wherein the variable loop originates from a peptide library.
27. The method of claim 24, wherein the variable loop has the amino acid sequence set
 forth in any one of SEQ ID Nos. 19, 21, 23.
- 20 28. The method of claim 22, wherein in step (a) 1, 2, 3 or all 4 codons encoding amino
 acid sequence positions 37, 46, 65 and 70 of human 4EBP1 are substituted by codons
 encoding any amino acid other than serine or threonine or are deleted.
- 25 29. The method of claim 22, wherein in step (a) the nucleic acid molecule encoding the
 4EBP1 scaffold is mutated such that 1 to 20 amino acids at the N-terminus, C-terminus
 or both of the 4EBP1 scaffold are deleted.
- 30 30. The method of claim 22, wherein step (c) further comprises:
 (a) providing as a given ligand a compound selected from the group consisting
 of a chemical compound in free or conjugated form that exhibits features of
 an immunological hapten, a peptide, a protein or another macromolecule,
 (b) contacting the plurality of protein aptamers with said ligand in order to allow
 formation of complexes between said ligand and protein aptamers having
35 binding affinity for said ligand, and

(c) removing protein aptamers having no or no substantial binding affinity.

31. The method according to claim 22, wherein the ligand is a protein or a fragment thereof.

32. The method according to claim 22, wherein the selection in step (c) is carried out under competitive conditions.

33. A protein aptamer obtainable by the method of any one of claims 22 to 32.

34. A nucleic acid molecule comprising a nucleotide sequence encoding a protein aptamer according to claim 3.

35. The nucleic acid molecule of claim 34 comprised in a vector.

36. The nucleic acid molecule of claim 34 comprised in a phagemid vector.

37. A host cell containing a nucleic acid molecule according to claim 34.

38. Method for the detection of a given non-natural ligand of human 4EBP1, comprising the steps of

(a) contacting a protein aptamer according to claim 1 with a sample suspected of containing the given ligand under suitable conditions, thereby allowing formation of a complex between the protein aptamer and the given ligand, and

(b) detecting the complexed protein aptamer by a suitable signal.

39. Method for the separation of a given non-natural ligand of human 4EBP1, comprising the steps of

(a) contacting protein aptamer according to claim 1 with a sample suspected to contain said ligand under suitable conditions, thereby allowing formation of a complex between the protein aptamer and the given ligand, and

(b) separating the protein aptamer/ligand complex from the sample.

40. The method according to claim 339, wherein the protein aptamer/ligand complex is bound onto a solid support.

41. Method for the targeting of a compound to a preselected site in an organism or tissue, comprising the steps of

(a) conjugating a protein aptamer according to claim 3 with said compound, and

5 (b) delivering the protein aptamer/compound complex to the preselected site.

