



(43) International Publication Date
3 October 2013 (03.10.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/148871 A1

(51) International Patent Classification:

C07K 19/00 (2006.01) *A61K 38/22* (2006.01)
C12N 15/62 (2006.01) *A61P 3/00* (2006.01)
C12N 15/63 (2006.01)

(21) International Application Number:

PCT/US2013/034152

(22) International Filing Date:

27 March 2013 (27.03.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/616,906 28 March 2012 (28.03.2012) US

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

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

Published:

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

(54) Title: ENGINEERED POLYPEPTIDES

(57) Abstract: Compounds are provided having *inter alia* good duration of action, high potency and/or convenient dosing regimens. The compounds are engineered polypeptides which incorporate an exendin domain in combination with a humanized chimeric seal leptin. Also provided are pharmaceutical compositions and methods of treatment for diseases including diabetes, overweight, obesity, Alzheimer's disease, short bowel syndrome, fatty liver disease, dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.



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ENGINEERED POLYPEPTIDES

CROSS-REFERENCES TO RELATED APPLICATIONS

- 5 **[0001]** This application claims the benefit of U.S. Provisional Pat. Appl. No. 61/616,906, filed March 28, 2012, which is incorporated herein by reference in its entirety and for all purposes.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

- [0001]** The Sequence Listing written in file 92494-868292_ST25.TXT, created on March
10 20, 2013, 500,808 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

- [0002]** Previously, we have demonstrated *inter alia* that leptin agonism is able to markedly and synergistically improve the therapeutic benefits already evident with administration of a GLP-1
15 agonist in non-alcohol steatohepatitis (NASH). Specifically, the combination of an ineffective dose of leptin with a moderately effective dose of [¹⁴L]-Exendin-4 (SEQ ID NO:1) leads to marked, synergistic improvements in food intake, body weight or adiposity, liver weight, lipid or plasma total cholesterol, or triglycerides. Accordingly, diseases amenable to such treatment include lipodystrophy, dyslipidemia, hyperlipidemia, overweight, obesity, hypothalamic
20 amenorrhea, Alzheimer's disease, leptin deficiency, fatty liver disease, diabetes (including type I and type II), NASH, nonalcoholic fatty liver disease (NAFLD), metabolic syndrome X, and Huntington's Disease, or combinations thereof.

- [0003]** There remains a need to develop additional polypeptide modalities providing leptin and GLP-1 agonist biological activity for use in the above described metabolic diseases, conditions
25 and disorders. Accordingly, there are provided engineered polypeptides having both leptin and GLP-1 agonist activities to treat the above conditions and methods for producing and using them.

- [0004]** Each patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety and for all purposes.

BRIEF SUMMARY OF THE INVENTION

- 30 **[0005]** In a first aspect, there is provided an engineered polypeptide including a first peptide hormone domain (HD1) which includes an exendin domain sequence, and a second peptide hormone domain (HD2) which includes a humanized chimeric seal leptin sequence. Within the

engineered polypeptide, HD1 is covalently bonded to HD2 through a bond, or through a linker L1, as described herein.

[0006] In another aspect, there is provided a method for treating a disease in a subject including administering an engineered polypeptide as disclosed herein to a subject in need thereof in an amount effective to treat the disease. The disease can be diabetes, overweight, obesity, Alzheimer's disease, short bowel syndrome, fatty liver disease, dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.

[0007] In another aspect, there is provided a pharmaceutical composition including an engineered polypeptide disclosed herein and a pharmaceutically acceptable excipient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] NOT APPLICABLE.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0009] "Obesity" and "overweight" refer to mammals having a weight greater than normally expected, and may be determined by, e.g., physical appearance, body mass index (BMI) as known in the art, waist-to-hip circumference ratios, skinfold thickness, waist circumference, and the like. The Centers for Disease Control and Prevention (CDC) define overweight as an adult human having a BMI of 25 to 29.9; and define obese as an adult human having a BMI of 30 or higher. Additional metrics for the determination of obesity exist. For example, the CDC states that a person with a waist-to-hip ratio greater than 1.0 is overweight.

[0010] "Lean body mass" refers to the fat-free mass of the body, i.e., total body weight minus body fat weight is lean body mass. Lean body mass can be measured by methods such as hydrostatic weighing, computerized chambers, dual-energy X-ray absorptiometry, skin calipers, magnetic resonance imaging (MRI) and bioelectric impedance analysis (BIA) as known in the art.

[0011] "Mammal" refers to warm-blooded animals that generally have fur or hair, that give live birth to their progeny, and that feed their progeny with milk. Mammals include humans; companion animals (e.g., dogs, cats); farm animals (e.g., cows, horses, sheep, pigs, goats); wild animals; and the like. In one embodiment, the mammal is a female. In one embodiment, the mammal is a female human. In one embodiment, the mammal is a male. In one embodiment, the mammal is a male human. In one embodiment, the mammal is a cat or dog. In one embodiment, the mammal is a diabetic mammal, e.g., a human having type 2 diabetes. In one

embodiment, the mammal is an obese diabetic mammal, e.g., an obese mammal having type 2 diabetes. The term “subject” in the context of methods described herein refers to a mammal.

[0012] “Fragment” in the context of polypeptides refers herein in the customary chemical sense to a portion of a polypeptide. For example, a fragment can result from N-terminal deletion or C-terminal deletion of one or more residues of a parent polypeptide, and/or a fragment can result from internal deletion of one or more residues of a parent polypeptide. “Fragment” in the context of an antibody refers to a portion of an antibody which can be linked to a biologically active molecule to modulate solubility, distribution within a subject, and the like. For example, Exendin-4(1-30) describes a biologically active fragment of Exendin-4 where the exendin C-terminal “tail” of amino acids 31-39 is deleted. The term “parent” in the context of polypeptides refers, in the customary sense, to a polypeptide which serves as a reference structure prior to modification, e.g., insertion, deletion, addition and/or substitution. The term “conjugate” in the context of engineered polypeptides described herein refers to covalent linkage between component polypeptides, e.g., HD1, HD2, linkers and the like. The term “fusion” in the context of engineered polypeptides described herein refers to covalent linkage between component polypeptides, e.g., HD1, HD2 and the like, via either or both terminal amino or carboxy functional group of the peptide backbone. Engineered polypeptides can be synthetically or recombinantly made. Typically, fusions are made using recombinant biotechnology, however, can also be made by chemical synthesis and conjugation methods.

[0013] “Analog” as used herein in the context of polypeptides refers to a compound that has insertions, deletions and/or substitutions of amino acids relative to a parent compound. “Analog sequence” as used herein in the context of polypeptides refers to an amino acid sequence that has insertions, deletions and/or substitutions of amino acids relative to a parent amino acid sequence (e.g., wild-type sequence, native sequence). An analog may have superior stability, solubility, efficacy, half-life, and the like. In some embodiments, an analog is a compound having at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or even higher, sequence identity to the parent compound. In a preferred embodiment the analog has from 1 to 5 amino acid modifications selected independently from an insertion, deletion, addition and substitution. In any of the embodiments herein, the exendin analog can have from 1 to 5 amino acid modifications selected independently from any one or combination of an insertion, deletion, addition and substitution, and preferably retains at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or even higher, sequence identity to the parent compound, and even more preferably at least 80%, 85%, 90%, 95%, 98%, or even higher, sequence identity to the parent compound, and preferably the parent compound is Exendin-4,

Exendin-4(1-38), Exendin-4(1-37), Exendin-4(1-36), Exendin-4(1-35), Exendin-4(1-34), Exendin-4(1-33), Exendin-4(1-32), Exendin-4(1-31), Exendin-4(1-30), Exendin-4(1-29) or Exendin-4(1-28), and most preferably the parent compound has the sequence of Exendin-4. In one embodiment at least amino acids corresponding to positions 1, 4, 6, 7 and 9 of Exendin-4 are those as in native Exendin-4, and further the one to five modifications are conservative amino acid substitutions at positions other than positions 1, 4, 6, 7 and 9 of Exendin-4. For example, in yet a further embodiment of the embodiments herein, an exendin analog retains the amino acid at least as found in position 3, 4, 6, 5, 7, 8, 9, 10, 11, 13, 15, 18, 19, 22, 23, 25, 26, and/or 30 of Exendin-4, and further preferably has no more than 1 to 5 of the remaining positions substituted with another amino acid, most preferably a chemically conservative amino acid. In all of the analogs herein, any substitution or modification at positions 1 and/or 2 will retain resistance to DPP-IV cleavage while retaining or improving insulinotropic activity as is known in the art for Exendin-4 analogs, such as desamino-histidyl-Exendin-4. As customary in the art, the term “conservative” in the context of amino acid substitutions refers to substitution which maintains properties of charge type (e.g., anionic, cationic, neutral, polar and the like), hydrophobicity or hydrophilicity, bulk (e.g., van der Waals contacts and the like), and/or functionality (e.g., hydroxy, amine, sulfhydryl and the like). Conversely, the term “non-conservative” refers to an amino acid substitution which is not conservative.

[0014] Unless indicated otherwise, the term “derivative” in the context of a compound disclosed herein refers to a compound afforded by chemical modification, e.g., by the bonding of one or more derivatizing moieties as described herein.

[0015] “Identity,” “sequence identity” and the like in the context of comparing two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 50% identity, preferably 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a sequence comparison algorithms as known in the art, for example BLAST or BLAST 2.0. This definition includes sequences that have deletions and/or additions, as well as those that have substitutions, as well as naturally occurring, e.g., polymorphic or allelic variants, and man-made variants. In preferred algorithms, account is made for gaps and the like, as known in the art. For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are

designated if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, 1981, *Adv. Appl. Math.* **2**:482, by the homology alignment algorithm of Needleman & Wunsch, 1970, *J. Mol. Biol.* **48**:443, by the search for similarity method of Pearson & Lipman, 1988, *Proc. Nat'l. Acad. Sci. USA* **85**:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection. See e.g., *Current Protocols in Molecular Biology* (Ausubel et al., eds. 1995 supplement)). Preferred examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., 1977, *Nuci. Acids Res.* **25**:3389-3402 and Altschul et al., 1990, *J. Mol. Biol.* **215**:403-410. BLAST and BLAST 2.0 are used, as known in the art, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the web site of the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *Id.*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, e.g., for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, 1989,

Proc. Natl. Acad. Sci. USA **89**:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0016] The term “IC₅₀” refers in the customary sense to the half maximal inhibitory concentration of a compound inhibiting a biological or biochemical function. Accordingly, in the context of receptor binding studies, IC₅₀ refers to the concentration of a test compound which competes half of a known ligand from a specified receptor. The term “EC₅₀” refers in the customary sense to the effective concentration of a compound which induces a response halfway between a baseline response and maximum response, as known in the art.

[0017] The term “about” in the context of a numeric value refers to +/- 10% of the numeric value.

[0018] The terms “peptide” and “polypeptide” in the context of components of the engineered polypeptides described herein are synonymous. As customary in the art, a lower case single-letter amino acid abbreviation (e.g., “a”) indicates a D-amino acid (e.g., D-Ala). In the nomenclature of side chain linked peptide compounds, square brackets (“[]”) indicate separate fragments and crosshatch (“#”) indicates linking positions.

II. Compounds

[0019] In a first aspect, there is provided an engineered polypeptide including a first peptide hormone domain (HD1) which includes an exendin domain sequence, and a second peptide hormone domain (HD2) which includes a humanized chimeric seal leptin sequence. Within the engineered polypeptide, HD1 is covalently bonded to HD2 through a bond, or through a linker L1, as described herein. The term “engineered polypeptide” refers, in the customary sense, to polypeptides and derivatives thereof which are not naturally occurring polypeptides, and/or which have been synthesized by chemical or biological processes, or combinations thereof. The term “exendin domain” refers to a polypeptide having the sequence of an exendin, an exendin analog, an exendin active fragment, or an exendin analog active fragment, and derivatives thereof, as disclosed herein. Thus, the term exendin domain expressly refers to an exendin, an exendin analog, an exendin active fragment, or an exendin analog active fragment. Unless indicated otherwise, the term “sequence” in the context of a polypeptide refers to the amino acid sequence of the polypeptide. Thus, the term “exendin domain sequence” refers to the amino acid sequence of an exendin domain, and the term “humanized chimeric seal leptin sequence” refers to the amino acid sequence of a humanized chimeric seal leptin. The term “active fragment” refers, in the customary sense, to a fragment of a parent polypeptide, which fragment demonstrates biological activity, e.g., binding, agonism or antagonism, in a biological assay.

The terms “biologically active compound” and the like refer, in the customary sense, to compounds, e.g., polypeptides, non-polypeptides, and the like, which can provide biological activity. The terms “humanized chimeric seal leptin” and the like refer to polypeptides having significant sequence identity, e.g., 50% to 95% sequence identity, to a seal leptin, a seal leptin analog, an active fragment of a seal leptin, an active fragment of a seal leptin analog, and derivatives thereof, wherein at least one region of contiguous amino acids of a parent seal leptin has been replaced by a corresponding region from human leptin. Thus, the term humanized chimeric seal leptin expressly refers to a seal leptin, a seal leptin analog, a seal leptin active fragment, or a seal leptin analog active fragment, wherein at least one region of contiguous amino acids of a parent seal leptin has been replaced by a corresponding region from human leptin. The terms “corresponding region” and the like in the context of the comparison of polypeptides refer, in the customary sense, to an alignment of polypeptides, as known in the art. It is understood that the absolute numbering of corresponding amino acids can differ between compared polypeptides. A “linker” as used herein refers to a divalent chemical moiety which can covalently bond to both HD1 and HD2. Thus, the term “peptide linker” refers to a divalent peptide which bonds together two chemical entities, e.g., HD1 and HD2.

[0020] In one embodiment, the engineered polypeptide has greater binding at a human leptin receptor relative to seal leptin binding.

[0021] In one embodiment, the engineered polypeptide has greater solubility relative to human leptin solubility.

[0022] In one embodiment, linker L1 is a bond. In one embodiment, linker L1 is a linker as described herein which covalently links HD1 and HD2.

[0023] In one embodiment, the engineered polypeptide includes HD1 as an N-terminal moiety and HD2 as a C-terminal moiety. The term “N-terminal moiety” refers, in the customary sense, to the relative positioning of a region, e.g., HD1, of a polypeptide, e.g., an engineered polypeptide disclosed herein, toward the N-terminal of the polypeptide. Conversely, the term “C-terminal moiety” refers, in the customary sense, to the relative positioning of a region of a polypeptide toward the C-terminal of the polypeptide. Accordingly, in one embodiment, the engineered polypeptide has the structure HD1-HD2. In one embodiment, the engineered polypeptide has the structure HD1-L1-HD2.

[0024] It is understood that absent an express indication of the N-terminus and/or C-terminus of an engineered polypeptide set forth herein, the engineered polypeptide is to be read in the N-terminus to C-terminus orientation. For example, where HD1 and HD2 are as defined herein, the

terms HD1-HD2, HD1-L1-HD2, and the like mean, in the absence of an express indication of the N-terminus and/or the C-terminus, that the HD1 resides at the N-terminus of the engineered polypeptide, and the HD2 resides at the C-terminus. Conversely, if the N-terminus and/or C-terminus is expressly indicated, then the engineered polypeptide is to be read according to the express indication of the termini. For example, the terms HD1_{C-term}-HD2, HD1-L1-HD2_{N-term} and the like mean that HD2 resides at the N-terminus of the engineered polypeptide, and HD1 resides at the C-terminus.

[0025] In one embodiment, the HD1 sequence includes an exendin domain sequence, as disclosed herein. In one embodiment, the HD1 sequence consists of an exendin domain sequence, as disclosed herein.

[0026] Polypeptide components contemplated for use in the compounds and methods described herein, e.g., HD1 and HD2, include exendin domains and humanized chimeric seal leptins. The structure and function of exendins and leptins is described following.

[0027] Exendins. The exendins are peptides that are found in the salivary secretions of the Gila monster and the Mexican Bearded Lizard, which are reptiles endogenous to Arizona and Northern Mexico. Exendin-3 is present in the salivary secretions of *Heloderma horridum* (Mexican Beaded Lizard), and Exendin-4 is present in the salivary secretions of *Heloderma suspectum* (Gila monster). See Eng et al, 1990, *J. Biol. Chem.*, **265**:20259-62; Eng et al, 1992, *J. Biol. Chem.*, **267**:7402-7405.

[0028] The sequences of Exendin-3 and Exendin-4, respectively, follow:
HSDGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH₂ (SEQ ID NO:2);
HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH₂ (SEQ ID NO: 3).

[0029] An Exendin-4 peptide analog has been reported that is a full-length C-terminally amidated Exendin-4 peptide analog with a single nucleotide difference at position 14 compared to native Exendin-4. See e.g., Hargrove *et al.*, 2007, *Regulatory Peptides*, **141**:113-119. The sequence of [¹⁴Leu]Exendin-4 is HGEGTFTSDLSKQLEEEAVRLFIEWLKNGGPSSGAPPPS-NH₂ (SEQ ID NO:1).

[0030] Another Exendin-4 peptide analog is a chimera of the first 32 amino acids of Exendin-4 having amino acid substitutions at positions 14 and 28 followed by a 5 amino acid sequence from the C-terminus of a non-mammalian (frog) GLP1: [¹⁴Leu, ²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37) with sequence: HGEGTFTSDLSKQLEEEAVRLFIEWLKQGGPSKEIIS (SEQ ID NO:4).

[0031] Also known in the art are C-terminally truncated, biologically active forms of Exendin-4, such as Exendin-4(1-28) (SEQ ID NO:5), Exendin-4(1-29) (SEQ ID NO:6), Exendin-4(1-30) (SEQ ID NO:7), Exendin-4(1-31) (SEQ ID NO:8), Exendin-4(1-32) (SEQ ID NO:9) and their amidated forms. These exendin analogs are suitable as exendin domains of the engineered polypeptides disclosed herein.

[0032] As is customary in the art, square brackets (i.e., "[]") in a peptidic compound name indicate substitution of the residue or chemical feature within the square brackets. For example, [¹⁴Leu]Exendin-4, [¹⁴Leu]Ex-4, and the like refer to Exendin-4 having leucine at position 14. The numeric position of an amino acid can be indicated by prepended or postpended numbers in a variety of ways routinely employed in the art. For example, the terms ¹⁴Leu, Leu14, 14Leu, Leu¹⁴ and the like, are synonymous in referring to a leucine at position 14.

[0033] It is understood that in some embodiments a C-terminal amide, or other C-terminal capping moiety can be present in compounds described herein. Unless indicated otherwise, each polypeptide disclosed herein having a C-terminal is understood to represent both C-terminal acid and C-terminal amide forms of the polypeptide, unless such C-terminal capping would prevent formation of an engineered polypeptide disclosed herein. Accordingly, HD1 would not be C-terminally capped if HD2 or a linker to HD2 is attached at the C-terminal of HD1.

[0034] The exendins have some sequence similarity to several members of the glucagon-like peptide (GLP-1) family, the highest homology (53%) being to GLP-1(7-36)NH₂. See e.g., Goke et al, 1993, *J. Biol. Chem.*, **268**:19650-55. The sequence of GLP-1(7-37)NH₂ (also sometimes referred to as "GLP-1") is HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG (SEQ ID NO:10). This peptide has an insulinotropic effect stimulating insulin secretion from pancreatic beta-cells. It is understood, however, that exendins are not GLP-1 homologs. For example, pharmacological studies have led to reports that Exendin-4 can act at GLP-1 receptors *in vitro* on certain insulin-secreting cells, however, it has also been reported that Exendin-4 may act at receptors not acted upon by GLP-1. Moreover, Exendin-4 shares some but not all biological properties *in vivo* with GLP-1, and it has a significantly longer duration of action than GLP-1. Based on their insulinotropic activities, the use of Exendin-3 and Exendin-4 for the treatment of diabetes mellitus and the prevention of hyperglycemia has been proposed (Eng, U.S. Pat. No. 5,424,286, incorporated herein by reference in its entirety and for all purposes), and indeed, Exendin-4 has been approved in the United States and in Europe for use as a therapeutic for treating type 2 diabetes.

[0035] Thus, without wishing to be bound by any theory, it is believed that exendins are not the species homolog of mammalian GLP-1 as was reported by Chen and Drucker who cloned the exendin gene from the Gila monster. See e.g., *J. Biol. Chem.* 1997, **272**:4108-15. Moreover, the observation that the Gila monster also has separate genes for proglucagons (from which GLP-1 is processed), that are more similar to mammalian proglucagon than exendin, indicated that exendins are not merely species homologs of GLP-1.

[0036] Methods for regulating gastrointestinal motility using exendin agonists are described in U.S. Pat. No. 6,858,576 (i.e., based on U.S. application Ser. No. 08/908,867 filed Aug. 8, 1997, which is a continuation-in-part of U.S. application Ser. No. 08/694,954 filed Aug. 8, 1996).

Methods for reducing food intake using exendin agonists are described in U.S. Pat. No. 6,956,026 (i.e., based on U.S. application Ser. No. 09/003,869, filed Jan. 7, 1998, which claims the benefit of U.S. Application Nos. 60/034,905 filed Jan. 7, 1997, 60/055,404 filed Aug. 7, 1997, 60/065,442 filed Nov. 14, 1997, and 60/066,029 filed Nov. 14, 1997).

[0037] Novel exendin agonist compound sequences useful in the engineered polypeptides described herein are described in WO 99/07404 (i.e., PCT/US98/16387 filed Aug. 6, 1998), in WO 99/25727 (i.e., PCT/US98/24210, filed Nov. 13, 1998), in WO 99/25728 (i.e., PCT/US98/24273, filed Nov. 13, 1998), in WO 99/40788, in WO 00/41546, and in WO 00/41548, which are incorporated herein by reference and for all purposes along with their granted U.S. patent counterparts. Methods to assay for exendin activities in vitro and in vivo, as known in the art, including insulinotropic, food intake inhibition activity and weight loss activity, are described herein and also in the above references and other references recited herein.

[0038] Certain exemplary exendins, exendin agonists, and exendin analog agonists include exendin fragments: Exendin-4(1-28) (SEQ ID NO:5), Exendin-4(1-29) (SEQ ID NO:6), Exendin-4(1-30) (SEQ ID NO:7), Exendin-4(1-31) (SEQ ID NO:8) and Exendin-4(1-32) (SEQ ID NO:9). Analogs thereof include substitution at the ¹⁴Met position (i.e., ¹⁴Met) with a non-oxidizing amino acid, e.g., leucine. Examples include [¹⁴Leu]Exendin-4(1-28) (SEQ ID NO:11), [¹⁴Leu]Exendin-4(1-29) (SEQ ID NO:12), [¹⁴Leu]Exendin-4(1-30) (SEQ ID NO:13), [¹⁴Leu]Exendin-4(1-31) (SEQ ID NO:14) and [¹⁴Leu]Exendin-4(1-32) (SEQ ID NO:15).