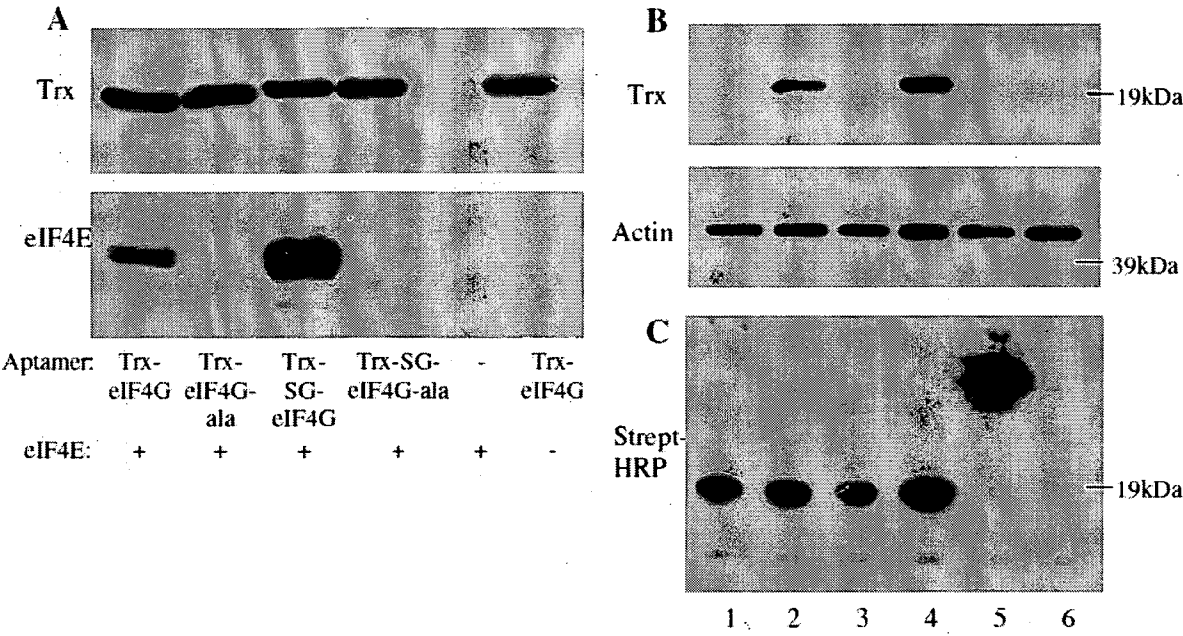


Figure 1

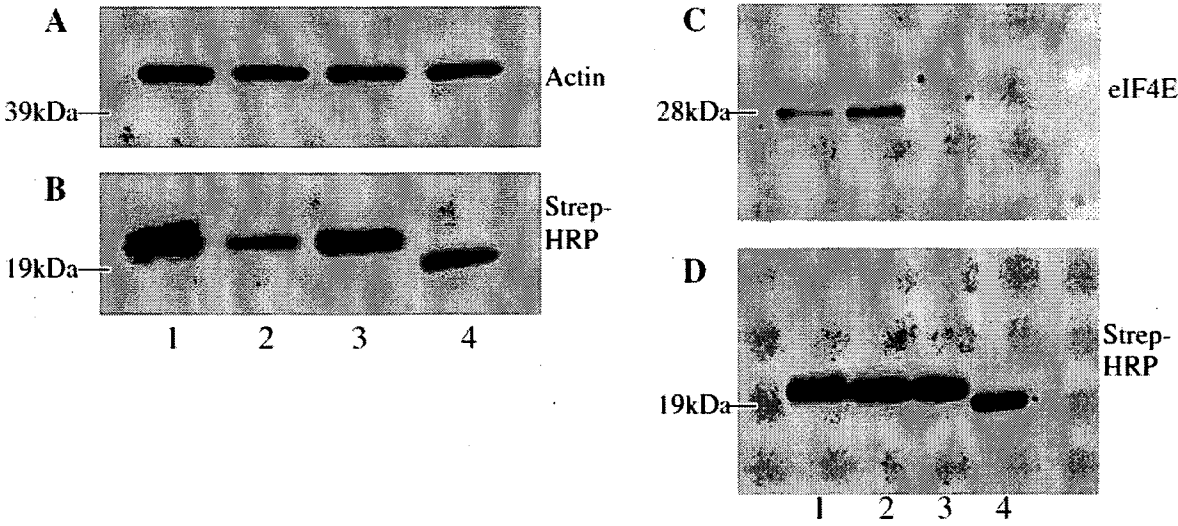


Figure 2

2/7

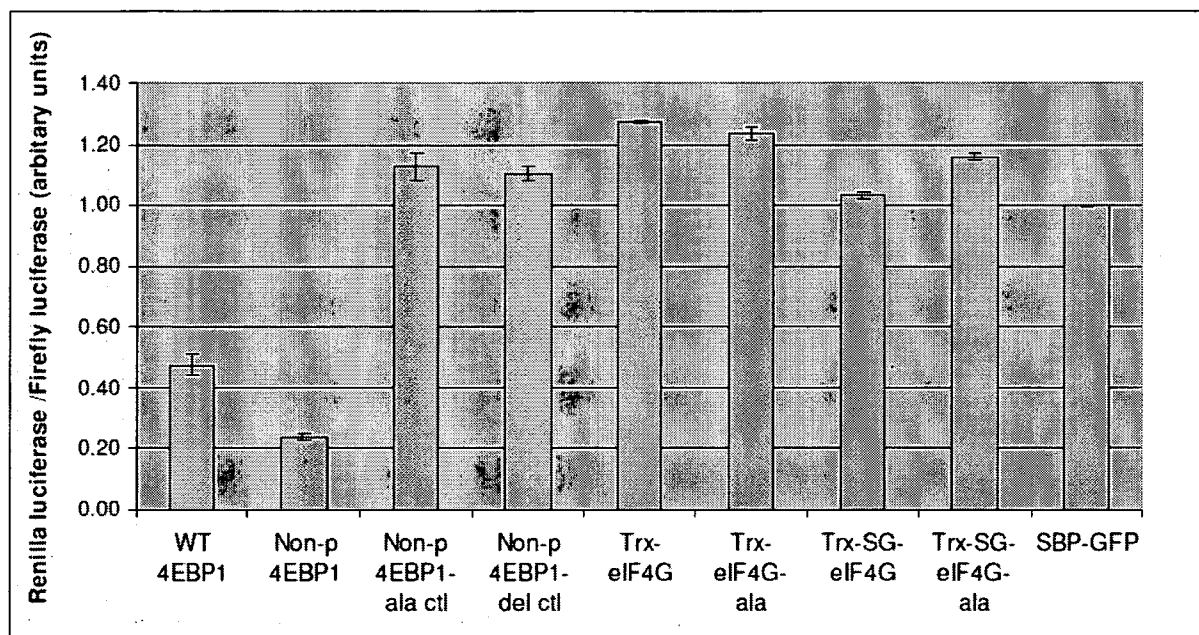


Figure 3

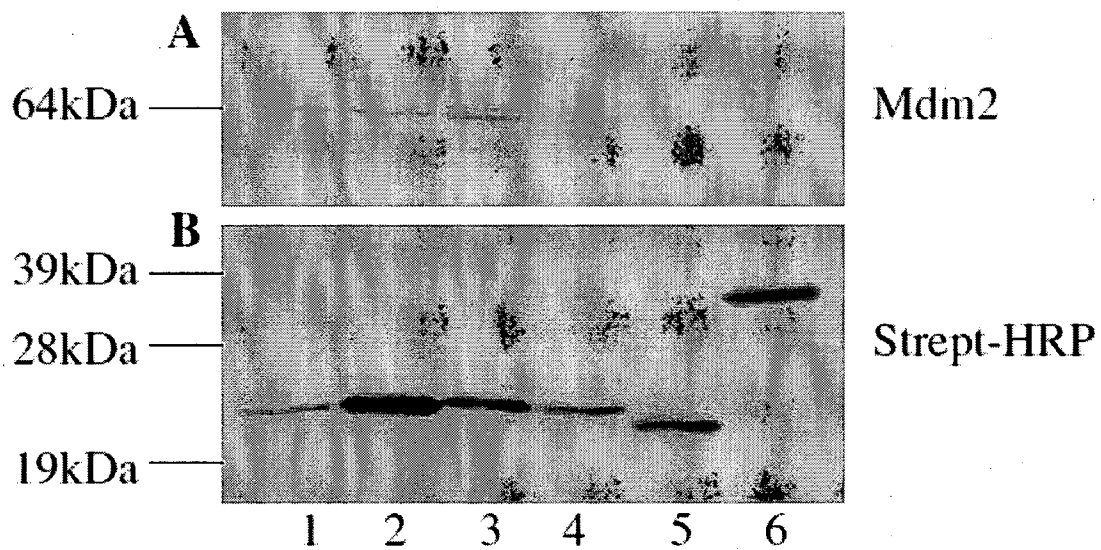


Figure 4

3/7

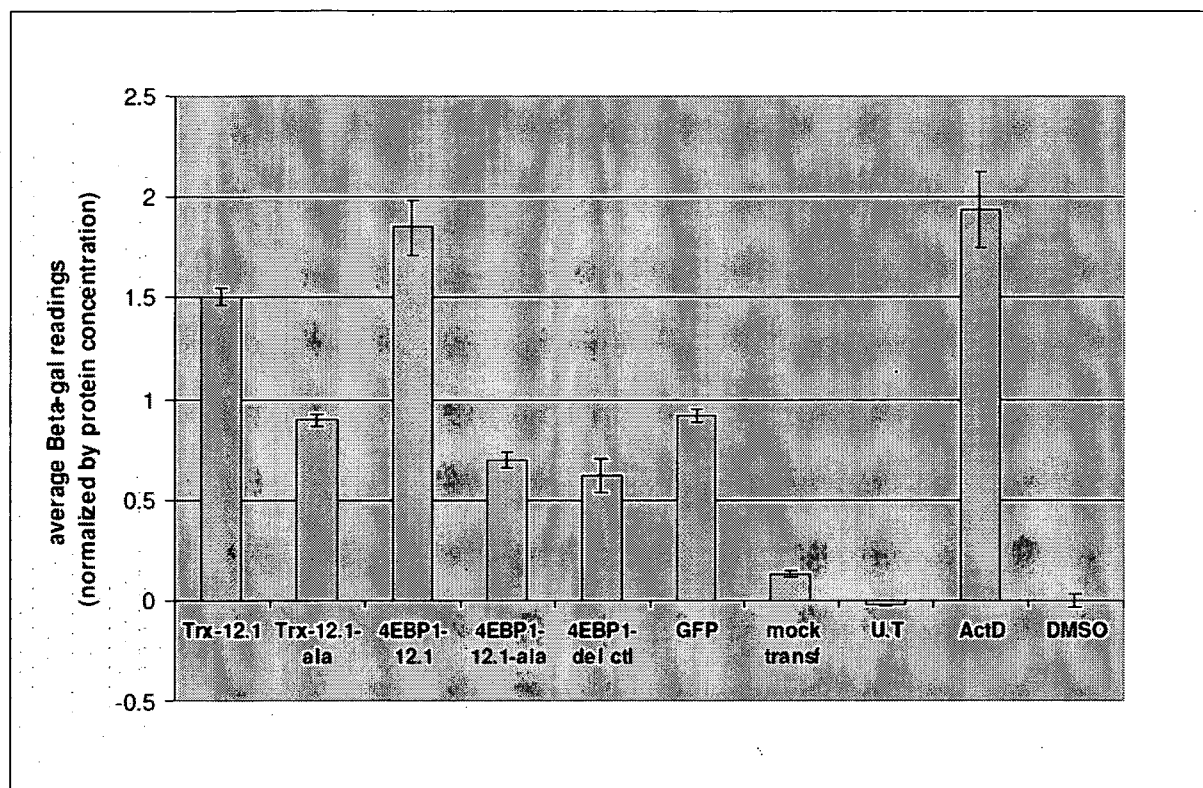


Figure 5

4/7

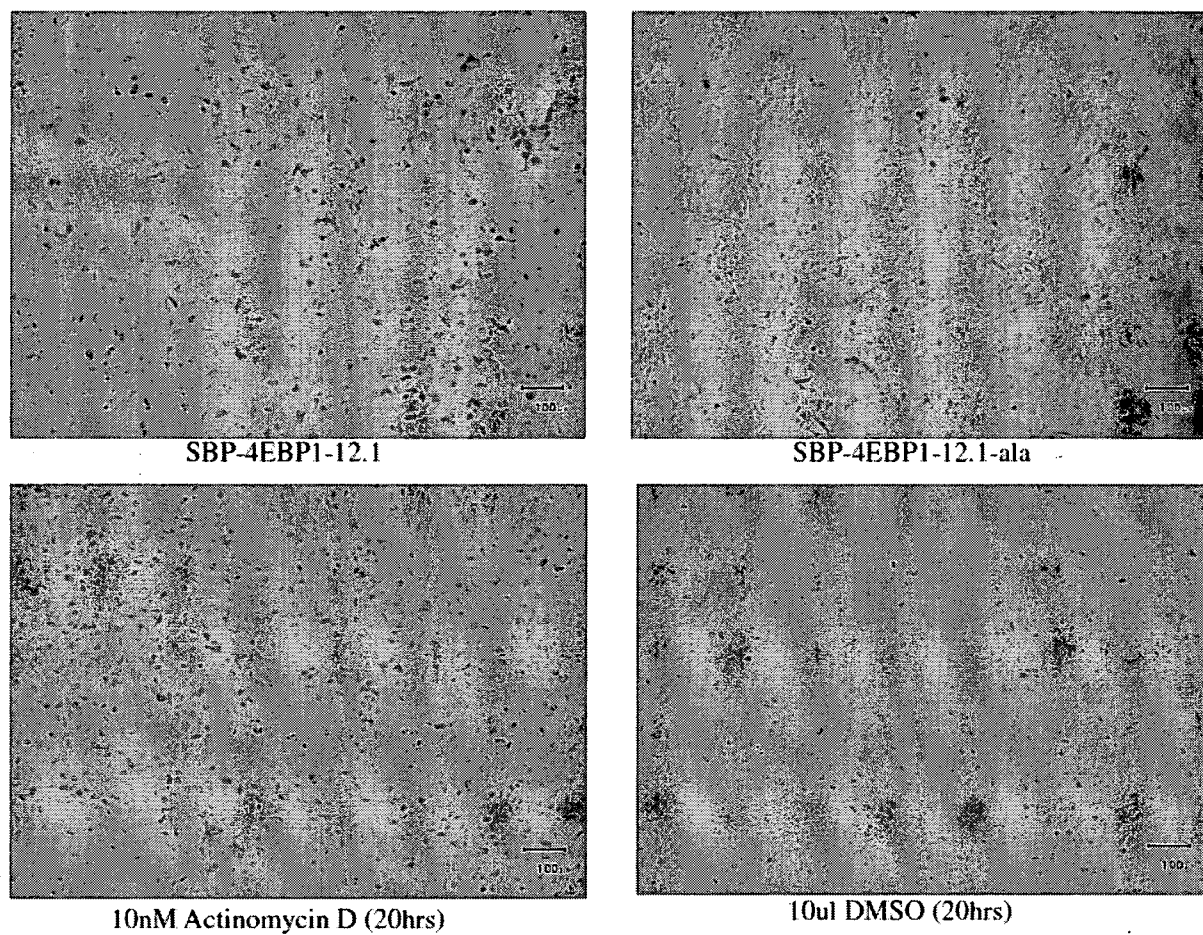


Figure 6

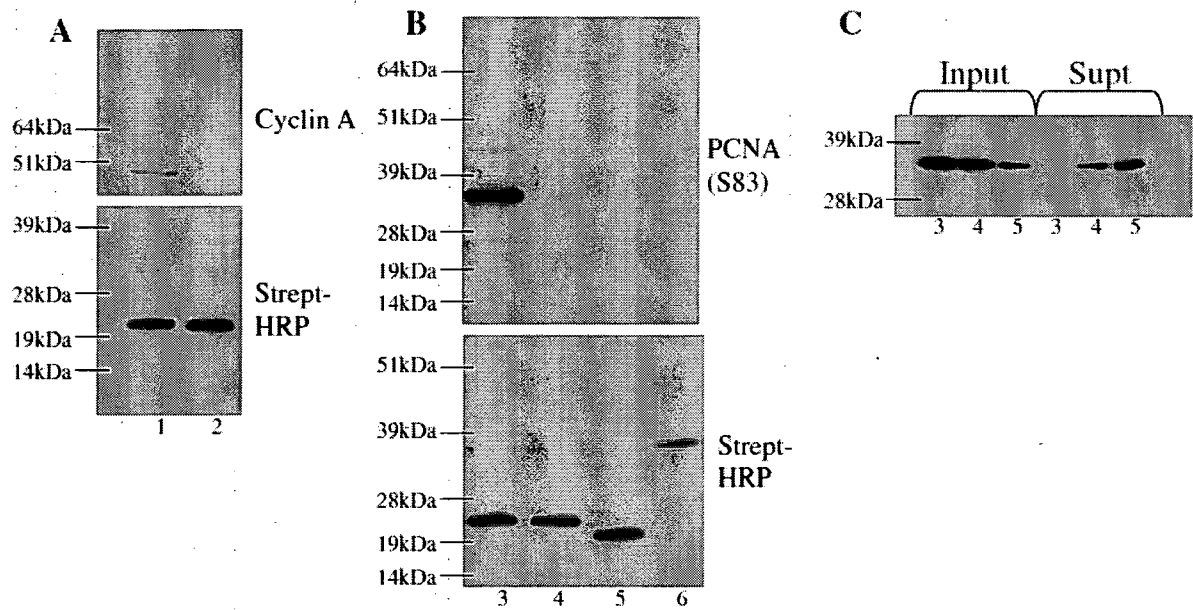


Figure 7

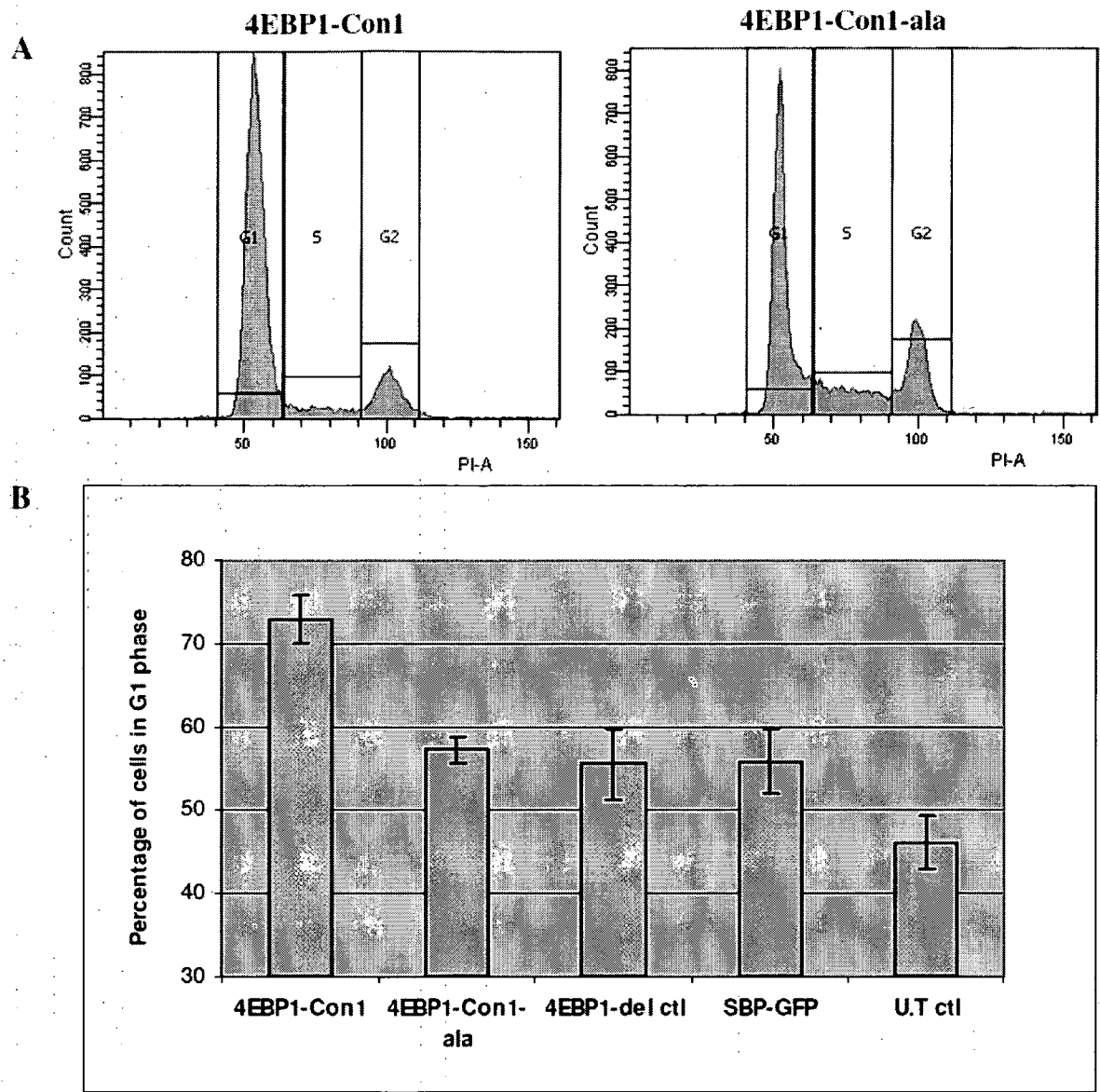


Figure 8

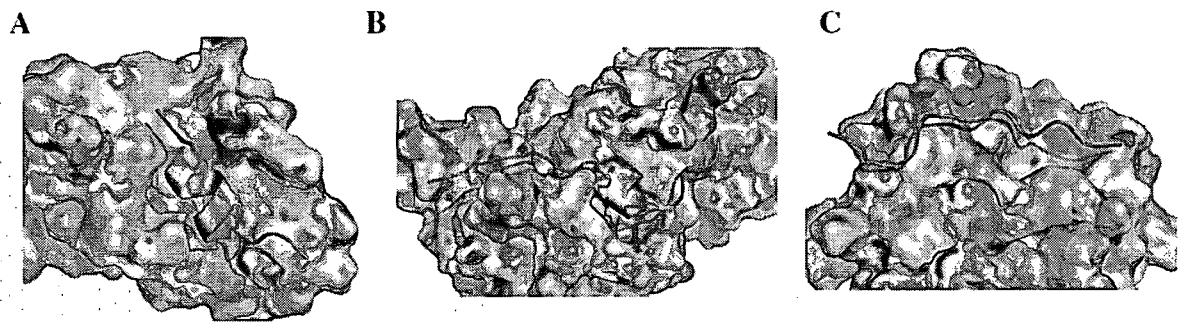


Figure 9

7/7

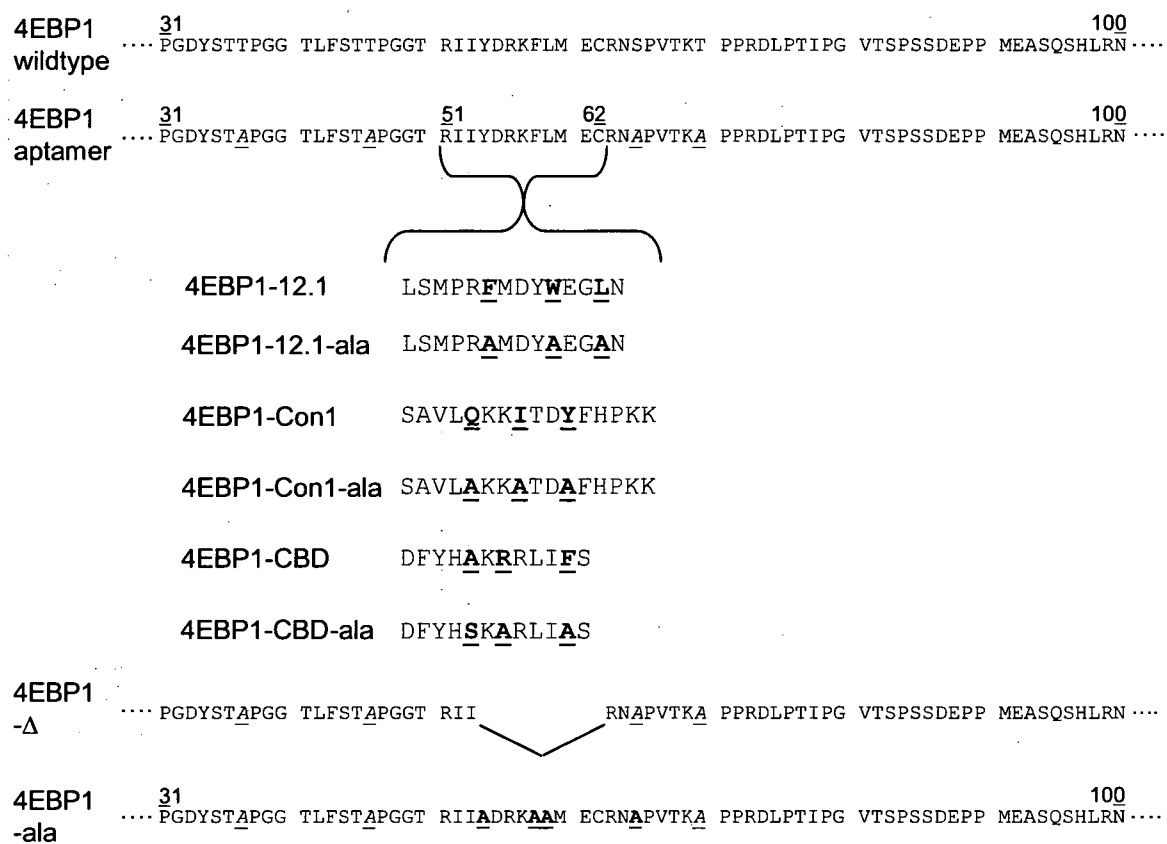


Figure 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2010/000462

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 15/115 (2010.01)

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)

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 practicable, search terms used)

Registry, CAPLUS, Medline, Wpids, Biosis: Keywords - 4E Binding Protein 1, mutate, modify, aptamer, scaffold, structure, framework and like terms

Genome Quest live Sequence Search based on SEQ ID NO. 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	See H. Y. & and Lane. D. P., "A Novel Unstructured Scaffold Based on 4EBP1 Enables the Functional Display of a Wide Range of Bioactive Peptides" J. Mol. Biol. (2010) 404, 819-831, Available online 7 October 2010 See whole doc. esp. abstract and pages 822 - page 828 1st paragraph	1-41
X	Brown, C. J., et al "Rational Design and Biophysical Characterization of Thioredoxin-Based Aptamers: Insights into Peptide Grafting" J. Mol. Biol. (2010) 395, 871-883 Available online 3 November 2009 See abstract	1, 2 and 33



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search
9 February 2011

Date of mailing of the international search report

15 FEB 2011

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