[0039] Exendin analog agonists for use as exendin domains in the engineered polypeptides described herein include those described in US Patent No. 7,223,725 (incorporated herein by reference and for all purposes), such as compounds of the formula: Xaa₁ Xaa₂ Xaa₃ Gly Xaa₅ Xaa₆ Xaa₇ Xaa₈ Xaa₉ Xaa₁₀ Xaa₁₁ Xaa₁₂ Xaa₁₃ Xaa₁₄ Xaa₁₅ Xaa₁₆ Xaa₁₇ Ala Xaa₁₉ Xaa₂₀ Xaa₂₁ Xaa₂₂ Xaa₂₃ Xaa₂₄ Xaa₂₅ Xaa₂₆ Xaa₂₇ Xaa₂₈-Z₁; wherein Xaa₁ is His, Arg or Tyr; Xaa₂ is Ser,

- Gly, Ala or Thr; Xaa₃ is Ala, Asp or Glu; Xaa₅ is Ala or Thr; Xaa₆ is Ala, Phe, Tyr; Xaa₇ is Thr or Ser; Xaa₈ is Ala, Ser or Thr; Xaa₉ is Asp or Glu; Xaa₁₀ is Ala, Leu, Ile, Val, or Met; Xaa₁₁ is Ala or Ser; Xaa₁₂ is Ala or Lys; Xaa₁₃ is Ala or Gln; Xaa₁₄ is Ala, Leu, Ile, Val or Met; Xaa₁₅ is Ala or Glu; Xaa₁₆ is Ala or Glu; Xaa₁₇ is Ala or Glu; Xaa₁₉ is Ala or Val; Xaa₂₀ is Ala or Arg;
- 5 Xaa₂₁ is Ala or Leu; Xaa₂₂ is Ala, Phe, Tyr; Xaa₂₃ is Ile, Val, Leu, or Met; Xaa₂₄ is Ala, Glu or Asp; Xaa₂₅ is Ala, Trp, Phe, Tyr; Xaa₂₆ is Ala or Leu; Xaa₂₇ is Ala or Lys; Xaa₂₈ is Ala or Asn. Z₁ is absent, Gly, Gly-Gly, Gly-Gly-Xaa₃₁, Gly-Gly-Xaa₃₁-Ser, Gly-Gly-Xaa₃₁-Ser-Ser (SEQ ID NO:391), Gly-Gly-Xaa₃₁-Ser-Ser-Gly (SEQ ID NO:392), Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala (SEQ ID NO:393), Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala-Xaa₃₆ (SEQ ID NO:394),
- 10 Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala-Xaa₃₆-Xaa₃₇ (SEQ ID NO:395), or Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala-Xaa₃₆-Xaa₃₇-Xaa₃₈ (SEQ ID NO:396); wherein Xaa₃₁, Xaa₃₆, Xaa₃₇ and Xaa₃₈ are independently Pro or are absent. It is expressly contemplated that each exendin analog agonist can be a C-terminal acid or C-terminal amine. In any and each of the exendin analogs described above, also specifically contemplated are those wherein a replacement
- 15 for the histidine corresponding to Xaa₁ is made with any of D-histidine, desamino-histidine, 2-amino-histidine, beta-hydroxy-histidine, homohistidine, N-alpha-acetyl-histidine, alpha-fluoromethyl-histidine, alpha-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine, 4-pyridylalanine, 4-imidazoacetyl, des-amino-histidyl (imidazopropionyl), beta-hydroxy-imidazopropionyl, N-dimethyl-histidyl or beta-carboxy-imidazopropionyl. Further specifically
- 20 contemplated herein are exendin analogs described herein wherein a replacement for the glycine at Xaa₂ is made with any of D-Ala, Val, Leu, Lys, Aib, (1-amino cyclopropyl) carboxylic acid, (1-aminocyclobutyl) carboxylic acid, l-aminocyclopentyl)carboxylic acid, (1-aminocyclohexyl)carboxylic acid, (1-aminocycloheptyl)carboxylic acid, or (1-amino cyclooctyl)carboxylic acid.
- 25 **[0040]** According to one embodiment, exemplary compounds include those of the above formula wherein: Xaa₁ is His or Arg; Xaa₂ is Gly or Ala; Xaa₃ is Asp or Glu; Xaa₅ is Ala or Thr; Xaa₆ is Ala or Phe; Xaa₇ is Thr or Ser; Xaa₈ is Ala, Ser or Thr; Xaa₉ is Asp or Glu; Xaa₁₀ is Ala, or Leu; Xaa₁₁ is Ala or Ser; Xaa₁₂ is Ala or Lys; Xaa₁₃ is Ala or Gln; Xaa₁₄ is Ala or Leu; Xaa₁₅ is Ala or Glu; Xaa₁₆ is Ala or Glu; Xaa₁₇ is Ala or Glu; Xaa₁₉ is Ala or Val; Xaa₂₀ is Ala or Arg;
- 30 Xaa₂₁ is Ala or Leu; Xaa₂₂ is Phe; Xaa₂₃ is Ile, Val; Xaa₂₄ is Ala, Glu or Asp; Xaa₂₅ is Ala, Trp or Phe; Xaa₂₆ is Ala or Leu; Xaa₂₇ is Ala or Lys; Xaa₂₈ is Ala or Asn; Z₁ is absent, Gly, Gly-Gly, Gly-Gly-Xaa₃₁, Gly-Gly-Xaa₃₁-Ser, Gly-Gly-Xaa₃₁-Ser-Ser, Gly-Gly-Xaa₃₁-Ser-Ser-Gly, Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala, Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala-Xaa₃₆, Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala-Xaa₃₆-Xaa₃₇, Gly-Gly-Xaa₃₁-Ser-Ser-Gly-Ala-

Xaa₃₆-Xaa₃₇-Xaa₃₈; wherein Xaa₃₁, Xaa₃₆, Xaa₃₇ and Xaa₃₈ being independently Pro or is absent, provided that no more than three of Xaa₃, Xaa₅, Xaa₆, Xaa₈, Xaa₁₀, Xaa₁₁, Xaa₁₂, Xaa₁₃, Xaa₁₄, Xaa₁₅, Xaa₁₆, Xaa₁₇, Xaa₁₉, Xaa₂₀, Xaa₂₁, Xaa₂₄, Xaa₂₅, Xaa₂₆, Xaa₂₇ and Xaa₂₈ are Ala. It is expressly contemplated that each exendin analog agonist can be a C-terminal acid or C-terminal amine. In any and each of the exendin analogs described above, also specifically contemplated are those wherein a replacement for the histidine corresponding to position Xaa1 is made with any of D-histidine, desamino-histidine, 2-amino-histidine, beta-hydroxy-histidine, homohistidine, N-alpha-acetyl-histidine, alpha-fluoromethyl-histidine, alpha-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine, 4-pyridylalanine, 4-imidazoacetyl, des-amino-histidyl (imidazopropionyl), beta-hydroxy-imidazopropionyl, N-dimethyl-histidyl or beta-carboxy-imidazopropionyl. Further specifically contemplated herein are exendin analogs described herein wherein a replacement for the glycine at Xaa 2 is made with any of D-Ala, Val, Leu, Lys, Aib, (1-aminocyclopropyl) carboxylic acid, (1-amino cyclobutyl)carboxylic acid, 1-aminocyclopentyl)carboxylic acid, (1-aminocyclohexyl) carboxylic acid, (1-amino cycloheptyl)carboxylic acid, or (1-aminocyclooctyl)carboxylic acid.

[0041] Other exemplary compounds include those set forth in WO 99/25727 identified therein as compounds 2-23. According to another embodiment, provided are compounds where Xaa₁₄ is Leu, Ile, or Val, more preferably Leu, and/or Xaa₂₅ is Trp, Phe or Tyr, more preferably Trp or Phe. It is believed that these compounds will be less susceptible to oxidative degradation, both in vitro and in vivo, as well as during synthesis of the compound.

[0042] Additional examples of exendin analogs suitable as exendin domains for use in the present engineered polypeptides include those described in United States Patent 6528486 published March 4, 2003 (incorporated herein by reference and for all purposes). Specifically, exendin analogs include those consisting of an exendin or exendin analog having at least 90% homology to Exendin-4 having optionally between one and five deletions at positions 34-39, and a C-terminal extension of a peptide sequence of 4-20 amino acid units covalently bound to said exendin wherein each amino acid unit in said peptide extension sequence is selected from the group consisting of Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, and Met. More preferably the extension is a peptide sequence of 4-20 amino acid residues, e.g., in the range of 4-15, more preferably in the range of 4-10 in particular in the range of 4-7 amino acid residues, e.g., of 4, 5, 6, 7, 8 or 10 amino acid residues, where 6 amino acid residues are preferred. Most preferably, according to U.S. Patent 6528486, the extension peptide contains at least one Lys residue, and is even more preferably from 3 to 7 lysines and even most preferably 6 lysines.

[0043] Exemplary exendin analogs useful as exendin domains include:

HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPSKKKKKKK [des-³⁶Pro]Exendin-4(1-39)-Lys₆) (SEQ ID NO:16);

KKKKKKKHGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAPPSKKKKKKK (H-Lys₆-[des
5 ³⁶Pro]Exendin-4(1-39)-Lys₆) (SEQ ID NO:17);

HGEGTFTSDLSKQMEEEEAVRLFIEWLWLKNGGPSSGAS (H-[des ³⁶Pro, ^{37,38}Pro]Exendin-4(1-39)-NH₂) (SEQ ID NO:18);

KKKKKKKHGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAS (H-(Lys)₆-[des ³⁶Pro,
^{37,38}Pro]Exendin-4(1-39) (SEQ ID NO:19);

10 NEEEEHHGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGAS (H-Asn-(Glu)₅-[des ³⁶Pro,
^{37,38}Pro]Exendin-4(1-39) (SEQ ID NO:20);

HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGASKKKKKK ([des ³⁶Pro,
^{37,38}Pro]Exendin-4(1-39)-(Lys)₆) (SEQ ID NO:21);

KKKKKKKHGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGASKKKKKK (H-(Lys)₆-[des
15 ³⁶Pro, ^{37,38}Pro]Exendin-4(1-39)-(Lys)₆) (SEQ ID NO:22); and

DEEEEEHHGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSSGASKKKKKK (Asn-(Glu)₅-[des
³⁶Pro, ^{37,38}Pro]Exendin-4(1-39)-(Lys)₆) (SEQ ID NO:23).

[0044] As customary in the art, repetition of an amino acid can be indicated by a subscripted number setting forth the number of repetitions; i.e., Lys₆, (Lys)₆ and the like refer to hexalysyl
20 (SEQ ID NO:24). As will be clear to the skilled artisan, in some contexts a subscripted number

can also indicate the position of a residue within a sequence; e.g., “AA₁ AA₂ AA₃” refers to amino acids 1-3 of a polypeptide sequence. In any and each of the exendin analogs described above, specifically contemplated are those wherein a replacement for the histidine corresponding to position 1 is made with any of D-histidine, desamino-histidine, 2-amino-histidine, beta-
25 hydroxy-histidine, homohistidine. N-alpha-acetyl-histidine, alpha-fluoromethyl-histidine, alpha-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine, 4-pyridylalanine, 4-imidazoacetyl, des-

amino-histidyl (or imidazopropionyl), beta-hydroxy-imidazopropionyl, N-dimethyl-histidyl or beta-carboxy-imidazopropionyl. Further specifically contemplated herein are exendin analogs described herein wherein a replacement for the glycine at position 2 is made with any of D-Ala,
30 Val, Leu, Lys, Aib, (1-aminocyclopropyl)carboxylic acid, (1-aminocyclobutyl)carboxylic acid, 1-aminocyclopentyl)carboxylic acid, (1-amino cyclohexyl)carboxylic acid, (1-aminocycloheptyl) carboxylic acid, or (1-aminocyclooctyl) carboxylic acid.

[0045] Further examples of exendin analogs suitable as exendin domains for use in the engineered polypeptide constructs are those described in published PCT application

WO2004035623 (incorporated herein by reference and for all purposes), particularly those comprised of naturally-occurring amino acids, which describes exendin analogs having at least one modified amino acid residue particularly at positions ¹³Gln, ¹⁴Met, ²⁵Trp or ²⁸Asn with reference to the corresponding positions of Exendin-4(1-39). Additional such analogs further
5 include a 1-7 amino acid C-terminal extension that includes at least one Lys amino acid and more preferably at least five Lys amino acid units such as six or seven Lys amino acid units.

[0046] Yet further examples of exendin analogs suitable as exendin domains for use in the engineered polypeptide constructs are those described in published PCT application WO/2010/120476, (incorporated herein by reference and for all purposes), which describes
10 exendin analogs having modified amino acid residues in the N-terminal portion of an exendin or exendin analog to create a high beta-turn characteristic in that region. For example, analogs are designed to mimic amino acid residues His₁ Gly₂ Glu₃ by creating a conformationally constrained region, include exendin analogs containing a thiazolidine-proline peptide mimetic at His₁ Gly₂ Glu₃, which can be used as a modification in Exendin-4 or other analogs described
15 herein.

[0047] In any and each of the exendins, exendin analogs and formulas described herein, specifically contemplated are those wherein a replacement for the histidine corresponding to position 1 is made with any of L-histidine, D-histidine, desamino-histidine, 2-amino-histidine, beta-hydroxy-histidine, homohistidine, N-alpha-acetyl-histidine, alpha-fluoromethyl-histidine,
20 alpha-methyl-histidine, 3-pyridylalanine, 2-pyridylalanine, 4-pyridylalanine, 4-imidazoacetyl, des-amino-histidyl (imidazopropionyl), beta-hydroxy-imidazopropionyl, N-dimethyl-histidyl or beta-carboxy-imidazopropionyl. For example, preferred exendin analogs for use in engineered polypeptide conjugates as described herein wherein the His1 position is modified are (4-imidazoacetyl) Exendin-4, (des-amino-histidyl) Exendin-4 (or (imidazopropionyl) Exendin-
25 4), (beta-hydroxy-imidazopropionyl) Exendin-4, (N-dimethyl-histidyl) Exendin-4 and (beta-carboxy-imidazopropionyl) Exendin-4. Further specifically contemplated herein are exendins or exendin analogs described herein wherein a replacement for the glycine at position 2 is made with any of D-Ala, Val, Leu, Lys, Aib, (1-aminocyclopropyl)carboxylic acid, (1-aminocyclobutyl)carboxylic acid, 1-aminocyclopentyl)carboxylic acid, (1-amino
30 cyclohexyl)carboxylic acid, (1-aminocycloheptyl)carboxylic acid, or (1-aminocyclooctyl) carboxylic acid.

[0048] Any of the above exendin analogs or their active fragments are suitable for use as exendin domains in the present engineered polypeptides, with or without a linker to HD2.

[0049] In some embodiments of any of the engineered polypeptides described herein, an exendin domain can have at least 65%, for example 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or even higher, sequence identity relative to a parent exendin sequence. In one embodiment, the parent exendin is Exendin-4, and the exendin analog may have at least 65%, for example 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or even higher, sequence identity relative to Exendin-4. Accordingly, in one embodiment, HD1 includes an exendin domain sequence having at least 65% identity with an Exendin-4 sequence (SEQ ID NO:3). In one embodiment, HD1 consists of an exendin domain sequence having at least 65% identity with an Exendin-4 sequence (SEQ ID NO:3).

[0050] In one embodiment, the exendin domain sequence is an Exendin-4 sequence (SEQ ID NO:3).

[0051] In one embodiment, the exendin domain sequence is a [¹⁴Leu]Exendin-4 sequence (SEQ ID NO:1).

[0052] In one embodiment, the exendin domain sequence has at least 90% identity with an Exendin-4(1-32) sequence (SEQ ID NO:15).

[0053] In one embodiment, the exendin domain sequence is a [¹⁴Leu]Exendin-4(1-32) sequence (SEQ ID NO:15).

[0054] In one embodiment, the exendin domain sequence is the sequence of Exendin-4(1-28) (SEQ ID NO:5), Exendin-4(1-29) (SEQ ID NO:6), Exendin-4(1-30) (SEQ ID NO:7), Exendin-4(1-31) (SEQ ID NO:8) or Exendin-4(1-32) (SEQ ID NO:9).

[0055] In one embodiment, the exendin domain sequence includes a sequence following: Exendin-4 (SEQ ID NO:3), [¹⁴Leu]Exendin-4 (SEQ ID NO:1), [¹⁴Leu, ²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37) (SEQ ID NO:4), [²⁸Gln]Exendin-4(1-32) (SEQ ID NO:25), [¹⁴Leu, ²⁸Gln]Exendin-4(1-32) (SEQ ID NO:26), [des-³⁶Pro]Exendin-4 (SEQ ID NO:27), [¹⁴Leu, ²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37)-Lys₆ (SEQ ID NO:4), Exendin-4-Lys₆ (SEQ ID NO:28), [²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37)-Lys₆ (SEQ ID NO:29), [¹⁴Leu, des-³⁸Pro]Exendin-4-Lys₆ (SEQ ID NO:30), or [des-³⁸Pro]Exendin-4-Lys₆ (SEQ ID NO:31).

[0056] In one embodiment, the exendin domain sequence has at least 70% identity with an Exendin-4 sequence (SEQ ID NO:3) or to a sequence selected from the group consisting of any of sequences Exendin-4 (SEQ ID NO:3), [¹⁴Leu]Exendin-4 (SEQ ID NO:1), [¹⁴Leu, ²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37) (SEQ ID NO:4), [²⁸Gln]Exendin-4(1-32) (SEQ ID NO:25), [¹⁴Leu, ²⁸Gln]Exendin-4(1-32) (SEQ ID NO:26), [des-³⁶Pro]Exendin-4 (SEQ ID

NO:27), [¹⁴Leu,²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37)-Lys₆ (SEQ ID NO:4), Exendin-4-Lys₆ (SEQ ID NO:28), [²⁸Gln]Exendin-4(1-32)-fGLP-1(33-37)-Lys₆ (SEQ ID NO:29), [¹⁴Leu,des-³⁸Pro]Exendin-4-Lys₆ (SEQ ID NO:30), or [des-³⁸Pro]Exendin-4-Lys₆ (SEQ ID NO:31).

[0057] In one embodiment, the exendin domain sequence includes a sequence following:

- 5 HEGTFTSDLSKQMEEEAVRLFIEWLKNNGPSSGAPPPS (SEQ ID NO:3),
 HEGTFTSDLSKQLEEEAVRLFIEWLKNNGPSSGAPPPS (SEQ ID NO:1),
 HEGTFTSDLSKQLEEEAVRLFIEWLKQGGPSKEIIS (SEQ ID NO:4),
 HEGTFTSDLSKQMEEEAVRLFIEWLKQGGPSKEIIS (SEQ ID NO:32),
 HEGTFTSDLSKQLEEEAVRLFIEWLKNNGPSSGAPPS (SEQ ID NO:33),
 10 HEGTFTSDLSKQMEEEAVRLFIEWLKNNGPSSGAPPS (SEQ ID NO:34),
 HEGTFTSDLSKQLEEEAVRLFIEWLKQGGPSKEIISKKKKKK (SEQ ID NO:35),
 HEGTFTSDLSKQMEEEAVRLFIEWLKNNGPSSGAPPPSKKKKKK (SEQ ID NO:36),
 HEGTFTSDLSKQMEEEAVRLFIEWLKQGGPSKEIISKKKKKK (SEQ ID NO:37),
 HEGTFTSDLSKQLEEEAVRLFIEWLKNNGPSSGAPPSKKKKKK (SEQ ID NO:38),
 15 HEGTFTSDLSKQMEEEAVRLFIEWLKNNGPSSGAPPSKKKKKK (SEQ ID NO:39).

[0058] In one embodiment, the exendin domain sequence consists of a sequence selected from (SEQ ID NO:3), (SEQ ID NO:1), (SEQ ID NO:4), and SEQ ID NOS:32-39.

- [0059]** In one embodiment, the exendin domain sequence has at least 70% identity with a sequence selected from (SEQ ID NO:3), (SEQ ID NO:1), (SEQ ID NO:4), and SEQ ID NOS:32-
 20 39.

[0060] In one embodiment, the exendin domain sequence includes from 1 to 5 amino acid modifications relative to an Exendin-4 sequence (SEQ ID NO:3), which 1 to 5 amino acid modifications are each independently selected from an insertion, deletion, addition or substitution.

- 25 **[0061]** In one embodiment, the exendin domain sequence includes from 1 to 5 amino acid modifications relative to an Exendin-4(1-32) sequence (SEQ ID NO:9), which 1 to 5 amino acid modifications are each independently selected from an insertion, deletion, addition or substitution.

- [0062]** In one embodiment, the exendin domain sequence includes from 1 to 5 amino acid
 30 modifications relative to an Exendin-4(1-28) sequence (SEQ ID NO:5), which 1 to 5 amino acid modifications are each independently selected from an insertion, deletion, addition or substitution.

[0063] Leptins. The term “leptin sequence” and “a leptin sequence” as used herein refer to leptins, leptin active fragments, leptin analogs, and leptin derivatives; and a leptin, a leptin active fragment, a leptin analog, and a leptin derivative; respectfully. Exemplary leptins include those which elicit one or more biological responses known in the art to be elicited when leptins are administered to subjects. *See*, e.g., published U.S. Patent application Nos. US 2007/0020284 and US 2008/0207512, U.S. Patent Nos. 6,309,853, and US 7,183,254, and PCT Published Application Nos. WO 96/005309, WO 98/28427, and WO 2009/064298), relating to a variety of biological responses including reduction of food intake, reduction of body weight, reduction of body weight gain, induction of satiety, reduction of caloric availability, reduction of caloric efficiency, reduction of metabolic plateau, increase in insulin sensitivity, reduction of hyperlipidemia, correction of dyslipidemia, reduction of hypertriglyceridemia, amelioration of obesity, amelioration of overweight, amelioration of diabetes mellitus (including type I diabetes, type II diabetes, and gestational diabetes), amelioration of insulin resistance, amelioration of lipodystrophy conditions associated therewith, as well as other biological responses known in the art to be elicited upon administration of a leptin.

[0064] Exemplary leptins include the compounds described in U.S. Patent Nos. US 5,594,101, US 5,851,995, US 5,691,309, US 5,580,954, US 5,554,727, US 5,552,523, US 5,559,208, US 5,756,461, US 6,309,853, published U.S. Patent application No. US 2007/0020284, and PCT Published Application Nos. WO 96/23517, WO 96/005309, WO 98/28427, WO 2004/039832, WO 98/55139, WO 98/12224, and WO 97/02004, each of which is incorporated herein in its entirety and for all purposes.

[0065] The term “leptin activity” includes leptin binding activity and leptin functional activity. The skilled artisan will recognize leptin analog compounds with leptin activity using suitable assays for measuring leptin binding or leptin functional activity. Leptin analog compounds can have an IC_{50} of about 200 nM or less, about 100 nM or less, or about 50 nM or less, or about 5 nM or less, or about 1 nM or less, in a leptin binding assay, such as that described herein. Leptin analog compounds can have an EC_{50} of about 20 nM or less, about 10 nM or less, about 5 nM or less, about 1 nM or less, or about 0.1 nM or less, in a leptin functional assay, such as that described herein.

[0066] Humanized Chimeric Seal Leptins, Fragments and Analogs Thereof. In one aspect of the present disclosure, a series of chimeric polypeptides are described. The terms “chimeric polypeptide” and “humanized chimeric seal leptin” are synonymous in the context of engineered polypeptides disclosed herein. These chimeric polypeptides are based on a wild type seal leptin polypeptide wherein at least one contiguous region of 1-30 amino acids of a wild type

seal leptin sequence has been replaced with a contiguous region of 1-30 amino acids of a mature human leptin sequence. A wild type seal leptin sequence includes the sequence of wild type seal leptin (SEQ ID NO:40) and the sequence of wild type seal leptin with an N-terminal methionine (SEQ ID NO:42). Accordingly, it is understood that SEQ ID NO:40 and SEQ ID NO:42 are seal
 5 leptins, SEQ ID NO:43 and SEQ ID NO:44 are human leptins, and the like.

[0067] A mature human leptin sequence, useful for chimerizing wild type seal leptin as provided herein, includes the following sequences: mature human leptins (SEQ ID NO:45), mature human leptins with N-terminal methionine (SEQ ID NO:46), mature human leptin form 1 (SEQ ID NO:43), mature human leptin form 2 (SEQ ID NO:47), mature human leptin form 3
 10 (SEQ ID NO:48), mature human leptin form 4 (SEQ ID NO:49), mature human leptin form 1 with N-terminal methionine (Metreleptin, or A100, SEQ ID NO:44), mature human leptin form 2 with N-terminal methionine (SEQ ID NO:50), mature human leptin form 3 with N-terminal methionine (SEQ ID NO:51), mature human leptin form 4 with N-terminal methionine (SEQ ID NO:52), A200 (SEQ ID NO:53), A300 (SEQ ID NO:54), A400 (SEQ ID NO:55), A500 (SEQ ID
 15 NO:56), and A100 variants (SEQ ID NOS:57-70). In some embodiments, a series of chimeric polypeptides are described wherein at least one contiguous region of 1-30 amino acids of a wild type seal leptin sequence (SEQ ID NO:40 or SEQ ID NO:42) has been replaced with a contiguous region of 1-30 amino acids of A100 (SEQ ID NO:44).

[0068] In one embodiment, the engineered polypeptide includes a humanized chimeric seal
 20 leptin sequence having the sequence of SEQ ID NO:40, wherein 5% to 55% of SEQ ID NO:40 is substituted with a corresponding human leptin sequence.

[0069] In one embodiment, the engineered polypeptide includes a humanized chimeric seal leptin sequence having the sequence of SEQ ID NO:41, wherein 5% to 55% of SEQ ID NO:41 is substituted with a corresponding human leptin sequence.

[0070] In any of the disclosed chimeric polypeptides, a contiguous region, e.g., 1-30, 2-30, 3-30, 4-30, 5-30, 6-30, 7-30, 8-30, 9-30, 10-30, of amino acids can include any naturally or non-naturally occurring amino acid. Any combination of amino acids can be employed without restriction. That is, two or more amino acids in a contiguous region can be replaced with a naturally occurring amino acid, a non-naturally occurring amino acid, a conservative
 30 substitution, a non-conservative substitution or any combination thereof. The term “conservative substitution” refers, as customary in the art, to an amino acid substitution which retains charge type and/or size, as known in the art. Conversely, the term “non-conservative substitution”

refers, as customary in the art, to an amino acid substitution which changes charge type and/or size, as known in the art.

[0071] The chimeric polypeptides described herein have demonstrated biological activity, in addition to enhanced physical properties. For example, a humanized chimeric seal leptin can have leptin activity in vitro and in vivo. A humanized chimeric seal leptins can also demonstrate enhanced stability and solubility compared to the mature human leptin polypeptides which are used to derive the sequence.

[0072] Representative leptins, leptin analogs, leptin active fragments, and leptin derivatives include the following:

[0073] **Unprocessed Full-length Human Leptin (i.e., includes 21-residue N-terminal signal sequence):**

MHWGTLGFLWLWPYLFYVQAVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLP WASGLETLD SLGGVLEASGYSTE VVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:71)

[0074] **Mature Human leptins (with N-terminal 21 amino acid signal sequence removed):**

VPIQKVQDDTKTLIKTIVTRINDISH-Xaa-Xaa-SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLP WASGLETLD SLGGVLEASGYSTE VVALSRLQGSLQDMLWQLDLSPGC , wherein: Xaa at position 27 is T or A ; and Xaa at position 28 is Q or absent (SEQ ID NO:45).

[0075] **Mature Human leptins with N-terminal methionine:**

MVPIQKVQDDTKTLIKTIVTRINDISH-Xaa-Xaa-SVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLP WASGLETLD SLGGVLEASGYSTE VVALSRLQGSLQDMLWQLDLSPGC , wherein: Xaa at position 28 is T or A ; and Xaa at position 29 is Q or absent (SEQ ID NO:46).

[0076] **Mature Human Leptin form 1:**

VPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLP WASGLETLD SLGGVLEASGYSTE VVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:43).

[0077] **Mature Human Leptin form 1 with N-terminal methionine (also known as**

Metreleptin, or A100):

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV

YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYS
TEVVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:44).

[0078] Mature Human Leptin form 2:

VPIQKVQDDTKTLIKTIVTRINDISHAQSVSSKQKVTGLDFIPGLHPILTL SKMDQTLAVY
5 QQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:47).

[0079] Mature Human Leptin form 2 with N-terminal methionine:

MVPIQKVQDDTKTLIKTIVTRINDISHAQSVSSKQKVTGLDFIPGLHPILTL SKMDQTLAVY
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYS
10 TEVVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:50).

[0080] Mature Human Leptin form 3:

VPIQKVQDDTKTLIKTIVTRINDISHTSVSSKQKVTGLDFIPGLHPILTL SKMDQTLAVYQ
QILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYSTE
VVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:48).

[0081] Mature Human Leptin form 3 with N-terminal methionine:

MVPIQKVQDDTKTLIKTIVTRINDISHTSVSSKQKVTGLDFIPGLHPILTL SKMDQTLAVY
QQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:51).

[0082] Mature Human Leptin form 4:

VPIQKVQDDTKTLIKTIVTRINDISHASVSSKQKVTGLDFIPGLHPILTL SKMDQTLAVYQ
20 QILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYSTE
VVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:49).

[0083] Mature Human Leptin form 4 with N-terminal methionine:

MVPIQKVQDDTKTLIKTIVTRINDISHASVSSKQKVTGLDFIPGLHPILTL SKMDQTLAVY
25 QQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPWASGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:52).

[0084] Leptin A100 Variants: Variants of Leptin A100 with the following residue substitutions follow:

[0085] Substitutions D41E, H98S, W101Q, D109E, G113E, M137I, W139Q and G146E:

30 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLEFIPGLHPILTL SKMDQTLAVY
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCSLPQASGLETLESLGEVLEASGYST
EVVALSRLQGSLQDILQQDLDSPEC (SEQ ID NO:57).

[0086] Substitutions H98S, W101Q, A102T, G113E, M137I, W139Q, and G146E:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCSLPQASGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDILQQLDLSPEC (SEQ ID NO:58).

5 [0087] Substitutions H98S, W101Q, G113E, M137I, W139Q, and G146E:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCSLPQASGLETLD SLGEVLEASGYST
EVVALSRLQGSLQDILQQLDLSPEC (SEQ ID NO:59).

[0088] Substitutions W101Q, G113E, M137I, W139Q, and G146E:

10 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPQASGLETLD SLGEVLEASGYST
EVVALSRLQGSLQDILQQLDLSPEC (SEQ ID NO:60).

[0089] Substitutions H98S, W101Q, M137I, W139Q, and G146E:

15 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCSLPQASGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDILQQLDLSPEC (SEQ ID NO:61).

[0090] Substitutions W101Q, G113E, M137I, W139Q, L143V, and G146E:

20 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPQASGLETLD SLGEVLEASGYST
EVVALSRLQGSLQDILQQLDVSPEC (SEQ ID NO:62).

[0091] Substitutions H98S, W101Q, A102T, M137I, W139Q, and G146E:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCSLPQTSGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDILQQLDLSPEC (SEQ ID NO:63).

25 [0092] Substitutions H98S, W101Q, D109E, G113E, and G146E:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCSLPQASGLETLESLGEVLEASGYST
EVVALSRLQGSLQDMLWQLDLSPEC (SEQ ID NO:64).

[0093] Substitutions W101Q, M137I, W139Q, and G146E:

30 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLA FSKSCHLPQASGLETLD SLGGVLEASGYS
TEVVALSRLQGSLQDILQQLDLSPEC (SEQ ID NO:65).

[0094] Substitutions W101Q, M137I, W139Q, L143V, and G146E:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLAFSK SCHLPQASGLETLD SLGGVLEASGYS
TEVVALSRLQGSLQDILQQLDVSPEC (SEQ ID NO:66).

5 [0095] Substitutions H98S, W101Q, A102T, M137I, W139Q, L143V, and G146E:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLAFSK SCSLPQTSGLETLD SLGGVLEASGYST
EVVALSRLQGSLQDILQQLDVSPEC (SEQ ID NO:67).

[0096] Substitutions H98S, W101Q, A102T, G113E, and G146E:

10 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLAFSK SCSLPQTSGLETLD SLGEVLEASGYST
EVVALSRLQGSLQDMLWQLDLSPEC (SEQ ID NO:68).

[0097] Substitutions W101Q, G113E, and W139Q:

15 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLAFSK SCHLPQASGLETLD SLGEVLEASGYST
EVVALSRLQGSLQDMLQQLDLSPGC (SEQ ID NO:69).

[0098] Substitutions W101Q, G113E, W139Q, and G146E:

20 MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMP SRNVIQISNDLENLRDLLHVLAFSK SCHLPQASGLETLD SLGEVLEASGYST
EVVALSRLQGSLQDMLQQLDLSPGC (SEQ ID NO:70).

[0099] **Leptin A200:** Leptin A200 is an Fc antibody fragment condensation product with leptin, as known in the art. See e.g., Lo *et al.*, 2005, *Protein Eng. Design & Selection*, **18**:1-10. The amino acid sequence of A200 is as follows:

25 MDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
PVLDSGDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGKVPIQKV
QDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSKMDQTLAVYQQILTS
MPSRNVIQISNDLENLRDLLHVLAFSK SCHLPWASGLETLD SLGGVLEASGYSTEVALS
30 RLQGSLQDMLWQLDLSPEC (SEQ ID NO:53)

[0100] **Leptin A300:** Leptin A300 is metreleptin with substitutions W101Q and W139Q (N-terminal ¹Met counted as residue 1):

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLKMDQTLAV
YQQILTSMPSRNVIIQISNDLENLRDLLHVLAFSKSKCHLPQASGLETLDLGGVLEASGYS
TEVVALSRLQGSLQDMLQQLDLSPGC (SEQ ID NO:54).

[0101] Leptin A400: Leptin A400 is metreleptin with the serine residue at position 78

5 replaced with a cysteine residue, as set forth following:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLKMDQTLAV
YQQILTSMPSRNVIIQICNDLENLRDLLHVLAFSKSKCHLPWASGLETLDLGGVLEASGYS
TEVVALSRLQGSLQDMLWQLDLSPGC (SEQ ID NO:55); to which a 20 kilodalton (kDa)
PEG moiety has been attached via the cysteine residue at position 78.

10 **[0102] Leptin A500:** Research by a number of investigators including the inventors has
focused on the effects on aggregation of residue substitution in leptin. *See e.g., Ricci et al.,*
2006. "Mutational approach to improve physical stability of protein therapeutics susceptible to
aggregation: Role of altered conformation in irreversible precipitation," Book Chapter. In:
MISBEHAVING PROTEINS: PROTEIN (MIS)FOLDING, AGGREGATION, AND STABILITY, Murphy RM,
15 Tsai AM, Eds., New York. Springer. pp. 331-350, which is incorporated herein by reference and
for all purposes. Accordingly, leptin A500 with sequence following has been used in certain
compounds and methods described herein:

MVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLEFIPGLHPILTLKMDQTLAV
YQQILTSMPSRNVIIQISNDLENLRDLLHVLAFSKSKCHLPQASGLETLESLLGGVLEASGYST
20 EVVALSRLQGSLQDMLQQLDLSPGC (SEQ ID NO:56).

[0103] In one embodiment, the HD2 sequence of the engineered polypeptide includes an
analog of the humanized chimeric seal leptin sequence or an active fragment of an analog of the
humanized chimeric seal leptin sequence.

[0104] Seal leptin: A sequence of seal leptin follows:

25 PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQ
ILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:40). SEQ ID NO:40 is leptin from
Halichoerus grypus (gray seal). A single point substitution (T120A) is found in the leptin from
Phoca vitulina (harbor seal), with sequence:
30 PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQ
ILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSAEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:41).

[0105] Seal leptin with N-terminal methionine:

MPQIRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
 QILTSLSQSRVSVQIANDLANLRALLRLLASAKSCPVPVRARGSDTIKGLGNVLRASVHSTE
 VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:42).

- 5 **[0106]** Exemplary polypeptides incorporating sequence regions from seal leptin and human leptins, and analogs thereof, include the following.

[0107] A. Chimeric Polypeptides Incorporating Human Helix 1

[0108] The Helix 1 region of a mature human leptin polypeptide spans a contiguous region of 20 amino acids. Helix 1 and Helix 3 are antiparallel helices that form part of Binding Site II of
 10 leptin to its receptor. This site interacts with the cytokine receptor homology domain (CRH) of the leptin receptor and is thought to be a major receptor binding site, but not involved in receptor activation. See, for example, Peelman et al., 2004, *J. Biol. Chem.* **279**:41038. Unless indicated otherwise, references herein to helices and other secondary structural features (e.g., Helix 1, Helix 2, Helix 3, Helix 4, AB Loop, Loop 3-4, and the like) refer to regions within an X-ray
 15 crystallographic model of human leptin and the corresponding regions within seal leptins, as known in the art. Accordingly, Helix 1 of seal leptin corresponds to Helix 1 of human leptin, Helix 2 of seal leptin corresponds to Helix 2 of human leptin, Helix 3 of seal leptin corresponds to Helix 3 of human leptin, Helix 4 of seal leptin corresponds to Helix 4 of human leptin, AB Loop of seal leptin corresponds to AB Loop of human leptin, and Loop 3-4 of seal leptin
 20 corresponds to Loop 3-4 of human leptin. Using the numbering of metreleptin (A100, SEQ ID NO:44): human Helix 1 encompasses residues 5-25; human Helix 2 encompasses residues 52-67; human Helix 3 encompasses residues 73-94; human Helix 4 encompasses residues 122-143; the human AB Loop encompasses residues 25-51; and the human Loop 3-4 encompasses residues 95-121.

25 **[0109]** In one embodiment, the present disclosure relates to chimeric polypeptides that are based on wild type seal leptin with an incorporated Helix 1 sequence from mature human leptin. In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 3-22 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino
 30 acids at positions 5-24 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence set forth in SEQ ID NO:72 following.

[0110] Seal leptin with amino acids 3-22 replaced with amino acids 5-24 (helix 1) of metreleptin, respectively:

PIQKVQDDTKTLIKTIVTRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
 QILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE
 VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:72).

[0111] In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild
 5 type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the
 contiguous region spanning the amino acids at positions 3-22 of SEQ ID NO:42 has been
 replaced with a contiguous region spanning the amino acids at positions 5-24 of A100 (SEQ ID
 NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID
 NO:73 following.

10 [0112] **Seal leptin with N-terminal methionine, and with amino acids 3-22 replaced with
 amino acids 5-24 (helix 1) of metreleptin, respectively:**

MPIQKVQDDTKTLIKTIVTRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATY
 QQILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHST
 EVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:73).

15 [0113] B. Chimeric Polypeptides Incorporating Human Helix 2

[0114] The Helix 2 region of a mature human leptin polypeptide spans a region of 16
 contiguous amino acids. This helix is buried in the 4-helix bundle as described in the
 crystallographic literature. See e.g., Zhang et al., 1997, *Nature* 387:206.

[0115] In one embodiment, the present disclosure relates to chimeric polypeptides that are
 20 based on wild type seal leptin with an incorporated Helix 2 sequence from mature human leptin.
 In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal
 leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at
 positions 50-65 of SEQ ID NO:40 has been replaced with a contiguous region spanning the
 amino acids at positions 52-67 of A100 (SEQ ID NO:44). In one embodiment, a chimeric
 25 polypeptide includes the sequence described in SEQ ID NO:74 following.

[0116] Seal leptin with amino acids 50-65 replaced with amino acids 52-67 (helix 2) of
 metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSKMDQTLAVYQ
 QILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE
 30 VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:74).

[0117] In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild
 type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the

contiguous region spanning the amino acids at positions 50-65 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 52-67 of A100 (SEQ ID NO:44). In some embodiments, a chimeric polypeptide includes the sequence described in SEQ ID NO:75.

- 5 [0118] Seal leptin with N-terminal methionine, and with amino acids 50-65 replaced with amino acids 52-67 (helix 2) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLKMDQTLAVY
QQILTSLSQRSVVQIANDLANLRALLRLLASAKSCPVPVRARGSDTIKGLGNVLRASVHST
EVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:75).

- 10 [0119] C. Chimeric Polypeptides Incorporating Human Helix 3

[0120] The Helix 3 region of a mature human leptin polypeptide spans a contiguous region of 22 amino acids. Helix 3 and Helix 1 are antiparallel helices that form part of Binding Site II of leptin to its receptor. This site interacts with the cytokine receptor homology domain (CRH) of the leptin receptor and is thought to be a major receptor binding site, but not involved in receptor
15 activation. *See, e.g.,* Peelman et al., 2004, J. Biol. Chem. 279:41038.

[0121] In one embodiment, the present disclosure relates to chimeric polypeptides that are based on wild type seal leptin with an incorporated helix 3 sequence from mature human leptin. In some embodiments, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino
20 acids at positions 71-92 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:76 following.

[0122] Seal leptin with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

25 PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQ
ILTSLSQSRNVIQISNDLENLRDLLHVLAFSKSCPVPVRARGSDTIKGLGNVLRASVHSTEVV
ALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:76).

[0123] In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the
30 contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID

NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:77 following.

[0124] Seal leptin with N-terminal methionine, and with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

5 MPIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPRARGSDTIKGLGNVLRASVHSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:77).

[0125] D. Chimeric Polypeptides Incorporating Human Helix 4

10 [0126] The Helix 4 region of a mature human leptin polypeptide spans a contiguous region of 22 amino acids. Helix 4 is thought to form parts of Binding Site I and Binding Site III of leptin, both of which are important for receptor activation. See, for example, Peelman et al., 2004, *Id.*

[0127] In one embodiment, the present disclosure relates to chimeric polypeptides that are based on wild type seal leptin with an incorporated helix 4 sequence from mature human leptin. In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal
15 leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 120-141 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 122-143 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:78 following.

20 [0128] Seal leptin with amino acids 120-141 replaced with amino acids 122-143 (helix 4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQ
ILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPRARGSDTIKGLGNVLRASVHSTEV
VALSRLQGSLLQDMLWQLDLNPGC (SEQ ID NO:78).

[0129] In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild
25 type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 120-141 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 122-143 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:79 following.

30 [0130] Seal leptin with N-terminal methionine, and with amino acids 120-141 replaced with amino acids 122-143 (helix 4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ

QILTSLQSRVSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE
VVALSRLQGSLQDMLWQLDLNPGC (SEQ ID NO:79).

[0131] E. Chimeric Polypeptides Incorporating Human AB Loop

[0132] The AB Loop region of a mature human leptin polypeptide spans a contiguous region of 27 amino acids. The AB Loop is thought to form part of Binding Site III as well as a small portion of Binding Site I of leptin. See, for example, Peelman et al., 2004, J. Biol. Chem. 279: 41038. This region also contains the absolutely conserved motif GLDFIP.

[0133] In one embodiment, the present disclosure relates to chimeric polypeptides that are based on wild type seal leptin with an incorporated AB Loop sequence from mature human leptin. In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:80 following.

[0134] Seal leptin with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQI
LTSLQSRVSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVV
ALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:80).

[0135] In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:81 following.

[0136] Seal leptin with N-terminal methionine, and with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQ
QILTSLQSRVSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:81).

[0137] F. Chimeric Polypeptides Incorporating Human Loop 3-4

[0138] The Loop 3-4 region of a mature human leptin polypeptide spans a contiguous region of 27 amino acids. Loop 3-4 is thought to contain a part of Binding Site III of leptin to its receptor. See, for example, Peelman et al., 2004, *Id.*

[0139] In one embodiment, the present disclosure relates to chimeric polypeptides that are based on wild type seal leptin with an incorporated Loop 3-4 sequence from mature human leptin. In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:44). In one embodiment, a chimeric polypeptide includes the sequence described in SEQ ID NO:82 following.

[0140] Seal leptin with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQ
ILTSLQSRSVVQIANDLANLRALLRLLASAKSCHLPWASGLETLDLGGVLEASGYSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:82).

[0141] In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:44). In some embodiments, a chimeric polypeptide includes the sequence described in SEQ ID NO:83 following.

[0142] Seal leptin with N-terminal methionine, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRSVVQIANDLANLRALLRLLASAKSCHLPWASGLETLDLGGVLEASGYSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:83).

[0143] G. Combination Humanized Chimeric Seal Leptins

[0144] In one embodiment, a chimeric combination polypeptide is included in the humanized chimeric seal leptin. The term “chimeric combination polypeptide” refers to a chimeric peptide wherein two or more contiguous regions of 1-30 amino acids of a wild type seal leptin sequence (for example, SEQ ID NO:40 or SEQ ID NO:42) have been replaced at each region with a corresponding contiguous region of 1-30 amino acids of a mature human leptin sequence.

Chimeric combination polypeptides can be engineered to demonstrate enhanced physical properties compared to the mature human leptin polypeptides which are used to derive the sequences, while retaining the biological activity of human leptin.

[0145] In one embodiment, the present disclosure relates to chimeric combination polypeptides that are based on wild type seal leptin with an incorporated helix 1 sequence and an incorporated helix 3 sequence from mature human leptin. In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 3-22 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 5-24 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:84 following.

[0146] Seal leptin with amino acids 3-22 replaced with amino acids 5-24 (helix 1) of metreleptin, and amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

PIQKVQDDTKTLIKTIVTRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPRARGSDTIKGLGNVLRASVHSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:84)

[0147] In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 3-22 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 5-24 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:85 following.

[0148] Seal leptin with N-terminal methionine, and with amino acids 3-22 replaced with amino acids 5-24 (helix 1) of metreleptin, and amino acids 72-93 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

MPIQKVQDDTKTLIKTIVTRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATY
QQILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPRARGSDTIKGLGNVLRASVHSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:85).

[0149] In one embodiment, the present disclosure relates to chimeric combination polypeptides that are based on wild type seal leptin with an incorporated helix 3 sequence and an incorporated AB Loop sequence from mature human leptin. In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:86 following.

[0150] Seal leptin with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, and with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQI
LTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPRARGSDTIKGLGNVLRASVHSTEVV
ALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:86).

[0151] In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:87 following.

[0152] Seal leptin with N-terminal methionine, and with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, and with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPRARGSDTIKGLGNVLRASVHSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:87).

[0153] In one embodiment, the present disclosure relates to chimeric combination polypeptides that are based on wild type seal leptin with an incorporated helix 3 sequence and an incorporated Loop 3-4 sequence from mature human leptin. In one embodiment, a chimeric combination

polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:4). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:88 following.

[0154] Seal leptin with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQ
ILTSLQSRNVIQISNDLENLRDLLHVLAFSK SCHLPWASGLETLDLGGVLEASGYSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:88).

[0155] In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:89 following.

[0156] Seal leptin with N-terminal methionine, with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFSK SCHLPWASGLETLDLGGVLEASGYSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:89).

[0157] In one embodiment, the present disclosure relates to chimeric combination polypeptides that are based on wild type seal leptin with an incorporated AB Loop sequence and an incorporated helix 4 sequence from mature human leptin. In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 25-51

of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 120-141 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 122-143 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:90 following.

- 5 **[0158]** Seal leptin with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, and with amino acids 120-141 replaced with amino acids 122-143 (helix 4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQI
LTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVV
10 ALSRLQGSLQDMLWQLDLNPGC (SEQ ID NO:90).

- [0159]** In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 120-141 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 122-143 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:91 following.

- [0160]** Seal leptin with N-terminal methionine, with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, and with amino acids 120-141 replaced with amino acids 122-143 (helix 4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQ
QILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE
VVALSRLQGSLQDMLWQLDLNPGC (SEQ ID NO:91).

- 25 **[0161]** In one embodiment, the present disclosure relates to chimeric combination polypeptides that are based on wild type seal leptin with an incorporated AB Loop sequence and an incorporated Loop 3-4 sequence from mature human leptin. In one embodiment, a chimeric polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at

positions 95-121 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:92 following.

[0162] Seal leptin with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQI
LTSLQSRSVVQIANDLANLRALLRLLASAKSCHLPWASGLETLDLGGVLEASGYSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:92).

[0163] In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:93 following.

[0164] Seal leptin with N-terminal methionine, with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQ
QILTSLQSRSVVQIANDLANLRALLRLLASAKSCHLPWASGLETLDLGGVLEASGYSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:93).

[0165] In one embodiment, the present disclosure relates to chimeric combination polypeptides that are based on wild type seal leptin with an incorporated AB Loop sequence, an incorporated Loop 3-4 sequence, and an incorporated helix 3 sequence from mature human leptin. In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide (SEQ ID NO:40), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44), the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44). In one

embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:94 following.

[0166] Seal leptin with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, and with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQI
LTSLQSRNVIQISNDLENLRDLLHVLAFSKSchLPWASGLETLDLGGVLEASGYSTEVV
ALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:94).

[0167] In one embodiment, a chimeric combination polypeptide includes the amino acid sequence of a wild type seal leptin polypeptide with an N-terminal methionine (SEQ ID NO:42), wherein the contiguous region spanning the amino acids at positions 23-49 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 25-51 of A100 (SEQ ID NO:44), the contiguous region spanning the amino acids at positions 93-119 of SEQ ID NO:42 has been replaced with a contiguous region spanning the amino acids at positions 95-121 of A100 (SEQ ID NO:44), and the contiguous region spanning the amino acids at positions 71-92 of SEQ ID NO:40 has been replaced with a contiguous region spanning the amino acids at positions 73-94 of A100 (SEQ ID NO:44). In one embodiment, a chimeric combination polypeptide includes the sequence described in SEQ ID NO:95 following.

[0168] Seal leptin with N-terminal methionine, with amino acids 23-49 replaced with amino acids 25-51 (AB loop) of metreleptin, with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, and with amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFSKSchLPWASGLETLDLGGVLEASGYSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:95).

[0169] In one embodiment, the chimeric polypeptides provided by the invention contain a Cys to Ser amino acid substitution at position 30 of the wild type seal polypeptide sequence. According to some embodiments, the following chimeric polypeptides or chimeric combination polypeptides are provided:

[0170] Seal leptin with amino acids 30 and 3-22 replaced with amino acids 32 and 5-24 (helix 1) of metreleptin, respectively:

PIQKVQDDTKTLIKTIVTRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ

QILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE
VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:96).

[0171] Seal leptin with N-terminal methionine, and with amino acids 30 and 3-22 replaced with amino acids 32 and 5-24 (helix 1) of metreleptin, respectively:

5 MPIQKVQDDTKTLIKTIVTRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATY
QQILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHST
EVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:97).

[0172] Seal leptin with amino acids 30 and 50-65 replaced with amino acids 32 and 52-67 (helix 2) of metreleptin, respectively:

10 PIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSKMDQTLAVYQQ
ILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:98).

[0173] Seal leptin with N-terminal methionine, and with amino acids 30 and 50-65 replaced with amino acids 32 and 52-67 (helix 2) of metreleptin, respectively:

15 MPIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSKMDQTLAVY
QQILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHST
EVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:99).

[0174] Seal leptin with amino acids 30 and 71-92 replaced with amino acids 32 and 73-94 (helix 3) of metreleptin, respectively:

20 PIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQI
LTSLQSRNVIQISNDLENLRDLLHVLAFAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVV
ALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:100).

[0175] Seal leptin with N-terminal methionine, and with amino acids 30 and 71-92 replaced with amino acids 32 and 73-94 (helix 3) of metreleptin, respectively:

25 MPIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFAFSKSCPVPARGSDTIKGLGNVLRASVHSTEV
VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:101).

[0176] Seal leptin with amino acids 30 and 120-141 replaced with amino acids 32 and 122-143 (helix 4) of metreleptin, respectively:

30 PIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQI
LTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVV
ALSRLQGSQDMLWQLDLNPGC (SEQ ID NO:102).

[0177] Seal leptin with N-terminal methionine, and with amino acids 30 and 120-141 replaced with amino acids 32 and 122-143 (helix 4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRSVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTE

5 VVALSRLQGSLQDMLWQLDLNPGC (SEQ ID NO:103).

[0178] Seal leptin with amino acids 30 and 93-119 replaced with amino acids 32 and 95-121 (loop 3-4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQI
LTSLQSRSVVQIANDLANLRALLRLLASAKSCHLPWASGLETLDLGGVLEASGYSTE

10 VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:104).

[0179] Seal leptin with N-terminal methionine, and with amino acids 30 and 93-119 replaced with amino acids 32 and 95-121 (loop 3-4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRSVVQIANDLANLRALLRLLASAKSCHLPWASGLETLDLGGVLEASGYSTE

15 VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:105).

[0180] Seal leptin with amino acid 30 replaced with amino acid 32, amino acids 3-22 replaced with amino acids 5-24 (helix 1) of metreleptin, and amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

PIQKVQDDTKTLIKTIVTRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
QILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTE

20 VALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:106)

[0181] Seal leptin with N-terminal methionine, and with amino acid 30 replaced with amino acid 32, amino acids 3-22 replaced with amino acids 5-24 (helix 1) of metreleptin, and amino acids 72-93 replaced with amino acids 73-94 (helix 3) of metreleptin, respectively:

MPIQKVQDDTKTLIKTIVTRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATY
QQILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTE

25 VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:107).

[0182] Seal leptin with amino acid 30 replaced with amino acid 32, amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

PIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQI
LTSLQSRNVIQISNDLENLRDLLHVLAFSKCHLPWASGLETLDLGGVLEASGYSTE

30 ALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:108).

[0183] Seal leptin with N-terminal methionine, with amino acid 30 replaced with amino acid 32, amino acids 71-92 replaced with amino acids 73-94 (helix 3) of metreleptin, and with amino acids 93-119 replaced with amino acids 95-121 (loop 3-4) of metreleptin, respectively:

MPIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQ
 5 QILTSLQSRNVIQISNDLENLRDLLHVLAFSKSchLPWASGLETLDSLGGVLEASGYSTE
 VVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:109).

[0184] It is understood that each of the polypeptides disclosed herein are also contemplated to include a methionine at the N-terminus in frame with the naturally-occurring first amino acid thereof, e.g., Met-Exendin-4, which is Exendin-4 with an added N-terminal methionine. It is
 10 further understood that where a C-terminal Gly appears in a engineered polypeptide sequence set forth herein, the residue may be lost during subsequent amidation. Some embodiments are intermediates in synthesis, for example, such as those having a “His tag” which is used for affinity purification as is known in the art, and that can optionally be subsequently removed to yield a mature engineered polypeptide suitable for therapeutic use.

[0185] Accordingly, in one embodiment HD2 has at least 50% identity with an amino acid sequence selected from the group consisting of: SEQ ID NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ
 20 ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

[0186] In one embodiment HD2 has at least 90% identity with an amino acid sequence selected from the group consisting of: SEQ ID NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ
 25 ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

[0187] In one embodiment, HD2 of the engineered polypeptide has the amino acid sequence of SEQ ID NO:77.

[0188] In one embodiment HD2 has an amino acid sequence selected from the group consisting of: SEQ ID NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

[0189] In one embodiment, there is provided an engineered polypeptide wherein the HD2 sequence includes a seal leptin sequence (SEQ ID NO:40); wherein at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence has been replaced with at least one contiguous region of from 8 to 30 amino acids of a human leptin sequence (SEQ ID NO:44); wherein the seal leptin sequence comprises a seal leptin Helix 1 sequence, a seal leptin Helix 2 sequence, a seal leptin Helix 3 sequence, a seal leptin Helix 4 sequence, a seal leptin AB Loop sequence, and a seal leptin Loop 3-4 sequence; and wherein the human leptin sequence comprises a human leptin Helix 1 sequence, a human leptin Helix 2 sequence, a human leptin Helix 3 sequence, a human leptin Helix 4 sequence, a human leptin AB Loop sequence, and a human leptin Loop 3-4 sequence

[0190] In one embodiment, at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence corresponds to the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence.

[0191] In one embodiment, the engineered polypeptide further includes from 1 to 5 amino acid modifications not encompassed within the at least one contiguous region of 8 to 30 amino acids of the seal leptin sequence, the 1 to 5 amino acid modifications each independently selected from an insertion, deletion, addition or substitution.

[0192] In one embodiment, at least one of the 1 to 5 amino acid modification not encompassed within the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence is substitution of cysteine at a position corresponding to position 30 of the seal leptin sequence.

[0193] In one embodiment, the seal leptin Helix 1 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 1 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence, and the seal leptin Helix 1 sequence is replaced by the human leptin Helix 1 sequence.

[0194] In one embodiment, the seal leptin Helix 2 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 2 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence, and the seal leptin Helix 2 sequence is replaced by the human leptin Helix 2 sequence.

[0195] In one embodiment, the seal leptin Helix 3 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 3 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence, and the seal leptin Helix 3 sequence is replaced by the human leptin Helix 3 sequence.

[0196] In one embodiment, the seal leptin Helix 4 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 4 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence, and the seal leptin Helix 4 sequence is replaced by the human leptin Helix 4 sequence.

[0197] In one embodiment, the seal leptin AB Loop sequence includes the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin AB Loop sequence includes the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence, and the seal leptin AB Loop sequence is replaced by the human leptin AB Loop sequence.

[0198] In one embodiment, the seal leptin Loop 3-4 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Loop 3-4 sequence includes the at least one contiguous region of from 8 to 30 amino acids of the human leptin sequence, and the seal leptin Loop 3-4 sequence is replaced by the human leptin Loop 3-4 sequence.

[0199] In one embodiment, the engineered polypeptide includes two contiguous regions of from 8 to 30 amino acids of the seal leptin sequence which have replaced with two contiguous regions of from 8 to 30 amino acids of a human leptin sequence (SEQ ID NO:44).

5 [0200] In one embodiment, a first contiguous region of from 8 to 30 amino acids of the seal leptin sequence has been replaced with a first contiguous region of from 8 to 30 amino acids of the human leptin sequence, and a second contiguous region of from 8 to 30 amino acids of the seal leptin sequence has been replaced with a second contiguous region of from 8 to 30 amino acids of the human leptin sequence.

10 [0201] In one embodiment, the seal leptin Helix 1 sequence includes the first contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 1 sequence includes the first contiguous region of 8 to 30 amino acids of the human leptin sequence, the seal leptin Helix 3 sequence includes the second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and the human leptin Helix 3 sequence includes the second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

15 [0202] In one embodiment, the seal leptin Helix 3 sequence includes the first contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 3 sequence includes the first contiguous region of 8 to 30 amino acids of the human leptin sequence, the seal leptin AB Loop sequence includes the second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and the human leptin AB Loop sequence includes
20 the second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0203] In one embodiment, the seal leptin Helix 3 sequence includes the first contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 3 sequence includes the first contiguous region of 8 to 30 amino acids of the human leptin sequence, the seal leptin Loop 3-4 sequence includes the second contiguous region of from 8 to
25 30 amino acids of said seal leptin sequence, and the human leptin Loop 3-4 sequence includes the second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0204] In one embodiment, the seal leptin Helix 4 sequence includes the first contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Helix 4 sequence includes the first contiguous region of 8 to 30 amino acids of the human leptin
30 sequence, the seal leptin AB Loop sequence includes the second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and the human leptin AB Loop sequence includes the second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0205] In one embodiment, the seal leptin AB Loop sequence includes the first contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin AB Loop sequence includes the first contiguous region of 8 to 30 amino acids of the human leptin sequence, the seal leptin Loop 3-4 sequence includes the second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and the human leptin Loop 3-4 sequence includes the second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0206] In one embodiment, a first contiguous region of from 8 to 30 amino acids of the seal leptin sequence has been replaced with a first contiguous region of from 8 to 30 amino acids of the human leptin sequence, a second contiguous region of from 8 to 30 amino acids of the seal leptin sequence has been replaced with a second contiguous region of from 8 to 30 amino acids of the human leptin sequence, and a third contiguous region of from 8 to 30 amino acids of the seal leptin sequence has been replaced with a third contiguous region of from 8 to 30 amino acids of the human leptin sequence.

[0207] In one embodiment, the seal leptin AB Loop sequence includes the first contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin AB Loop sequence includes the first contiguous region of 8 to 30 amino acids of the human leptin sequence, the seal leptin Loop 3-4 sequence includes the second contiguous region of from 8 to 30 amino acids of the seal leptin sequence, the human leptin Loop 3-4 sequence includes the second contiguous region of from 8 to 30 amino acids of the human leptin sequence, the seal leptin Helix 3 sequence includes the third contiguous region of from 8 to 30 amino acids of the seal leptin sequence, and the human leptin Helix 3 sequence includes the third contiguous region of 8 to 30 amino acids of the human leptin sequence.

[0208] **Linkers.** In some embodiments, engineered polypeptides are provided having a linker L1, as described herein, covalently linking a polypeptide hormone domains HD1 and HD2. In some embodiments, a first linker (L1) covalently links HD1 within the engineered polypeptide. In some embodiments, L1 is a bond. In some embodiments, the polypeptide hormone domain (e.g., HD1 as described herein) can be covalently linked to the HD2 peptide via a peptide linker. Any linker is optional; i.e., any linker may simply be a bond. In one embodiment the linker includes from 1 to 30 amino acids linked by peptide bonds. The amino acids can be selected from the 20 naturally occurring (i.e., physiological) amino acids. Alternatively, non-natural amino acids can be incorporated either by chemical synthesis, post-translational chemical modification or by in vivo incorporation by recombinant expression in a host cell. Some of these amino acids may be glycosylated. In another embodiment the 1 to 30 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine, and further from aspartate and

glutamate. In a further embodiment the linker is made up of a majority of amino acids that are sterically unhindered, such as glycine, alanine and/or serine. "Sterically unhindered" refers, in the customary sense, to a amino acid having a small side chain, e.g., 0-2 non-hydrogen atoms, such that steric hinderance is minimized relative to amino acids having larger side chains, e.g., Leu, Trp, Tyr, Phe, and the like. Polyglycines are particularly useful, e.g. (Gly)₃, (Gly)₄, (Gly)₅, as are polyalanines, poly(Gly-Ala) and poly(Gly-Ser). Charged polyglycines can be useful, and include e.g., poly (Gly_n-Glu) (SEQ ID NO:110), poly(Gly_n-Lys), (SEQ ID NO:111), poly(Gly_n-Asp) (SEQ ID NO:112), and poly(Gly_n-Arg) (SEQ ID NO:113) motifs (where n can be 1 to 6). Other specific examples of linkers include (Gly)₃Lys(Gly)₄ (SEQ ID NO:114); (Gly)₃AsnGlySer(Gly)₂ (SEQ ID NO:115); (Gly)₃Cys(Gly)₄ (SEQ ID NO:116); and GlyProAsnGlyGly (SEQ ID NO:117). Combinations of Gly and Ala are particularly useful as are combination of Gly and Ser. Thus, in a further embodiment the peptide linker is selected from the group of a glycine rich peptide, e.g., Gly-Gly-Gly; the sequences [Gly-Ser]_n (SEQ ID NO:118), [Gly- Gly- Ser]_n (SEQ ID NO:119), [Gly-Gly-Gly- Ser]_n (SEQ ID NO:120) and [Gly-Gly-Gly-Gly-Ser]_n (SEQ ID NO:121), where n is 1, 2, 3, 4, 5 or 6, for example [Gly-Gly-Gly-Gly Ser]₃. "Glycine rich peptide" refers to a polypeptide which includes a plurality of glycine residues, preferably a majority of glycine residues, more preferably a preponderance of glycine residues.

[0209] In certain embodiments, charged linkers may be used. Such charges linkers may be contain a significant number of acidic residues (e.g., Asp, Glu, and the like), or may contain a significant number of basic residues (e.g., Lys, Arg, and the like), such that the linker has a pI lower than 7 or greater than 7, respectively. As understood by the artisan, and all other things being equal, the greater the relative amount of acidic or basic residues in a given linker, the lower or higher, respectively, the pI of that linker will be. Such linkers may impart advantageous properties to the engineered polypeptides disclosed herein, such as modifying the peptides pI (isoelectric point) which can in turn improve solubility and/or stability characteristics of such polypeptides at a particular pH, such as at physiological pH (e.g., between pH 7.2 and pH 7.6, inclusive), or in a pharmaceutical composition including such polypeptides. As is known in the art, solubility for a peptide can be improved by formulation in a composition having a pH that is at least or more than plus or minus one pH unit from the pI of the peptide.

[0210] For example, an "acidic linker" is a linker that has a pI of less than 7; between 6 and 7, inclusive; between 5 and 6, inclusive; between 4 and 5, inclusive; between 3 and 4, inclusive; between 2 and 3, inclusive; or between 1 and 2, inclusive. Similarly, a "basic linker" is a linker that has a pI of greater than 7; between 7 and 8, inclusive; between 8 and 9, inclusive; between 9

and 10, inclusive; between 10 and 11, inclusive; between 11 and 12 inclusive, or between 12 and 13, inclusive. In certain embodiments, an acidic linker contains a sequence selected from the group of [Gly-Glu]_n (SEQ ID NO:122); [Gly-Gly-Glu]_n (SEQ ID NO:123); [Gly-Gly-Gly-Glu]_n (SEQ ID NO:124); [Gly-Gly-Gly-Gly-Glu]_n (SEQ ID NO:125), [Gly-Asp]_n (SEQ ID NO:126);
 5 [Gly-Gly-Asp]_n (SEQ ID NO:127); [Gly-Gly-Gly-Asp]_n (SEQ ID NO:128); [Gly-Gly-Gly-Gly-Asp]_n (SEQ ID NO:129), where n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more; for example, [Gly-Gly-Glu]₆. In certain embodiments, a basic linker will contain a sequence that is selected from the group of [Gly-Lys]_n (SEQ ID NO:130); [Gly-Gly-Lys]_n (SEQ ID NO:131); [Gly-Gly-Gly-Lys]_n (SEQ ID NO:132); [Gly-Gly-Gly-Gly-Lys]_n (SEQ ID NO:133), [Gly-Arg]_n (SEQ ID NO:134);
 10 [Gly-Gly-Arg]_n (SEQ ID NO:135); [Gly-Gly-Gly-Arg]_n (SEQ ID NO:136); [Gly-Gly-Gly-Gly-Arg]_n (SEQ ID NO:137) where n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more; for example, [Gly-Gly-Lys]₆.

[0211] Additionally, linkers may be prepared which possess certain structural motifs or characteristics, such as an alpha helix. For example, such a linker may contain a sequence that is
 15 selected from the group of [Glu-Ala-Ala-Ala-Lys]_n (SEQ ID NO:138), where n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more; for example, [Glu-Ala-Ala-Ala-Lys]₃, [Glu-Ala-Ala-Ala-Lys]₄, or [Glu-Ala-Ala-Ala-Lys]₅. One in the art can readily determine helix content of any particular linker sequence.

[0212] A biocompatible linker other than a peptide linker may be used to covalently attach
 20 terminii of HD1 and HD2, a side chain of HD1 with a terminus of HD2, a side chain of HD2 with a terminus of HD1, or a side chain of HD1 with a side chain of HD2. The linker can be a biocompatible polymer, preferably water soluble, and more preferably about 50kDa to about 5000kDa, or about 50KDa to 500kDa, or about 100kDa to 500kDa. An exemplary
 25 biocompatible, water soluble polymer linker is a PEG linker, such as $-(CH_2-CH_2-O)_n-$ where n is such that the PEG linker can have a molecular weight of 100 to 5000 kDa, preferably 100 to 500 kDa. Such a linker may be $-NH-CH_2-CH_2-(O-CH_2-CH_2)_n-O-CH_2-CO-$, where n is such that the PEG linker molecular weight is 100kDa to 5000kDa, preferably 10kDa to 500kDa. Other
 biocompatible polymers can be used, such as including but not limited to polysaccharides, polypropylene glycol, and co-polymers of propylene and ethylene glycols. Typically such a
 30 linker will include a reactive group at each end that can be the same or different reactive group. Such linkers with reactive groups are known and available. Preferably the reactive group is reactive with either an N-terminal amino or C-terminal carboxy group of a peptide. For example, a reactive group can be an a butylaldehyde, a propionaldehyde, an aldehyde, a succinimide or a maleimide moiety, as is known in the art. Less preferred are alkyl linkers such

as $-\text{NH}-(\text{CH}_2)_n-\text{C}(\text{O})-$, wherein $n = 2-20$, and which can be further substituted by any group that does not sterically-hinder peptide function, such as a lower alkyl (e.g., $\text{C}_1\text{-C}_6$), lower acyl, halogen, CN, and NH_2 .

[0213] It is also to be understood that linkers suitable for use in accordance with the invention may possess one or more of the characteristics and motifs described above and herein. For example, a linker may include an acidic linker as well as a structural motif, such as an alpha helix. Similarly, a linker may include a basic linker and a structural motif, such as an alpha helix. A linker may include an acidic linker, a basic linker, and a structural motif, such as an alpha helix. Additionally, it is also to be understood that engineered polypeptides in accordance with the invention may possess more than one linker, and each such linker may possess one or more of the characteristics described herein.

[0214] The linkers described herein are exemplary, and linkers within the scope of this invention may be much longer and may include other residues.

[0215] Accordingly, in one embodiment, the engineered polypeptide includes a linker L1 which is a peptide linker including from 1 to 30 amino acids.

[0216] In one embodiment, linker L1 includes amino acids selected from the 20 naturally occurring amino acids. In one embodiment, linker L1 consists of amino acids selected from the 20 naturally occurring amino acids.

[0217] In one embodiment, linker L1 includes a non-natural amino acid incorporated by chemical synthesis, post-translational chemical modification or by in vivo incorporation by recombinant expression in a host cell.

[0218] In one embodiment, the amino acids of linker L1 are selected from glycine, alanine, proline, asparagine, glutamine, and lysine.

[0219] In one embodiment, linker L1 includes a majority of amino acids that are sterically unhindered. The term "sterically unhindered" refers, in the customary sense, to relative conformational freedom of an amino acid due to reduced side chain bulk or side chain to backbone constraint.

[0220] In one embodiment, linker L1 includes polyglycine, polyalanine, poly(Gly-Ala) or poly(Gly-Ser).

[0221] In one embodiment, linker L1 includes the sequence $(\text{Gly})_3$, $(\text{Gly})_4$ (SEQ ID NO:139), or $(\text{Gly})_5$ (SEQ ID NO:140).

[0222] In one embodiment, linker L1 includes the sequence GGG, GGS, GGGG (SEQ ID NO:139), or GGGS (SEQ ID NO:141).

[0223] In one embodiment, linker L1 includes the sequence (Gly)₃Lys(Gly)₄ (SEQ ID NO:114); (Gly)₃AsnGlySer(Gly)₂ (SEQ ID NO:115); (Gly)₃Cys(Gly)₄ (SEQ ID NO:116); or GlyProAsnGlyGly (SEQ ID NO:117).

[0224] In one embodiment, linker L1 includes combinations of Gly and Ala.

[0225] In one embodiment, linker L1 includes combinations of Gly and Ser.

[0226] In one embodiment, linker L1 is a glycine rich peptide.

[0227] In one embodiment, linker L1 includes an N-terminal dipeptide, which N-terminal dipeptide includes amino acids residues T, A, S or G. In one embodiment, linker L1 includes an N-terminal TG dipeptide.

[0228] In one embodiment, linker L1 includes a C-terminal dipeptide, which C-terminal dipeptide includes amino acids residues T, A, S or G. In one embodiment, linker L1 includes a C-terminal AS dipeptide. In one embodiment, linker L1 includes an N-terminal TG dipeptide and a C-terminal AS dipeptide.

[0229] In one embodiment, linker L1 includes a sequence selected from the group consisting of TG-(GGG)₁ (SEQ ID NO:142), TG-(GGGG)₁ (SEQ ID NO:143), TG-(GGGGG)₁ (SEQ ID NO:144), TG-(GGG)₂ (SEQ ID NO:145), TG-(GGGGGGG)₁ (SEQ ID NO:146), TG-(GGGG)₂ (SEQ ID NO:147), TG-(GGG)₃ (SEQ ID NO:148), (GGG)₁-AS (SEQ ID NO:149), (GGGG)₁-AS (SEQ ID NO:150), (GGGGG)₁-AS (SEQ ID NO:151), (GGG)₂-AS (SEQ ID NO:152), (GGGGGGG)₁-AS (SEQ ID NO:153), (GGGG)₂-AS (SEQ ID NO:154), (GGG)₃-AS (SEQ ID NO:155), TG-(GGG)₁-AS (SEQ ID NO:156), TG-(GGGG)₁-AS (SEQ ID NO:157), TG-(GGGGG)₁-AS (SEQ ID NO:158), TG-(GGG)₂-AS (SEQ ID NO:159), TG-(GGGGGGG)₁-AS (SEQ ID NO:160), TG-(GGGG)₂-AS (SEQ ID NO:161), and TG-(GGG)₃-AS (SEQ ID NO:162).

[0230] In one embodiment, linker L1 includes a sequence selected from the group consisting of TG-(GGS)₁ (SEQ ID NO:163), TG-(GGGS)₁ (SEQ ID NO:164), TG-(GGGGS)₁ (SEQ ID NO:165), TG-(GGS)₂ (SEQ ID NO:166), TG-(GGGGGS)₁ (SEQ ID NO:167), TG-(GGGS)₂ (SEQ ID NO:168), TG-(GGS)₃ (SEQ ID NO:169), (GGS)₁-AS (SEQ ID NO:170), (GGGS)₁-AS (SEQ ID NO:171), (GGGGS)₁-AS (SEQ ID NO:172), (GGS)₂-AS (SEQ ID NO:173), (GGGGGS)₁-AS (SEQ ID NO:174), (GGGS)₂-AS (SEQ ID NO:175), (GGS)₃-AS (SEQ ID NO:176), TG-(GGS)₁-AS (SEQ ID NO:177), TG-(GGGS)₁-AS (SEQ ID NO:178), TG-

(GGGGS)₁-AS (SEQ ID NO:179), TG-(GGS)₂-AS (SEQ ID NO:180), TG-(GGGGGGS)₁-AS (SEQ ID NO:181), TG-(GGGS)₂-AS (SEQ ID NO:182), and TG-(GGS)₃-AS (SEQ ID NO:183).

[0231] In one embodiment, linker L1 consists of a sequence selected from the group consisting of TG-(GGG)₁ (SEQ ID NO:142), TG-(GGGG)₁ (SEQ ID NO:143), TG-(GGGGG)₁ (SEQ ID NO:144), TG-(GGG)₂ (SEQ ID NO:145), TG-(GGGGGGG)₁ (SEQ ID NO:146), TG-(GGGG)₂ (SEQ ID NO:147), TG-(GGG)₃ (SEQ ID NO:148), (GGG)₁-AS (SEQ ID NO:149), (GGGG)₁-AS (SEQ ID NO:150), (GGGGG)₁-AS (SEQ ID NO:151), (GGG)₂-AS (SEQ ID NO:152), (GGGGGGG)₁-AS (SEQ ID NO:153), (GGGG)₂-AS (SEQ ID NO:154), (GGG)₃-AS (SEQ ID NO:155), TG-(GGG)₁-AS (SEQ ID NO:156), TG-(GGGG)₁-AS (SEQ ID NO:157), TG-(GGGGG)₁-AS (SEQ ID NO:158), TG-(GGG)₂-AS (SEQ ID NO:159), TG-(GGGGGGG)₁-AS (SEQ ID NO:160), TG-(GGGG)₂-AS (SEQ ID NO:161), and TG-(GGG)₃-AS (SEQ ID NO:162).

[0232] In one embodiment, linker L1 consists of a sequence selected from the group consisting of TG-(GGS)₁ (SEQ ID NO:163), TG-(GGGS)₁ (SEQ ID NO:164), TG-(GGGGS)₁ (SEQ ID NO:165), TG-(GGS)₂ (SEQ ID NO:166), TG-(GGGGGGS)₁ (SEQ ID NO:167), TG-(GGGS)₂ (SEQ ID NO:168), TG-(GGS)₃ (SEQ ID NO:169), (GGS)₁-AS (SEQ ID NO:170), (GGGS)₁-AS (SEQ ID NO:171), (GGGGS)₁-AS (SEQ ID NO:172), (GGS)₂-AS (SEQ ID NO:173), (GGGGGGS)₁-AS (SEQ ID NO:174), (GGGS)₂-AS (SEQ ID NO:175), (GGS)₃-AS (SEQ ID NO:176), TG-(GGS)₁-AS (SEQ ID NO:177), TG-(GGGS)₁-AS (SEQ ID NO:178), TG-(GGGGS)₁-AS (SEQ ID NO:179), TG-(GGS)₂-AS (SEQ ID NO:180), TG-(GGGGGGS)₁-AS (SEQ ID NO:181), TG-(GGGS)₂-AS (SEQ ID NO:182), and TG-(GGS)₃-AS (SEQ ID NO:183).

[0233] In some embodiments, an engineered polypeptide described herein is superior to a corresponding compound having a different moiety that can extend plasma half-life (e.g., PEG or of Fc or albumin) conjugated with a hormone domain(s). In this context, the term “superior” refers to a variety of functional properties which could be weighed in the evaluation of a treatment for a disease or disorder. For example, the engineered polypeptide described herein could require less biologically active (hormone domain) component, for example 1X, 2X, 3X, 4X, 5X, or even less, than the corresponding compound having a different moiety conjugated with the hormone domain(s). For further example, the engineered polypeptide described herein could have higher potency, for example, 1.5X, 2X, 3X, 4X, 5X, 10X, 20X, 50X, or even higher potency.

[0234] Engineered polypeptide compounds contemplated herein include the compounds as set forth in Table 1 following. Compounds with sequence of SEQ ID NOS:184-375 include an

exendin domain which is Exendin-4(1-28), [¹⁴L]Exendin-4(1-28), Exendin-4(1-32), or [¹⁴L]Exendin-4(1-32). A linker is present, having the sequence TGGGGAS, TGGGSAS, TGGGGGAS, TGGGGSAS, TG-(GGGG)₄-AS, and TG-(GGGS)₄-AS. The humanized chimeric seal leptin component of the engineered polypeptides has the sequence of SEQ ID NO:72, SEQ ID NO:94, SEQ ID NO:74, SEQ ID NO:98, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:78, or SEQ ID NO:102.

Table 1.

Engineered polypeptide	SEQ ID NO:
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQKVQDDTKTLIKTIVTRINDISP PQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	184
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISH TQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCHLPWASGLETLDLGGVLEASGYSTEVVALSRLKAALQDMLRQLDRNP GC	185
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVCSRPRVAGLDFIPRVQSVRTLKMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	186
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTLKMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	187
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	188
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	189
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRSVVQIANDLANLRAL LRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSQDMLWQLDLNP GC	190
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRSVVQIANDLANLRAL LRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSQDMLWQLDLNP GC	191
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQKVQDDTKTLIKTIVTRINDISP PQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	192
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISHT	193

Engineered polypeptide	SEQ ID NO:
QSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLQSRNVIQISNDLENLRDLLH VLAFSK SCHLPWASGLETLDLGGVLEASGYSTEVALSRLKAALQDMLRQLDRNPG C	
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISPP QGVC SRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRN PGC	194
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRN PGC	195
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HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRNVIQISNDLENLRDLL HVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRNP GC	197
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISPP QGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRSVVQIANDLANLRAL LRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLQGSQDMLWQLDLNP GC	198
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRSVVQIANDLANLRAL LRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLQGSQDMLWQLDLNP GC	199
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQKVQDDTKTLIKTIVTRINDIS PPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDR NPGC	200
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HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDIS PPQGVCSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDR NPGC	202
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HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDIS PPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRNVIQISNDLENLRD LLHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRN	204

Engineered polypeptide	SEQ ID NO:
PGC	
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HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDIS PPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLQSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSLQDMLWQLD LNPGC	206
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDIS PPQGVSSRPVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLQSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSLQDMLWQLD LNPGC	207
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQKVQDDTKTLIKTIVTRINDIS PPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLQSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDR NPGC	208
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDIS HTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLQSRNVIQISNDLENLRDL LHVLAFSKSCHLPWASGLETLDSLGGVLEASGYSTEVVALSRLKAALQDMLRQLDRN PGC	209
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDR NPGC	210
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	211
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRNVIQISNDLENLRDL LHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	212
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRNVIQISNDLENLRDL LHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	213
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSLQDMLWQLDL NPGC	214
HGEFTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSLQDMLWQLDL NPGC	215

Engineered polypeptide	SEQ ID NO:
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HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLQSRN VIQISNDLENLRDLLHVLAFSK SCHLPWASGLETDSLGGVLEASGYSTEVVALSRLKA ALQDMLRQLDRNPGC	217
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSR SVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRL KAALQDMLRQLDRNPGC	218
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSR SVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRL KAALQDMLRQLDRNPGC	219
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRN VIQISNDLENLRDLLHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKA ALQDMLRQLDRNPGC	220
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRN VIQISNDLENLRDLLHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKA ALQDMLRQLDRNPGC	221
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRS VVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQ GSLQDMLWQLDLNPGC	222
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSRS VVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQ GSLQDMLWQLDLNPGC	223
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQKVQDDT KTLIKTIVTRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLQSR SVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRL KAALQDMLRQLDRNPGC	224
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQRVQDDT KTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTL SGMDQILATYQQILTSLQSRN VIQISNDLENLRDLLHVLAFSK SCHLPWASGLETDSLGGVLEASGYSTEVVALSRLKA ALQDMLRQLDRNPGC	225
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQRVQDDT KTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLQSR SVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRL KAALQDMLRQLDRNPGC	226

Engineered polypeptide	SEQ ID NO:
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HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQRVQDDT KTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSN VIQISNDLENLRDLLHVLAFAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKA ALQDMLRQLDRNPGC	228
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQRVQDDT KTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSN VIQISNDLENLRDLLHVLAFAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKA ALQDMLRQLDRNPGC	229
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQRVQDDT KTLIKTIITRINDISPPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRS VVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQ GSLQDMLWQLDLNPGC	230
HGEGTFTSDLSKQMEEEEAVRLFIEWLKNTGGGGSGGGSGGGSGGGSSASPIQRVQDDT KTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRS VVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQ GSLQDMLWQLDLNPGC	231
HGEGTFTSDLSKQLEEEEAVRLFIEWLKNTGGGGASPIQKVQDDTKTLIKTIVTRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	232
HGEGTFTSDLSKQLEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISHT QSVSSKQKV TGLDFIPGLHPIL TSGMDQILATYQQILTSLSRSNVIQISNDLENLRDLLH VLAFAK SCHLPWASGLETDSLGGVLEASGYSTEVVALSRLKAALQDMLRQLDRNPG C	233
HGEGTFTSDLSKQLEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVCSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	234
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HGEGTFTSDLSKQLEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSNVIQISNDLENLRDLL HVLAFAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	236
HGEGTFTSDLSKQLEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP QGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSNVIQISNDLENLRDLL HVLAFAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	237
HGEGTFTSDLSKQLEEEEAVRLFIEWLKNTGGGGASPIQRVQDDTKTLIKTIITRINDISPP	238

Engineered polypeptide	SEQ ID NO:
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HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQKVQDDTKTLIKTIVTRINDISP QGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRSVVQIANDLANLRAL LRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	240
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISHT QSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLLH VLAFSKSCHLPWASGLETDSLGGVLEASGYSTEVVALSRLKAALQDMLRQLDRNPG C	241
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISP QGVCSRPRVAGLDFIPRVQSVRTLSGMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	242
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISP QGVSSRPRVAGLDFIPRVQSVRTLSGMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRN PGC	243
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISP QGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	244
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISP QGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNP GC	245
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGSASPIQRVQDDTKTLIKTIITRINDISP QGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSRSVVQIANDLANLRAL LRLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSQDMLWQLDLNP GC	246
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HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISH	249

Engineered polypeptide	SEQ ID NO:
TQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCHLPWASGLETLDLGGVLEASGYSTEVALSRLKAALQDMLRQLDRNP GC	
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HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRN PGC	251
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRNVIQISNDLENLRDL LHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRNP GC	252
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HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLQGSQDMLWQLDL NPGC	254
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLQGSQDMLWQLDL NPGC	255
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQKVQDDTKTLIKTIVTRINDIS PPQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDR NPGC	256
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISH TQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLL HVLAFSKSCHLPWASGLETLDLGGVLEASGYSTEVALSRLKAALQDMLRQLDRNP GC	257
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLSRSVVQIANDLANLR ALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDR NPGC	258
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPRVAGLDFIPRVQSVRTL SKMDQTLAVYQQILTSLSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRN PGC	259
HGEGTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTL SGMDQILATYQQILTSLSRNVIQISNDLENLRDL LHVLAFSKSCPVPARGSDTIKGLGNVLRASVHSTEVALSRLKAALQDMLRQLDRNP	260

Engineered polypeptide	SEQ ID NO:
GC	
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HGEFTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGSASPIQRVQDDTKTLIKTIITRINDISP PQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSLQDMLWQLDL NPGC	262
HGEFTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGSASPIQRVQDDTKTLIKTIITRINDISP PQGVSSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSQSRSVVQIANDLANLRA LLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRLQGSLQDMLWQLDL NPGC	263
HGEFTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQKVQDDT KTLIKTIVTRINDISPPQGVCSRPRVAGLDFIPRVQSVRTLSGMDQILATYQQILTSLSQR SVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRL KAALQDMLRQLDRNP	264
HGEFTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISHTQSVSSKQKVTGLDFIPGLHPILTLSGMDQILATYQQILTSLSQRN VIQISNDLENLRDLLHVLAFSKKSCHLPWASGLETLDLGGVLEASGYSTEVVALSRLKA ALQDMLRQLDRNP	265
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HGEFTFTSDLSKQLEEEAVRLFIEWLKNTGGGGGGGGGGGGGGGGGGGGASPIQRVQDDT KTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLKMDQTLAVYQQILTSLSQR SVVQIANDLANLRALLRLLASAKSCPVPARGSDTIKGLGNVLRASVHSTEVVALSRL KAALQDMLRQLDRNP	267
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Engineered polypeptide	SEQ ID NO:
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HGEGTFTSDLSKQMEEEEAVRLFIEWLKNGGPSTGGGGGASPIQRVQDDTKTLIKTIITRI	305

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Engineered polypeptide	SEQ ID NO:
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Engineered polypeptide	SEQ ID NO:
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Engineered polypeptide	SEQ ID NO:
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[0235] Derivatives. It is understood that engineered polypeptides disclosed herein include those that have been derivatized. Such derivatized engineered polypeptides include conjugation to one or more polymer moieties, such as polyethylene glycol (PEG) or fatty acid chains of various lengths (e.g., stearyl, palmitoyl, octanoyl, etc.), or by the addition of polyamino acids, such as poly-his, poly-arg, poly-lys, and poly-ala. Modifications can also include small molecules moieties, such as short alkyls and constrained alkyls (e.g., branched, cyclic, fused, adamantyl) and aromatic groups. The polymer moieties will typically have a molecular weight from about 500 to about 60,000 Daltons. The polymer may be linear or branched. Such derivatizations can take place at the N- or C-terminus or at a side chain of an amino acid residue, e.g. lysine epsilon amino group, aspartic acid, glutamic acid, cysteine sulfhydryl group, within the Polypeptide Conjugate. Alternatively, derivatization can occur at multiple sites throughout the conjugate polypeptide. To provide a site(s) for derivatization, substitution of one or more amino acids with, or addition of, a lysine, aspartic acid, glutamic acid or cysteine can be done. See for example U.S. Patents 5824784 and 5824778, which are incorporated by reference herein.

[0236] In one embodiment the engineered polypeptides can be conjugated to one, two, or three polymer moieties. In one embodiment, the engineered polypeptides are linked to one polyethylene glycol. Pegylating the engineered polypeptides can improve their aqueous solubility, increase plasma half-life, reduce immunogenicity and/or improve oral uptake. The polyethylene glycol can have a molecular weight from about 200 daltons to about 80,000 daltons; from about 5,000 daltons to about 60,000 daltons; from about 10,000 daltons to about 50,000 daltons; or from about 15,000 daltons to about 40,000 daltons. The polyethylene glycol

may be linear or branched. In one embodiment the pegylated engineered polypeptides includes a lysine side chain to which is covalently attached via the lysine epsilon amino group a polyethylene glycol moiety. In a further embodiment the total molecular weight of the PEG moiety is at least about 10,000 Daltons, at least about 20,000 Daltons, at least about 40,000 Daltons or at least about 60,000 Daltons.

[0237] In one embodiment, engineered polypeptides are linked to one or two polyethylene glycols, where the polyethylene glycol is further linked to a lipophilic moiety. In one embodiment, the polyethylene glycol in this case may have a molecular weight from about 200 to about 7,000 daltons or from about 500 to about 5,000 daltons. The lipophilic moiety may be an alkyl group (e.g., C₁₋₂₀ alkyl group; C₁₋₁₀ alkyl group; C₁₋₆ alkyl group; C₁₋₄ alkyl group), a fatty acid (e.g., C₄₋₂₈ fatty acid chain; C₈₋₂₄ fatty acid chain; C₁₀₋₂₀ fatty acid chain), cholesteryl, adamantyl, and the like. The alkyl group may be linear or branched, preferably linear. In one embodiment, the fatty acid is an acetylated fatty acid or an esterified fatty acid. The – (polyethylene glycol)-(lipophilic moiety) may be linked to the compound at a C-terminal amino acid residue, an N-terminal amino acid residue, an internal amino acid residue (e.g., an internal Lys amino acid residue), or a combination thereof (e.g., the compound is linked at the N-terminal and C-terminal amino acid residues).

[0238] In one embodiment, the engineered polypeptides are linked to a polyamino acid. Exemplary polyamino acids include poly-lysine, poly-aspartic acid, poly-serine, poly-glutamic acid, and the like. The polyamino acid may be in the D or L form, preferably the L form. The polyamino acids may comprise from 1 to 12 amino acid residues; from 2 to 10 amino acid residues; or from 2 to 6 amino acid residues.

[0239] In one embodiment, the engineered polypeptides are linked to a fatty acid. The fatty acid may be a C₄-C₂₈ fatty acid chain, a C₈-C₂₄ fatty acid chain, or a C₁₀-C₂₀ fatty acid chain. In one embodiment, the fatty acid is an acetylated fatty acid. In one embodiment, the fatty acid is an esterified fatty acid.

[0240] In one embodiment, the engineered polypeptides are linked to albumin. The albumin may be a recombinant albumin, serum albumin, or recombinant serum albumin. In another embodiment, the compounds are linked to an albumin-fatty acid (i.e., an albumin linked to a fatty acid).

[0241] In one embodiment, the engineered polypeptides are linked to an immunoglobulin or an immunoglobulin Fc region. The immunoglobulin may be IgG, IgE, IgA, IgD, or IgM. In one embodiment, the compounds are linked to an IgG Fc region or an IgM Fc region. The

immunoglobulin Fc region is (i) the heavy chain constant region 2(C_H2) of an immunoglobulin; (ii) the heavy chain constant region 3(C_H3) of an immunoglobulin; or (iii) both the heavy chain constant regions 2(C_H2) and 3(C_H3) of an immunoglobulin. The immunoglobulin Fc region may further comprise the hinge region at the heavy chain constant region. Other embodiments for the immunoglobulin Fc region that can be linked to extendin analog peptides are described in WO 2008/082274, the disclosure of which is incorporated by reference herein.

[0242] When the engineered polypeptides disclosed herein are covalently linked to one or more polymers, such as those described herein, any linking group known in the art can be used. The linking group may comprise any chemical group(s) suitable for linking the peptide to the polymer. Alternatively, engineered polypeptides can be directly attached to the polymer without any linking group. Exemplary linking groups include amino acids, maleimido groups, dicarboxylic acid groups, succinimide groups, or a combination of two or more thereof. Methods for linking peptides to one or more polymers are known in the art and described, for example, in US Patent No. 6,329,336; US Patent No. 6,423,685; US Patent No. 6,924,264; WO 2005/077072, WO 2007/022123, WO 2007/053946; WO 2008/058461; and WO 2008/082274, the disclosures of which are incorporated by reference herein.

[0243] In one embodiment, there is provided an engineered polypeptide including one or more duration enhancing moieties linked thereto, optionally through a linker. Linkage of the duration enhancing moiety to the peptide can be through a linker as described herein. Alternatively, linkage of the duration enhancing moiety to the peptide can be via a direct covalent bond. The duration enhancing moiety can be a water soluble polymer, or a long chain aliphatic group, as described herein. In some embodiments, a plurality of duration enhancing moieties are attached to the peptide, in which case each linker to each duration enhancing moiety is independently selected from the linkers described herein. The terms “duration enhancing moiety” and the like refer, in the customary sense, to chemical species which lengthen the duration of biological activity of the attached engineered polypeptide.

[0244] In one embodiment, a duration enhancing moiety is covalently bonded to an amino acid side chain of the peptide, or to a backbone atom or moiety thereof. Exemplary backbone moieties include a free amine at the N-terminal, and a free carboxyl or carboxylate at the C-terminal. In one embodiment, an amino acid side chain or a backbone atom or moiety is covalently bonded to a polyethylene glycol, a long chain aliphatic group, or a derivative thereof.

[0245] In one embodiment, the duration enhancing moiety is a water-soluble polymer. A “water soluble polymer” means a polymer which is sufficiently soluble in water under

physiologic conditions of *e.g.*, temperature, ionic concentration and the like, as known in the art, to be useful for the methods described herein. A water soluble polymer can increase the solubility of a peptide or other biomolecule to which such water soluble polymer is attached. Indeed, such attachment has been proposed as a means for improving the circulating life, water
5 solubility and/or antigenicity of administered proteins, *in vivo*. See, *e.g.*, U.S. Pat. No. 4,179,337; U.S. Published Appl. No. 2008/0032408. Many different water-soluble polymers and attachment chemistries have been used towards this goal, such as polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic
10 anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and the like.

[0246] In one embodiment, the duration enhancing moiety includes a polyethylene glycol. Polyethylene glycol ("PEG") has been used in efforts to obtain therapeutically usable peptides. See, *e.g.*, Zalipsky, S., 1995, *Bioconjugate Chemistry* 6:150-165; Mehvar, R., 2000, *J. Pharm.*
15 *Pharmaceut. Sci.* 3:125-136. As appreciated by one of skill in the art, the PEG backbone $[(CH_2CH_2-O)_n]$, n : number of repeating monomers] is flexible and amphiphilic. Without wishing to be bound by any theory or mechanism of action, the long, chain-like PEG molecule or moiety is believed to be heavily hydrated and in rapid motion when in an aqueous medium. This rapid motion is believed to cause the PEG to sweep out a large volume and prevents the
20 approach and interference of other molecules. As a result, when attached to another chemical entity (such as a peptide), PEG polymer chains can protect such chemical entity from immune response and other clearance mechanisms. As a result, pegylation can lead to improved drug efficacy and safety by optimizing pharmacokinetics, increasing bioavailability, and decreasing immunogenicity and dosing frequency. "Pegylation" refers to conjugation of a PEG moiety with
25 another compound. For example, attachment of PEG has been shown to protect proteins against proteolysis. See, *e.g.*, Blomhoff, H. K. *et al.*, 1983, *Biochim Biophys Acta* 757:202-208. Unless expressly indicated to the contrary, the terms "PEG," "polyethylene glycol polymer" and the like refer to polyethylene glycol polymer and derivatives thereof, including methoxy-PEG (mPEG).

[0247] Methods for attaching polymer moieties, such as PEG and related polymers, to reactive
30 groups found on a peptides and proteins are well known in the art. Typical attachment sites in proteins include primary amino groups, such as those on lysine residues or at the N-terminus, thiol groups, such as those on cysteine side-chains, and carboxyl groups, such as those on glutamate or aspartate residues or at the C-terminus. Common sites of attachment are to the

sugar residues of glycoproteins, cysteines or to the N-terminus and lysines of the target peptide. The terms “pegylated” and the like refer to covalent attachment of polyethylene glycol to a peptide or other biomolecule, optionally through a linker as described herein and/or as known in the art.

5 [0248] In one embodiment, a PEG moiety in an engineered polypeptide described herein has a nominal molecular weight within a specified range. The size of a PEG moiety is indicated by reference to the nominal molecular weight, typically provided in kilodaltons (kDa). The molecular weight is calculated in a variety of ways known in the art, including number, weight, viscosity and “Z” average molecular weight. It is understood that polymers, such as PEG and the
10 like, exist as a distribution of molecule weights about a nominal average value.

[0249] Exemplary of the terminology for molecular weight for PEGs, the term “mPEG40KD” refers to a methoxy polyethylene glycol polymer having a nominal molecular weight of 40 kilodaltons. Reference to PEGs of other molecular weights follows this convention. In some
15 embodiments, the PEG moiety has a nominal molecular weight in the range 10-100 kDa, 20-80 kDa, 20-60 kDa, or 20-40 kDa. In some embodiments, the PEG moiety has a nominal molecular weight of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or even 100 kDa. Preferably, the PEG moiety has a molecular weight of 20, 25, 30, 40, 60 or 80 kDa.

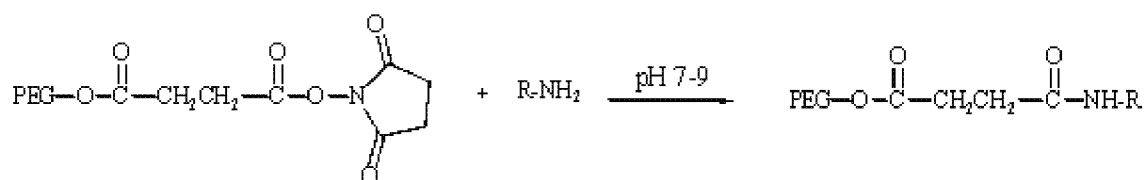
[0250] PEG molecules useful for derivatization of engineered polypeptides are typically classified into linear, branched and comb (*i.e.*, PolyPEG®) classes of PEGs, as known in the art.
20 Furthermore, the terms “two arm branched,” “Y-shaped” and the like refer to branched PEG moieties, as known in the art. The term “comb” in the context of PEGs, also known as “comb” or “comb-type” PEGs, refers to a variety of multi-arm PEGs attached to a backbone, typically poly(methacrylate), as known in the art.

[0251] Covalent attachment of PEG can be conveniently achieved by a variety of methods
25 available to one skilled in the synthetic chemical arts. For pegylation at backbone or side chain amine, PEG reagents are typically reacted under mild conditions to afford the pegylated compound. Optionally, additional steps including but not limited to reduction are employed. In a typical peptide-mPEG conjugation scheme, N-hydroxysuccinimide (NHS) functionalized mPEG can be mixed with peptide having a free amine in a suitable solvent (*e.g.*, dry DMF) under
30 nitrogen in the presence of DIPEA (*e.g.*, 3 equivalents per TFA counterion) for a suitable time (*e.g.*, 24 hrs). The conjugate can be precipitated by the addition of a precipitation reagent (*e.g.*, cold diethyl ether). The precipitate can be isolated by centrifugation and dissolved in water followed by lyophilization. Purification can be afforded by a variety of chromatographic

procedures (*e.g.*, MacroCap SP cation exchange column using gradient 0.5 M NaCl). Purity can be checked by SDS-PAGE. Mass spectrometry (*e.g.*, MALDI) can be used to characterize the conjugate after dialysis against water.

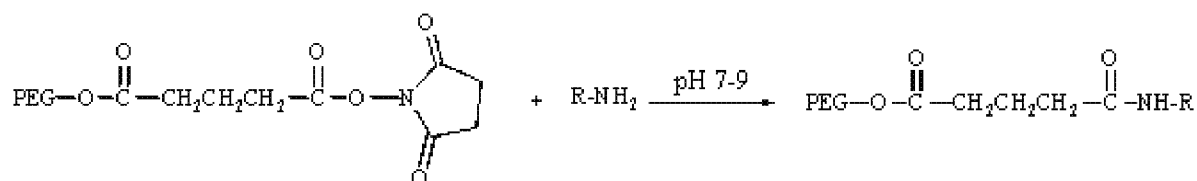
[0252] PEG-SS (succinimidyl succinate). PEG-SS reacts with amine groups under mild conditions to form the amide, as shown in Scheme 1. NHS functionalization provides amino reactive PEG derivatives that can react with primary amine groups at pH 7~9 to form stable amide bonds. Reaction can be finished in 1 hour or even less time. Exemplary reactions follow in Schemes 1 and 2.

Scheme 1.



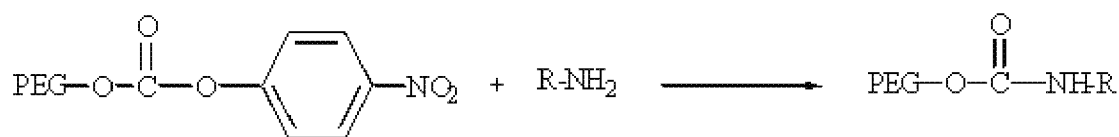
[0253] PEG-SG (succinimidyl glutarate). Similarly, PEG-SG reacts with amine groups to form the corresponding amide, as shown in Scheme 2.

Scheme 2.



[0254] PEG-NPC (p-nitrophenyl carbonate). PEG-NPC reacts with amine functionalities to form the relatively stable urethane functionality, as shown in Scheme 3.

Scheme 3.



[0255] PEG-isocyanate. As shown in Scheme 4, PEG-isocyanate can react with amine to form the resultant relatively stable urethane linkage.

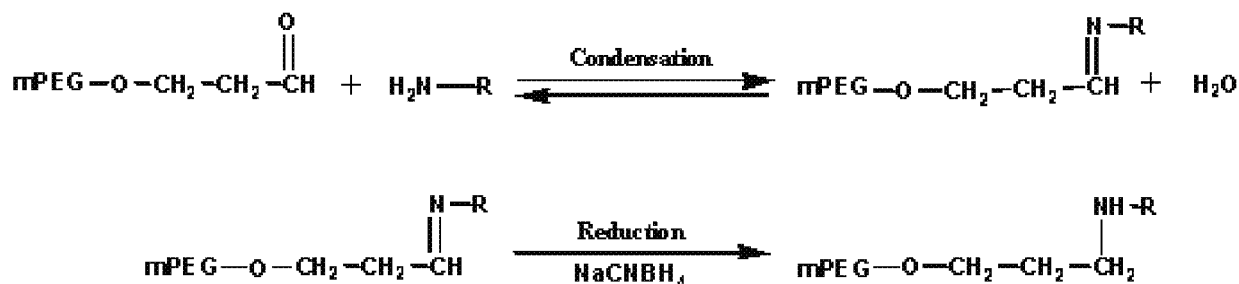
Scheme 4.



[0256] PEG-aldehyde. A variety of PEG-aldehyde reactions with amine can afford the imine, which can be further reduced to afford the pegylated amine. The reaction pH may be important for target selectivity. N-terminal amine pegylation may be at around pH 5. For example,

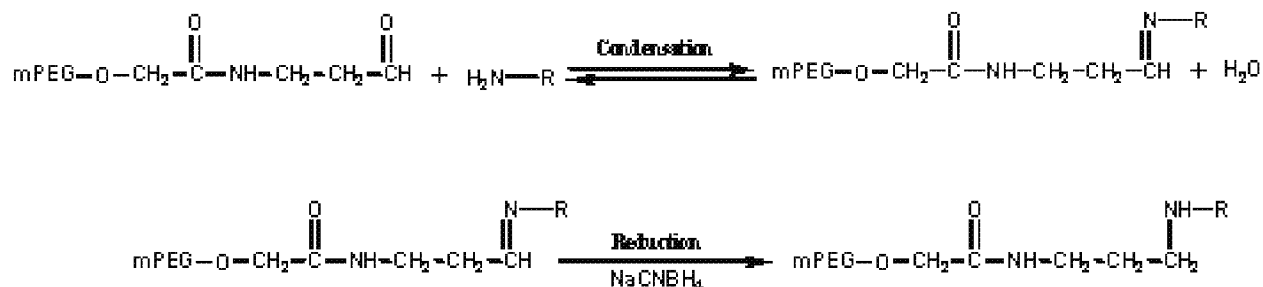
reaction of mPEG-propionaldehyde with peptide amine, followed by reduction affords the compound depicted in Scheme 5 following.

Scheme 5.



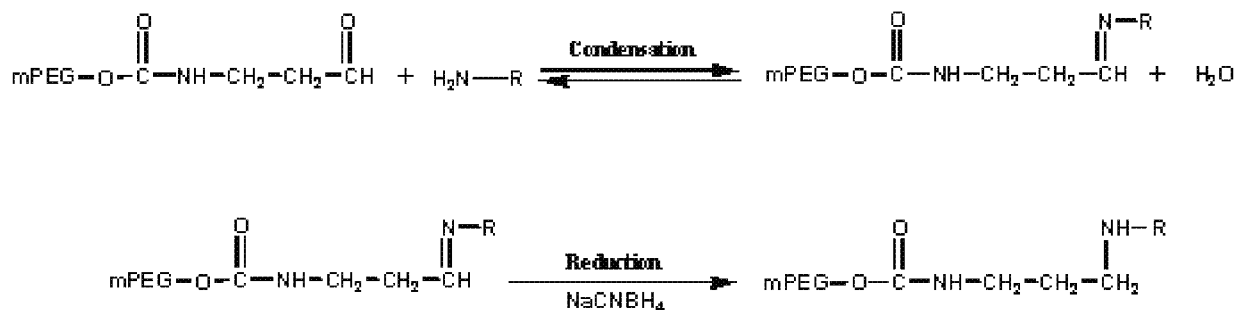
- 5 [0257] Similarly, condensation of mPEG-amide-propionaldehyde with amine and subsequent reduction can afford the compounds depicted in Scheme 6 following.

Scheme 6.

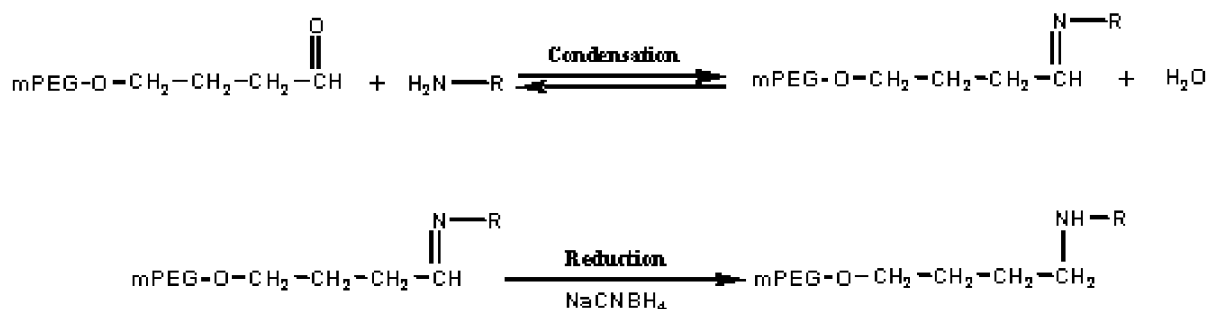


- 10 [0258] Reaction of mPEG-urethane-propionaldehyde with amine and subsequent reduction can afford the compounds depicted in Scheme 7 following.

Scheme 7.

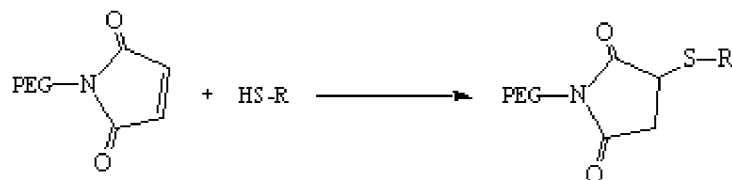


- [0259] Furthermore, reaction of mPEG-butylaldehyde with amine and subsequent reduction can afford the compounds depicted in Scheme 8 following.

Scheme 8.

[0260] Thiol pegylation: PEG-maleimide. Pegylation is conveniently achieved at free thiol groups by a variety of methods known in the art. For example, as shown in Scheme 9 following,

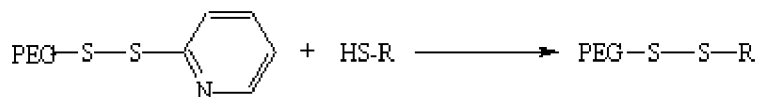
5 PEG-maleimide pegylates thiols of the target compound in which the double bond of the maleimic ring breaks to connect with the thiol. The rate of reaction is pH dependent and best conditions are found around pH 8.

Scheme 9.

[0261] PEG-vinylsulfone. Additionally, as depicted in Scheme 10 following, PEG-vinylsulfone is useful for the pegylation of free thiol.

Scheme 10.

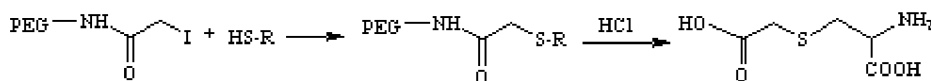
[0262] PEG-orthopyridyl-disulfide (OPSS). Formation of disulfide linked PEG to a peptide is achieved by a variety of methods known in the art, including the reaction depicted in Scheme 11 following. In this type of linkage, the resulting PEG conjugate can be decoupled from the peptide by reduction with, for example but not limited to, borohydride, small molecule dithiol (*e.g.*, dithioerythritol) and the like.

Scheme 11.

[0263] PEG-iodoacetamide. PEG-iodoacetamide pegylates thiols to form stable thioether bonds in mild basic media. This type of conjugation presents an interesting aspect in that by strong acid analysis the pegylated cysteine residue of the protein can give rise to

carboxymethylcysteine which can be evaluated by a standard amino acid analysis (for example, amino acid sequencing), thus offering a method to verify the occurrence of the reaction. A typical reaction scheme is depicted in Scheme 12 following.

Scheme 12.



[0264] In one embodiment, the duration enhancing moiety includes a long chain aliphatic group, and the resulting compound is a long chain peptide conjugate. Accordingly, the term “long chain engineered polypeptide conjugate” as used herein refers to an engineered polypeptide to which a long chain aliphatic group is attached, optionally through a linker. Thus, a further strategy for modulating the duration of activity and potency of peptide and protein therapeutic agents involves derivatizing with long chain aliphatic (*e.g.*, fatty acid) chains of various lengths, for example but not limited to C₆-C₂₄, C₈-C₂₀, C₁₀-C₁₈, C₁₂-C₁₆, and the like. A “fatty acid” as used herein means a long chain aliphatic moiety terminated with a carboxyl functionality. It is understood that long chain aliphatic groups can be fully hydrogenated or partially dehydrogenated. The term “C_x” (*e.g.*, C₆, C₈, and the like) refers to a carbon chain containing “x” carbon atoms. In some embodiments, the carboxyl functionality of a fatty acid is available for bonding with the peptide. Indeed, the acylation of amino groups is a common means employed for chemically modifying proteins, and general methods of acylation are known in the art and include the use of activated esters, acid halides, or acid anhydrides. *See, e.g., Methods of Enzymology* **25**:494-499 (1972), U.S. Patent No. 7,402,565 and RE37,971, each of which is incorporated herein by reference in its entirety and for all purposes. Such long chain conjugation may occur singularly at the N- or C-terminus or at the side chains of amino acid residues within the sequence of the peptide. Linkers may be employed between the long chain aliphatic groups or fatty acid groups and the peptide, as known in the art and described herein. There may be multiple sites available for bonding along the peptide. Substitution of one or more amino acids with lysine, aspartic acid, glutamic acid, or cysteine may provide additional sites for bonding. *See, e.g.*, U.S. Pat. Nos. 5,824,784 and 5,824,778. Fatty acid chain(s) may be linked to an amino, carboxyl, or thiol group, and may be linked by N or C terminus, or at the side chains of lysine, aspartic acid, glutamic acid, or cysteine, as known in the art and/or as described herein. The fatty acid moieties may be linked with diamine and dicarboxylic groups, as known in the art.

[0265] Methods for conjugation of long chain (*e.g.*, C₆-C₂₄) aliphatic groups, preferably fatty acid chains, to peptides are available to the skilled artisan. In one embodiment, the long chain

aliphatic group is C₁₆, C₁₈, C₂₀, C₂₂ or even C₂₄. In one embodiment, the long chain aliphatic group is fully hydrogenated. In some embodiments, the long chain aliphatic group contains one or more double bonds.

III. Methods of Design and Production

5 [0266] **Design of constructs.** The engineered polypeptides described herein can be designed at the amino acid level. These sequences can then be back translated using a variety of software products known in the art such that the nucleotide sequence is optimized for the desired expression host, e.g. based protein expression, codon optimization, restriction site content. For example, the nucleotide sequence can be optimized for *E. coli* based protein expression and for
10 restriction site content. Based on the nucleotide sequence of interest, overlapping oligonucleotides can be provided for multistep PCR, as known in the art. These oligonucleotides can be used in multiple PCR reactions under conditions well known in the art to build the cDNA encoding the protein of interest. For one example is 1X AmpliTaq® Buffer, 1.3 mM MgCl₂, 200uM dNTPs, 4 U AmpliTaq® Gold, 0.2 uM of each primer (AmpliTaq Gold, ABI), with
15 cycling parameters: (94C:30s, 58C:1 min, 72C:1min), 35 cycles.

[0267] Restriction sites can be added to the ends of the PCR products for use in vector ligation as known in the art. Specific sites can include NdeI and XhoI, such that the cDNA can then be in the proper reading frame in a pET45b expression vector (Novagen). By using these sites, any N-terminal His Tag that are in this vector can be removed as the translation start site would then
20 be downstream of the tag. Once expression constructs are completed, verification can be conduct by sequencing using e.g., T7 promoter primer, T7 terminator primer and standard ABI BigDye® Term v3.1 protocols as known in the art. Sequence information can be obtained from e.g., an ABI 3730 DNA Analyzer and can be analyzed using Vector NTI® v.10 software (Invitrogen). Expression constructs can be designed in a modular manner such that linker
25 sequences can be easily cut out and changed, as known in the art.

[0268] Protease recognition sites, known in the art or described herein, can be incorporated into constructs useful for the design, construction, manipulation and production of recombinant engineering polypeptides described herein.

[0269] **Exemplary constructs and expression.** Constructs useful in the production of
30 engineered polypeptides contemplated herein include constructs set forth following.

[0270] Codon optimized nucleotide sequences for all proteins can be generated by overlap PCR and subcloned into a modified pET32 vector (EK cleavage site replaced with TEV cleavage

site) at Kpn1 and Xho1 restriction sites. Sequence verified vector DNA can then be transformed to BL21 cells (Novagen), and induced at 30C O/N in MAGIC MEDIC™ autoinducing media (Invitrogen).

[0271] General methods of production. The engineered polypeptide compounds described herein may be prepared using biological, chemical, and/or recombinant DNA techniques that are known in the art. Exemplary methods are described herein and in US Patent No. 6,872,700; WO 2007/139941; WO 2007/140284; WO 2008/082274; WO 2009/011544; and US Publication No. 2007/0238669, the disclosures of which are incorporated herein by reference in their entireties and for all purposes. Other methods for preparing the compounds are set forth herein.

[0272] The engineered polypeptides compounds described herein may be prepared using standard solid-phase peptide synthesis techniques, such as an automated or semiautomated peptide synthesizer. Briefly and generally, HD1 and HD2 can be made separately and then conjugated together or can be made as a single polypeptide. Thus, HD1 and HD2 may alternatively be produced by non-biological peptide synthesis using amino acids and/or amino acid derivatives having reactive side-chains protected, the non-biological peptide synthesis including step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide according to the first aspect having reactive side-chains protected, removing the protecting groups from the reactive side-chains of the polypeptide, and folding of the polypeptide in aqueous solution. Thus, normal amino acids (e.g. glycine, alanine, phenylalanine, isoleucine, leucine and valine) and pre-protected amino acid derivatives are used to sequentially build a polypeptide sequence, in solution or on a solid support in an organic solvent. When a complete polypeptide sequence is built, the protecting groups are removed and the polypeptide is allowed to fold in an aqueous solution.

[0273] Without wishing to be bound by any theory, it is believed that the engineered polypeptides according to the present disclosure reversibly fold. Accordingly, the engineered polypeptide may be produced by a method including producing HD1 and HD2 according to any method, e.g. as described herein, such as by non-biological peptide synthesis, and conjugating the produced HD1 and HD2 components which then fold completely reversibly. This can be assessed by a variety of methods, e.g., circular dichroism spectra analysis. For example, one spectrum can be taken at 20°C and a second spectrum after heating to 90 °C followed by return to 20 °C. During this procedure the T_m, as known in the art, can determined and used to assess changes after folding of the denatured polypeptide.

[0274] Typically, using such techniques, an alpha-N-carbamoyl protected amino acid and an amino acid attached to the growing peptide chain on a resin are coupled at room temperature (RT) in an inert solvent (e.g., dimethylformamide, N-methylpyrrolidinone, methylene chloride, and the like) in the presence of coupling agents (e.g., dicyclohexylcarbodiimide, 1-

5 hydroxybenzo- triazole, and the like) in the presence of a base (e.g., diisopropylethylamine, and the like). The alpha-N-carbamoyl protecting group is removed from the resulting peptide-resin using a reagent (e.g., trifluoroacetic acid, piperidine, and the like) and the coupling reaction repeated with the next desired N-protected amino acid to be added to the peptide chain. Suitable N-protecting groups are well known in the art, such as t-butyloxycarbonyl (tBoc)

10 fluorenylmethoxycarbonyl (Fmoc), and the like. The solvents, amino acid derivatives and 4-methylbenzhydryl-amine resin used in the peptide synthesizer may be purchased from Applied Biosystems Inc. (Foster City, CA).

[0275] For chemical synthesis solid phase peptide synthesis can be used for the engineered polypeptides, since in general solid phase synthesis is a straightforward approach with excellent
15 scalability to commercial scale, and is generally compatible with relatively long engineered polypeptides. Solid phase peptide synthesis may be carried out with an automatic peptide synthesizer (Model 430A, Applied Biosystems Inc., Foster City, CA) using the NMP/HOBt (Option 1) system and tBoc or Fmoc chemistry (*See APPLIED BIOSYSTEMS USER'S MANUAL FOR THE ABI 430A PEPTIDE SYNTHESIZER*, Version 1.3B Jul. 1, 1988, section 6, pp. 49-70, Applied
20 Biosystems, Inc., Foster City, CA) with capping. Boc-peptide-resins may be cleaved with HF (-5°C to 0°C, 1 hour). The peptide may be extracted from the resin with alternating water and acetic acid, and the filtrates lyophilized. The Fmoc-peptide resins may be cleaved according to standard methods (e.g., *Introduction to Cleavage Techniques*, Applied Biosystems, Inc., 1990, pp. 6-12). Peptides may also be assembled using an Advanced Chem Tech Synthesizer (Model
25 MPS 350, Louisville, Ky.).

[0276] The compounds (exendins, humanized chimeric seal leptins, linkers, engineered polypeptides) described herein may also be prepared using recombinant DNA techniques using methods known in the art, such as Sambrook et al., 1989, *MOLECULAR CLONING: A
LABORATORY MANUAL*, 2d Ed., Cold Spring Harbor. Non-peptide compounds may be prepared
30 by art-known methods. For example, phosphate-containing amino acids and peptides containing such amino acids, may be prepared using methods known in the art, such as described in Bartlett et al, 1986, *Biorg. Chem.*, **14**:356-377. Compounds can be conjugated using art methods or as described herein

[0277] The engineered polypeptides may alternatively be produced by recombinant techniques well known in the art. See, e.g., Sambrook et al., 1989 (*Id.*). These engineered polypeptides produced by recombinant technologies may be expressed from a polynucleotide. One skilled in the art will appreciate that the polynucleotides, including DNA and RNA, that encode such engineered polypeptides may be obtained from the wild-type cDNA, e.g. Exendin-4, taking into consideration the degeneracy of codon usage, and may further engineered as desired to incorporate the indicated substitutions. These polynucleotide sequences may incorporate codons facilitating transcription and translation of mRNA in microbial hosts. Such manufacturing sequences may readily be constructed according to the methods well known in the art. See, e.g., WO 83/04053, incorporated herein by reference in its entirety and for all purposes. The polynucleotides above may also optionally encode an N-terminal methionyl residue. Non-peptide compounds useful in the present invention may be prepared by art-known methods. For example, phosphate-containing amino acids and peptides containing such amino acids may be prepared using methods known in the art. See, e.g., Bartlett and Landen, 1986, *Bioorg. Chem.* 14: 356-77.

[0278] A variety of expression vector/host systems may be utilized to contain and express a engineered polypeptide coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transfected with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (e.g., Ti or pBR322 plasmid); or animal cell systems. Mammalian cells that are useful in recombinant protein productions include but are not limited to VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, COS cells (such as COS-7), WI 38, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562 and 293 cells. Exemplary protocols for the recombinant expression of the protein are described herein and/or are known in the art.

[0279] As such, polynucleotide sequences are useful in generating new and useful viral and plasmid DNA vectors, new and useful transformed and transfected procaryotic and eucaryotic host cells (including bacterial, yeast, and mammalian cells grown in culture), and new and useful methods for cultured growth of such host cells capable of expression of the present engineered polypeptides. The polynucleotide sequences encoding engineered polypeptides herein may be useful for gene therapy in instances where underproduction of engineered polypeptides would be alleviated, or the need for increased levels of such would be met.

[0280] The present invention also provides for processes for recombinant DNA production of the present engineered polypeptides. Provided is a process for producing the engineered polypeptides from a host cell containing nucleic acids encoding the engineered polypeptide including: (a) culturing the host cell containing polynucleotides encoding the engineered polypeptide under conditions facilitating the expression of the DNA molecule; and (b) obtaining the engineered polypeptides.

[0281] Host cells may be prokaryotic or eukaryotic and include bacteria, mammalian cells (such as Chinese Hamster Ovary (CHO) cells, monkey cells, baby hamster kidney cells, cancer cells or other cells), yeast cells, and insect cells.

[0282] Mammalian host systems for the expression of the recombinant protein also are well known to those of skill in the art. Host cell strains may be chosen for a particular ability to process the expressed protein or produce certain post-translation modifications that will be useful in providing protein activity. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing, which cleaves a “prepro” form of the protein, may also be important for correct insertion, folding and/or function. Different host cells, such as CHO, HeLa, MDCK, 293, WI38, and the like, have specific cellular machinery and characteristic mechanisms for such post-translational activities, and may be chosen to ensure the correct modification and processing of the introduced foreign protein.

[0283] Alternatively, a yeast system may be employed to generate the engineered polypeptides of the present invention. The coding region of the engineered polypeptides DNA is amplified by PCR. A DNA encoding the yeast pre-pro-alpha leader sequence is amplified from yeast genomic DNA in a PCR reaction using one primer containing nucleotides 1-20 of the alpha mating factor gene and another primer complementary to nucleotides 255-235 of this gene (Kurjan and Herskowitz, 1982, *Cell*, **30**: 933-43). The pre-pro-alpha leader coding sequence and engineered polypeptide coding sequence fragments are ligated into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such that the promoter directs expression of a fusion protein consisting of the pre-pro-alpha factor fused to the mature engineered polypeptide. As taught by Rose and Broach, (Rose & Broach, 1990, *Meth. Enz.*, **185**: 234-79, Goeddel ed., Academic Press, Inc., San Diego, CA), the vector further includes an ADH2 transcription terminator downstream of the cloning site, the yeast “2-micron” replication origin, the yeast leu-2d gene, the yeast REP1 and REP2 genes, the *E. coli* beta-lactamase gene, and an *E. coli* origin of replication. The beta-lactamase and leu-2d genes provide for selection in bacteria and yeast, respectively. The leu-2d gene also facilitates increased copy number of the plasmid in yeast to

induce higher levels of expression. The REP1 and REP2 genes encode proteins involved in regulation of the plasmid copy number.

[0284] The DNA construct described in the preceding paragraph is transformed into yeast cells using a known method, e.g., lithium acetate treatment (Steams et al., 1990, *Meth. Enz.* **185**: 280-297). The ADH2 promoter is induced upon exhaustion of glucose in the growth media (Price et al., 1987, *Gene* **55**:287). The pre-pro-alpha sequence effects secretion of the fusion protein from the cells. Concomitantly, the yeast KEX2 protein cleaves the pre-pro sequence from the mature engineered polypeptides (Bitter et al., 1984, *Proc. Natl. Acad. Sci. USA* **81**:5330-5334).

[0285] Engineered polypeptides of the invention may also be recombinantly expressed in yeast, e.g. Pichia, using a commercially available expression system, e.g., the Pichia Expression System (Invitrogen, San Diego, CA), following the manufacturer's instructions. This system also relies on the pre-pro-alpha sequence to direct secretion, but transcription of the insert is driven by the alcohol oxidase (AOX1) promoter upon induction by methanol. The secreted engineered polypeptide is purified from the yeast growth medium by, e.g., the methods used to purify said engineered polypeptide from bacterial and mammalian cell supernatants.

[0286] Alternatively, the DNA encoding a engineered polypeptide may be cloned into a baculovirus expression vector, e.g., pVL1393 (PharMingen, San Diego, CA). This engineered-polypeptide-encoding vector is then used according to the manufacturer's directions (PharMingen) or known techniques to infect *Spodoptera frugiperda* cells, grown for example in sf9 protein-free media, and to produce recombinant protein. The protein is purified and concentrated from the media using methods known in the art, e.g. a heparin-Sepharose column (Pharmacia, Piscataway, New Jersey) and sequential molecular sizing columns (Amicon, Beverly, Massachusetts), and resuspended in appropriate solution, e.g. PBS. SDS-PAGE analysis can be used to characterize the protein, for example by showing a single band that confirms the size of the desired engineered polypeptide, as can full amino acid amino acid sequence analysis, e.g. Edman sequencing on a Proton 2090 Peptide Sequencer, or confirmation of its N-terminal sequence.

[0287] For example, the DNA sequence encoding the predicted mature engineered polypeptide may be cloned into a plasmid containing a desired promoter and, optionally, a leader sequence (see, e.g., Better et al., 1988, *Science* **240**:1041-1043). The sequence of this construct may be confirmed by automated sequencing. The plasmid is then transformed into *E. coli*, strain MC1061, using standard procedures employing CaCl₂ incubation and heat shock treatment of the bacteria (Sambrook et al., *Id.*). The transformed bacteria are grown in LB medium supplemented

with carbenicillin, and production of the expressed protein is induced by growth in a suitable medium. If present, the leader sequence will affect secretion of the mature engineered polypeptide and be cleaved during secretion. The secreted recombinant engineered polypeptide is purified from the bacterial culture media by the method described herein.

- 5 [0288] Alternatively, the engineered polypeptides may be expressed in an insect system. Insect systems for protein expression are well known to those of skill in the art. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The engineered polypeptide coding sequence is cloned into a nonessential region of the virus, such as the
- 10 polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of a engineered polypeptide will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which engineered polypeptide of the present invention is expressed (Smith et al., 1983, *J. Virol.* **46**:584; Engelhard et al., 1994, *Proc. Natl. Acad. Sci. USA* **91**:3224-3227).
- 15 [0289] In another example, the DNA sequence encoding the engineered polypeptides may be amplified by PCR and cloned into an appropriate vector, for example, pGEX-3X (Pharmacia, Piscataway, New Jersey). The pGEX vector is designed to produce a fusion protein including glutathione-S-transferase (GST), encoded by the vector, and a protein encoded by a DNA fragment inserted into the vector's cloning site. The primers for the PCR may be generated to
- 20 include, for example, an appropriate cleavage site. The recombinant fusion protein may then be cleaved from the GST portion of the fusion protein. The pGEX-3X/ engineered polypeptide construct is transformed into *E. coli* XL-1 Blue cells (Stratagene, La Jolla, CA), and individual transformants are isolated and grown at 37 degrees C in LB medium (supplemented with carbenicillin) to an optical density at wavelength 600 nm of 0.4, followed by further incubation
- 25 for 4 hours in the presence of 0.5 mM Isopropyl beta-D-Thiogalactopyranoside (Sigma Chemical Co., St. Louis, Missouri). Plasmid DNA from individual transformants is purified and partially sequenced using an automated sequencer to confirm the presence of the desired engineered polypeptide-encoding gene insert in the proper orientation.

- [0290] The fusion protein, when expected to be produced as an insoluble inclusion body in the
- 30 bacteria, may be purified as described above or as follows. Cells are harvested by centrifugation; washed in 0.15 M NaCl, 10 mM Tris, pH 8, 1 mM EDTA; and treated with 0.1 mg/mL lysozyme (Sigma Chemical Co.) for 15 min. at RT. The lysate is cleared by sonication, and cell debris is pelleted by centrifugation for 10 min. at 12,000xg. The fusion protein-containing pellet is resuspended in 50 mM Tris, pH 8, and 10 mM EDTA, layered over 50% glycerol, and

centrifuged for 30 min. at 6000xg. The pellet is resuspended in standard phosphate buffered saline solution (PBS) free of Mg^{++} and Ca^{++} . The fusion protein is further purified by fractionating the resuspended pellet in a denaturing SDS polyacrylamide gel (Sambrook et al., supra). The gel is soaked in 0.4 M KCl to visualize the protein, which is excised and
5 electroeluted in gel-running buffer lacking SDS. If the GST/engineered polypeptide fusion protein is produced in bacteria as a soluble protein, it may be purified using the GST Purification Module (Pharmacia Biotech).

[0291] The fusion protein may be subjected to digestion to cleave the GST from the mature engineered polypeptide. The digestion reaction (20-40 μ g fusion protein, 20-30 units human
10 thrombin (4000 U/mg (Sigma) in 0.5 mL PBS) is incubated 16-48 hrs. at RT and loaded on a denaturing SDS-PAGE gel to fractionate the reaction products. The gel is soaked in 0.4 M KCl to visualize the protein bands. The identity of the protein band corresponding to the expected molecular weight of the engineered polypeptide may be confirmed by partial amino acid
15 sequence analysis using an automated sequencer (Applied Biosystems Model 473A, Foster City, CA).

[0292] In a particularly exemplary method of recombinant expression of the engineered polypeptides of the present invention, mammalian 293 cells may be co-transfected with plasmids containing the engineered polypeptides cDNA in the pCMV vector (5' CMV promoter, 3' HGH poly A sequence) and pSV2neo (containing the neo resistance gene) by the calcium phosphate
20 method. In one embodiment, the vectors should be linearized with ScaI prior to transfection. Similarly, an alternative construct using a similar pCMV vector with the neo gene incorporated can be used. Stable cell lines are selected from single cell clones by limiting dilution in growth media containing 0.5 mg/mL G418 (neomycin-like antibiotic) for 10-14 days. Cell lines are
25 screened for engineered polypeptides expression by ELISA or Western blot, and high-expressing cell lines are expanded for large scale growth.

[0293] It is preferable that the transformed cells are used for long-term, high-yield protein production and as such stable expression is desirable. Once such cells are transformed with vectors that contain selectable markers along with the desired expression cassette, the cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective
30 media. The selectable marker is designed to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell.

[0294] A number of selection systems may be used to recover the cells that have been transformed for recombinant protein production. Such selection systems include, but are not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in tk-, hgp^{rt}- or ap^{rt}- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, that confers resistance to methotrexate; gpt, that confers resistance to mycophenolic acid; neo, that confers resistance to the aminoglycoside, G418; also, that confers resistance to chlorsulfuron; and hyg^{ro}, that confers resistance to hygromycin. Additional selectable genes that may be useful include trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine. Markers that give a visual indication for identification of transformants include anthocyanins, beta-glucuronidase and its substrate, GUS, and luciferase and its substrate, luciferin.

[0295] The engineered polypeptides of the present invention may be produced using a combination of both automated peptide synthesis and recombinant techniques. For example, either or both the HD1 and HD2, and optionally a linker, can be made synthetically or recombinantly and then ligated together using methods known in the art, such as “native chemical ligation” and known variations thereof in which an amide bond is formed joining the parent compounds. See, e.g., United States Patent No. 6326468, which is incorporated herein by reference and for all purposes. Alternatively, for example, an engineered polypeptide of the present invention may contain a combination of modifications including deletion, substitution, insertion and derivatization by PEGylation (or other moiety, e.g. polymer, fatty acyl chain, C-terminal amidation). Such an engineered polypeptide may be produced in stages. In the first stage, an intermediate engineered polypeptide containing the modifications of deletion, substitution, insertion, and any combination thereof, may be produced by recombinant techniques as described. Then after an optional purification step as described herein, the intermediate engineered polypeptide is PEGylated (or subjected to other chemical derivatization, e.g., acylation, C-terminal amidation) through chemical modification with an appropriate PEGylating reagent (e.g., from NeKtar Transforming Therapeutics, San Carlos, CA) to yield the desired engineered polypeptide derivative. One skilled in the art will appreciate that the above-described procedure may be generalized to apply to a engineered polypeptide containing a combination of modifications selected from deletion, substitution, insertion, derivation, and other means of modification well known in the art and contemplated by the present invention.

[0296] C-terminal amidation can be achieved by use of a glycine amino acid-C-terminally extended precursor, synthesized for example in yeast (e.g. Pichia) as alpha-factor fusion protein

that will be secreted into culture medium. After purification, the C-terminal glycine of the engineered polypeptide precursor can be converted to amide by enzymatic amidation, e.g. peptidylglycine alpha-amidating monooxygenase (PAM). See e.g., Cooper et al., 1989, *Biochem. Biophys. Acta*, **1014**:247-258. See also United States Patent 6319685, which is

5 incorporated herein by reference in its entirety and for all purposes, which teaches methods for enzymatic amidation, including an alpha-amidating enzyme from rat being sufficiently pure in alpha-amidating enzyme to exhibit a specific activity of at least about 25 mU per mg of protein, and being sufficiently free of proteolytic impurities to be suitable for use with substrates purified from natural sources or produced by recombinant DNA techniques.

10 **[0297]** Peptides may be purified by any number of methods known in the art, including as described herein. In one method peptides are purified by RP-HPLC (preparative and analytical) using a Waters Delta Prep 3000 system. A C4, C8 or C18 preparative column (10 μ , 2.2X25 cm; Vydac, Hesperia, CA) may be used to isolate peptides, and purity may be determined using a C4, C8 or C18 analytical column (5 μ , 0.46X25 cm; Vydac). Solvents (A=0.1% TFA/water and
15 B=0.1% TFA/CH₃CN) may be delivered to the analytical column at a flow rate of 1.0 ml/min and to the preparative column at 15 ml/min. Amino acid analyses may be performed on the Waters Pico Tag system and processed using the Maxima program. Peptides may be hydrolyzed by vapor-phase acid hydrolysis (115°C, 20-24 h). Hydrolysates may be derivatized and analyzed by standard methods (Cohen et al, THE PICO TAG METHOD: A MANUAL OF ADVANCED
20 TECHNIQUES FOR AMINO ACID ANALYSIS, pp. 11-52, Millipore Corporation, Milford, Mass. (1989)). Fast atom bombardment analysis may be carried out by M-Scan, Incorporated (West Chester, Pa.). Mass calibration may be performed using cesium iodide or cesium iodide/glycerol. Plasma desorption ionization analysis using time of flight detection may be carried out on an Applied Biosystems Bio-Ion 20 mass spectrometer.

25 **[0298] Engineered polypeptide expression assay.** Methods are available for assaying the level of protein expression by a host cell. Procedures useful for assaying the level of protein expression by a host cell are exemplified in the following typical protocol. About 25 μ l BL21 *E. coli* cells are transformed with 2 μ l plasmid DNA (expression vector for the engineered polynucleotide). Cells can be plated and incubated overnight at 37 degrees C or at room
30 temperature (RT) over a 48-hr period. A single colony can be selected and used to grow starter culture in 4 ml LB media with appropriate antibiotic for ~6 hrs. Glycerol stocks can be prepared by adding 100 μ l 80% sterile glycerol to 900 μ l stock, which can then be mixed gently and stored at -80C. A 250 μ l sample can be removed for TCP uninduced sample. An aliquot, for example, 2 ml of Magic media containing appropriate antibiotic can be inoculated with 5 μ l starter culture,

which can then be incubated overnight (up to 24 hrs) at 37C, 300 rpm. As known in the art, Magic Media is autoinducing. Alternatively, 60 ml Magic Media containing appropriate antibiotic can be inoculated with 60 ul starter culture in a 250ml or 125 ml Thompson flask, which can then be incubated overnight (up to 24 hrs) at 30C, 300rpm. After incubation, 250 ul culture can be removed from each tube and the cells pelleted. The cell can be resuspended in 1 ml 50 mM Tris pH 8, 150mM NaCl, to which can be added 0.1 volumes (100ul) POP culture reagent and 1 ul r-lysozyme (1:750 dilution in r-lysozyme buffer). The mixture can be mixed well and incubated at least 10 min at RT. The preparation can then be centrifuge 10 min at 14000 x G. The supernatant (soluble fraction) can be removed and retained, and samples can be prepared for gel analysis (15 ul + 5 ul LDS). The remaining inclusion body pellet can be resuspended in 1ml 1% SDS with sonication. The sample can be prepared for gel analysis (15ul + 5 ul LDS). For uninduced samples, 1.0 volumes POP culture reagent and 1 ul r-lysozyme (1:750 dilution in r-lysozyme buffer) can be added. The mixture can be mixed well and incubated at least 10 min at RT. These samples may not need to be centrifuged. The sample can then be prepared for gel analysis (15ul + 5 ul LDS). NU-PAGE gels (4-12%) non-reduced in 1X MES buffer can be run and stained with SIMPLYBLUE™ microwave protocol. Destaining can be conducted overnight, as known in the art. A gel image can be retained, and analyzed to determine protein expression levels.

[0299] Engineered polypeptides can be expressed and isolated as follows. A protein sequence of the desired engineered polypeptide can be designed and back translated using commercial software to a DNA sequence for cloning into an *E. coli* expression vector. Nucleic acid sequences can be either obtained as oligonucleotides and ligated using standard PCR amplification techniques, or can be digested from existing expression constructs using standard restriction enzymes and then ligated together. Sequences expressing the protein of interest can be placed in plasmid pET45 with a T7 promoter for inducible expression. After constructs are verified by sequencing, the vector DNA can be purified and transformed into an expression host, typically BL21(DE3). A single colony can be selected to grow a starter culture in 4 ml LB media for ~6 hrs. Glycerol stocks can be prepared by adding 100ul 80% glycerol to 900ul stock and stored at -80C. Optionally, 500 ul of un-induced sample can be retained for gel analysis. A 60 ml culture (e.g. MAGICMEDIA™ *E. coli* Expression Medium; Invitrogen, USA; see Glenn et al., *J. Biol. Chem.* 2008, **283**(19):12717-29) can be inoculated using 60ul starter culture in a 125ml Thompson flask and incubated at 30 degrees C overnight. Removed 250ul sample for analysis. The cells can be collected as a pellet by centrifuging, and frozen for later processing. Preparation of cell extract and first pass purification with Nickel resin can be performed as

follows. *E. coli* cell pellets can be completely resuspended in a volume of lysis buffer (50 mM TrisHCl, 150 mM NaCl, pH 8.0) equal to the starting culture volume. Cells can then be subjected to a microfluidizer (Microfluidics, MA) at 100 psi for three times. Cell extracts can be centrifuged for 30 minutes at 16,000 x g to remove debris. EGTA (150mM stock) can be added to the cell extract to a final concentration of 3 mM EGTA. The lysate can then be applied to a Ni-NTA SUPERFLOW™ column that has been washed and pre-equilibrated. Protein bound to the column can then be washed with lysis buffer plus EGTA (50 mM TrisHCl, 150 mM NaCl, pH8.0, 3 mM EGTA) before the bound protein is eluted with 50 mL of elution buffer (25 mM TrisHCl, 50 mM NaCl, 250 mM Imidazol, pH8.0). Cleavage of His-Tag and subsequent purification can be as follows. The eluted protein can be concentrated with Amicon® Ultra15 centrifugal filter unit (Millipore, USA) and then diluted with 25 mM TrisHCl, pH8.0, 50 mM NaCl to prepare for protease digestion which removes the HisTag from the N-terminus of the desired protein. Added can be 0.1% of β-mercaptoethanol and 1% of Turbo TEV protease (2 mg/mL, 10,000 units/mg; Excellgen, USA) to the protein solution, which can be mixed and incubated at room temperature for 4 hours and then at 4°C over night. An Ni-NTA SUPERFLOW™ column (Qiagen, USA) can be pre-equilibrated with 50 mM TrisHCl, 100 mM NaCl, 45 mM imidazole, pH8.0. The TEV digest reaction can be diluted 2-fold with 50 mM TrisHCl, 150 mM NaCl, pH8.0. The diluted digest reaction can be carefully applied to the top of Ni-NTA column and flow-through can be collected. To the column can be added 10 mL of 50 mM trisHCl, 100 mM NaCl, 45 mM imidazole, pH8.0 to elute any unbound protein. The eluted proteins from the column can be collected and combined, and then further purified using size exclusion chromatography (2x with Superdex® 75 HiLoad 26/60 column; GE Healthcare Biosciences, USA). Any remaining bacterial endotoxin can be removed using EndoTrap® Red (Lonza, Switzerland) according to manufacturer's instructions.

[0300] Inclusion Body preparation. For engineered polypeptides that are found in the inclusion body fraction, the following procedure can be beneficial. The cell pellet can be resuspended in a minimum of 100 ml Lysis buffer for each 50 ml culture. Upon the addition of 30ml, a 10ml pipette can be used to resuspend, then the tube can be washed out with an additional 70ml. The resuspended cell solution can be multiply run, e.g., 4 passes, through a microfluidizer@ 100 PSI (min) taking care to keep chamber in ice water through the entire process. The fluidized slurry can be centrifuged at 14000 x g, 20 min (e.g., JLA 10.5, 10,000rpm, using 250 ml nalgene bottles). The inclusion body pellet can be resuspended on ice in chilled lysis buffer with stir bar and stir plate for 1 hour at 4C after disruption with pipette tip. The pellet can be resuspended a second time in distilled H₂O with stir bar and stir plate for 1

hour at 4C after disruption with pipette tip, followed by centrifugation at 14000 x g, 15 min. The supernatant can be removed and discarded. The resultant can be stored at -80C.

[0301] Protein purification. As described herein, numerous methods are known for isolation of expressed polypeptides. Preferred are secreted engineered polypeptides. However, the following is one example if inclusion bodies are formed. Inclusion body pellets can be solubilized in appropriate volume of solubilization buffer (8M urea or 8M guanidine, 50 mM Tris, 10 mM DTT, pH 7.75) for 1 hour at RT. The solubilized pellets can be centrifuged for 20 min at 27 000g. Filtered (e.g., 0.4 um) supernatant can be transferred drop by drop into appropriate volume of refolding buffer (50 mM Tris-HCl, 1 M urea, 0.8 M arginine, 4 mM cysteine, 1 mM cystamine; pH 8) at RT. The result can then be placed at 4°C overnight or longer with gentle mixing. Samples can be concentrated and run on a gel filtration column (SUPERDEX™ 75 26/60) at 1-2 ml/min in 4C environment using a GE Healthsciences AKTA FPLC™. Appropriate protein containing fractions can be identified via SDS-PAGE, pooled and run through a second gel filtration column. Pooled protein can then be concentrated in Amicon filter to appropriate concentration and assayed for endotoxin levels using, e.g., ENDOSAFE® PTS™ Reader (Charles River), as known in the art. Once a protein sample has passed the endotoxin criteria, it can be sterile filtered, dispensed into aliquots and run through quality control assays. Quality control assays can include analytical HPLC-SEC, non reducing SDS PAGE and RP HPLC – MS to obtain approximate mass. Proteins can be obtained in 1xPBS (137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium phosphate, 1.4 mM monopotassium phosphate, pH7.2), distributed into aliquots and flash frozen for storage at -70 to -80 °C.

IV. Methods of Use and Treating Disease

[0302] Indications. A variety of diseases and disorders are contemplated to be beneficially treated by the engineered polypeptides and methods described herein.

[0303] Obesity and overweight. Obesity and its associated disorders including overweight are common and serious public health problems in the United States and throughout the world. Upper body obesity is the strongest risk factor known for type 2 diabetes mellitus and is a strong risk factor for cardiovascular disease. Obesity is a recognized risk factor for hypertension, atherosclerosis, congestive heart failure, stroke, gallbladder disease, osteoarthritis, sleep apnea, reproductive disorders such as polycystic ovarian syndrome, cancers of the breast, prostate, and colon, and increased incidence of complications of general anesthesia. *See, e.g., Kopelman, 2000, Nature 404:635-43.*

[0304] Obesity reduces life-span and carries a serious risk of the co-morbidities listed above, as well disorders such as infections, varicose veins, acanthosis nigricans, eczema, exercise intolerance, insulin resistance, hypertension hypercholesterolemia, cholelithiasis, orthopedic injury, and thromboembolic disease. *See e.g., Rissanen et al, 1990, Br. Med. J., 301:835-7.*

5 Obesity is also a risk factor for the group of conditions called insulin resistance syndrome, or "Syndrome X" and metabolic syndrome. The worldwide medical cost of obesity and associated disorders is enormous.

[0305] The pathogenesis of obesity is believed to be multi-factoral. A problem is that, in obese subjects, nutrient availability and energy expenditure do not come into balance until there is
10 excess adipose tissue. The central nervous system (CNS) controls energy balance and coordinates a variety of behavioral, autonomic and endocrine activities appropriate to the metabolic status of the animal. The mechanisms or systems that control these activities are broadly distributed across the forebrain (e.g., hypothalamus), hindbrain (e.g., brainstem), and spinal cord. Ultimately, metabolic (i.e., fuel availability) and cognitive (i.e., learned preferences)
15 information from these systems is integrated and the decision to engage in appetitive (food seeking) and consummatory (ingestion) behaviors is either turned on (meal procurement and initiation) or turned off (meal termination). The hypothalamus is thought to be principally responsible for integrating these signals and then issuing commands to the brainstem. Brainstem nuclei that control the elements of the consummatory motor control system (e.g., muscles
20 responsible for chewing and swallowing). As such, these CNS nuclei have literally been referred to as constituting the "final common pathway" for ingestive behavior.

[0306] Neuroanatomical and pharmacological evidence support that signals of energy and nutritional homeostasis integrate in forebrain nuclei and that the consummatory motor control system resides in brainstem nuclei, probably in regions surrounding the trigeminal motor
25 nucleus. There are extensive reciprocal connection between the hypothalamus and brainstem. A variety of CNS-directed anti-obesity therapeutics (e.g., small molecules and peptides) focus predominantly upon forebrain substrates residing in the hypothalamus and/or upon hindbrain substrates residing in the brainstem.

[0307] Obesity remains a poorly treatable, chronic, essentially intractable metabolic disorder.

30 Accordingly, a need exists for new therapies useful in weight reduction and/or weight maintenance in a subject. Such therapies would lead to a profound beneficial effect on the subject's health.

[0308] **Diabetes and cardiovascular disease.** Diabetes mellitus is recognized as a complex, chronic disease in which 60% to 70% of all case fatalities among diabetic patients are a result of cardiovascular complications. Diabetes is not only considered a coronary heart disease risk equivalent but is also identified as an independent predictor of adverse events, including recurrent myocardial infarction, congestive heart failure, and death following a cardiovascular incident. The adoption of tighter glucose control and aggressive treatment for cardiovascular risk factors would be expected to reduce the risk of coronary heart disease complications and improve overall survival among diabetic patients. Yet, diabetic patients are two to three times more likely to experience an acute myocardial infarction than non-diabetic patients, and diabetic patients live eight to thirteen years less than non-diabetic patients.

[0309] Understanding the high risk nature of diabetic/acute myocardial infarction patients, the American College of Cardiology/American Heart Association (“ACC/AHA”) clinical practice guidelines for the management of hospitalized patients with unstable angina or non-ST-elevation myocardial infarction (collectively referred to as “ACS”) recently recognized that hospitalized diabetic patients are a special population requiring aggressive management of hyperglycemia. Specifically, the guidelines state that glucose-lowering therapy for hospitalized diabetic/ACS patients should be targeted to achieve preprandial glucose less than 10 mg/dL, a maximum daily target than 180 mg/dL, and a post-discharge hemoglobin A_{1c} less than 7%.

[0310] In a nationwide sample of elderly ACS patients, it was demonstrated that an increase in 30-day mortality in diabetic patients corresponded with the patients having higher glucose values upon admission to the hospital. See “Diabetic Coronary Artery Disease & Intervention,” *Coronary Therapeutics* 2002, Oak Brook, IL, September 20, 2002. There is increasing evidence that sustained hyperglycemia rather than transient elevated glucose upon hospital admission is related to serious adverse events. Although the ideal metric for hyperglycemia and vascular risk in patients is not readily known, it appears that the mean glucose value during hospitalization is most predictive of mortality. In a separate study of ACS patients from over forty hospitals in the United States, it was found that persistent hyperglycemia, as opposed to random glucose values upon admission to the hospital, was more predictive of in-hospital mortality. See *Acute Coronary Syndrome Summit: A State of the Art Approach*, Kansas City, MO, September 21, 2002. Compared with glucose values upon admission, a logistic regression model of glucose control over the entire hospitalization was most predictive of mortality. There was nearly a two-fold increased risk of mortality during hospitalization for each 10 mg/dL increase in glucose over 120 mg/dL. In a smaller cohort of consecutive diabetic/ACS patients, there was a graded increase in mortality at one year with increasing glucose levels upon hospital admission. In the

hospital setting, the ACC/AHA guidelines suggest initiation of aggressive insulin therapy to achieve lower blood glucose during hospitalization.

[0311] Lipid regulation diseases. Dyslipidemia is a disruption in the normal lipid component in the blood. It is believed that prolonged elevation of insulin levels can lead to dyslipidemia.

5 Hyperlipidemia is the presence of raised or abnormal levels of lipids and/or lipoproteins in the blood. Fatty liver disease, e.g., nonalcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver disease ranging from simple fatty liver (steatosis), to nonalcoholic steatohepatitis (NASH), to cirrhosis (irreversible, advanced scarring of the liver). All of the stages of NAFLD have in common the accumulation of fat (fatty infiltration) in the liver cells
10 (hepatocytes).

[0312] Additionally, without wishing to be bound by any theory, it is believed that relative insulin deficiency in type 2 diabetes, glucose toxicity, and increased hepatic free fatty acid burden through elevated delivery from intra-abdominal adipose tissue via the portal vein, are implicated as possible causes in fatty liver disorders. Indeed, it has been hypothesized that eating
15 behavior is the key factor driving the metabolic syndrome of obesity with its many corollaries, including NASH. Accordingly, treatments aimed at decreasing food intake and increasing the number of small meals, as has already been demonstrated in type 2 diabetes, may effectively treat and prevent NASH. Drugs that promote insulin secretion and weight loss, and delay gastric emptying are also effective at improving glucose tolerance and thus may improve fatty liver with
20 its attendant hyperinsulinemia. Thus, use of exendins, exendin analog agonists, exendin derivative agonists, particularly Exendin-4, can be well suited as a treatment modality for this condition. Accordingly, engineered polypeptides described herein which include an exendin or biologically active (hormone domain) peptide component, or fragment or analog thereof, can be useful in the treatment of fatty liver disorders.

25 **[0313] Alzheimer's disease.** Alzheimer's disease (AD), as known in the art, is associated with plaques and tangles in the brain which include dysregulation of the A-beta protein. Stimulation of neuronal GLP-1 receptors has been reported to play an important role in regulating neuronal plasticity and cell survival. GLP-1 has been reported to induce neurite outgrowth and to protect against excitotoxic cell death and oxidative injury in cultured neuronal
30 cells. GLP-1 and Exendin-4 were reported to reduce endogenous levels of amyloid-beta peptide (A-beta protein) in mouse brain and to reduce levels of beta-amyloid precursor protein (beta-APP) in neurons. *See, e.g., Perry et al., 2004, Curr. Drug Targets* **5(6)**:565-571. Treatment with the engineered compounds disclosed herein can provide benefit to the therapeutic targets associated with Alzheimer's disease.

[0314] Parkinson's disease. Parkinson's disease (PD) is the synonym of "primary parkinsonism", i.e. isolated parkinsonism due to a neurodegenerative process without any secondary systemic cause. Parkinsonism is characterized by symptoms of tremor, stiffness, and slowing of movement caused by loss of dopamine. Without wishing to be bound by any theory, it is believed that Exendin-4 may act as a survival factor for dopaminergic neurons by functioning as a microglia-deactivating factor and suggest that Exendin-4 may be a valuable therapeutic agent for neurodegenerative diseases such as PD.

[0315] Metabolic syndrome X. Metabolic Syndrome X is characterized by insulin resistance, dyslipidemia, hypertension, and visceral distribution of adipose tissue, and plays a pivotal role in the pathophysiology of type 2 diabetes. It has also been found to be strongly correlated with NASH, fibrosis, and cirrhosis of the liver. Accordingly, engineered polypeptides described herein can be useful in the treatment of metabolic syndrome X.

[0316] Steroid induced diabetes. Glucocorticoids are well known to affect carbohydrate metabolism. In response to exogenous glucocorticoid administration, increased hepatic glucose production and reduced insulin secretion and insulin-stimulated glucose uptake in peripheral tissues is observed. Furthermore, glucocorticoid treatment alters the proinsulin(P1)/immunoreactive insulin(IRI) ratio, as known in the art. Typical characteristics of the hyperglycemia induced by glucocorticoids in subjects without diabetes include a minimal elevation of fasting blood glucose, exaggerated postprandial hyperglycemia, insensitivity to exogenous insulin, and non-responsiveness to metformin or sulfonylurea therapy. Accordingly, engineered polypeptides described herein which include an exendin biologically active (hormone domain) peptide component, or fragment or analog thereof, can be useful in the treatment of steroid induced diabetes.

[0317] Human Immunodeficiency Virus (HIV) Treatment-Induced Diabetes. Shortly after the introduction of human immunodeficiency virus (HIV)-1 protease inhibitors (PIs) into routine clinical use, reports linking PT use with the development of hyperglycemia began to appear. While approximately 1% to 6% of HIV-infected subjects who are treated with PIs will develop diabetes mellitus, a considerably larger proportion will develop insulin resistance and impaired glucose tolerance. Accordingly, engineered polypeptides described herein which include an exendin biologically active (hormone domain) peptide component, or fragment or analog thereof, can be useful in the treatment of HIV treatment-induced diabetes.

[0318] Latent Autoimmune Diabetes in Adults (LADA). Progressive autoimmune diabetes, also known as latent autoimmune diabetes in adults (LADA), is thought to be present in

approximately 10% of patients diagnosed with type 2 diabetes. LADA patients have circulating antibodies to either islet cell cytoplasmic antigen or, more frequently, glutamic acid decarboxylase. These subjects exhibit clinical features characteristic of both type 1 and type 2 diabetes. Although insulin secretion is better preserved in the slowly progressing than in the rapidly progressing form of autoimmune diabetes, insulin secretion tends to deteriorate with time in LADA subjects. Accordingly, engineered polypeptides described herein which include an exendin biologically active (hormone domain) peptide component, or fragment or analog thereof, can be useful in the treatment of LADA.

[0319] Hypoglycemia Unawareness (HU). Defective glucose counterregulation can occur even after only a single recent episode of hypoglycemia. Subjects who experience repeated episodes of hypoglycemia often lose their capacity to recognize the symptoms typically associated with hypoglycemia or impending insulin shock, a condition called "hypoglycemia unawareness". Because the-patient doesn't appreciate his or her own status, blood glucose levels can then fall so low that serious neurological problems ensue, including coma and seizure. Accordingly, engineered polypeptides described herein which include an exendin biologically active (hormone domain) peptide component, or fragment or analog thereof, can be useful in the treatment of HU.

[0320] Restrictive Lung Disease. GLP 1 receptor has been localized in the lung. Exendins can elicit a biological response via GLP-1 receptor. In particular, sarcoidosis is a systemic granulomatous disease that frequently involves the lung. Although classically thought of as a restrictive lung disease, airway obstruction has become a recognized feature of the disease in the past years. Sarcoidosis can affect the airway at any level and when the involvement includes small airways, it can resemble more common obstructive airway diseases, such as asthma and chronic bronchitis. Accordingly, engineered polypeptides described herein which include an exendin biologically active (hormone domain) peptide component, or fragment or analog thereof, can be useful in the treatment of restrictive lung disease because such hormone domain peptide can improve elasticity of lung or delay rigidity.

[0321] Short Bowel Syndrome (SBS). Exendin-4 has been reported as effective for the treatment of short bowel syndrome. See Kunkel et al. Neurogastroenterol. Motil. (2011). SBS is a serious clinical disorder characterized by diarrhea and nutritional deprivation. Glucagon-like peptide-1 (GLP-1), produced by L-cells in the ileum, regulates proximal gut transit. When extensive ileal resection occurs, as in SBS, GLP-1 levels may be deficient. Exenatide improved the nutritional state and intestinal symptoms of patients with SBS. Accordingly, SBS patients are amenable to treatment with the engineered polypeptides described herein. Improvement in

bowel frequency and form and obtaining bowel movements that are no longer meal-related can be achieved. An additional benefit is that total parenteral nutrition can be stopped. These compounds herein will provide substantial improvement in the bowel habits, nutritional status and quality of life of SBS patients, and further may reduce the need for parenteral nutrition and small bowel transplant.

[0322] Non-alcoholic steatohepatitis (NASH) and Non-alcohol Fatty Liver Disease

(NAFLD). NASH is now considered to be one of the most common liver diseases in western countries. Fatty infiltration is a typical response of the liver to a wide array of noxious stimuli, including hypoxia, toxins, systemic inflammation, malignancies, and various metabolic derangements. Although NASH itself is generally considered to be a benign condition, it may lead to liver fibrosis, cirrhosis, and ultimately failure. NASH is a subcategory of NAFLD characterized histologically by macrovesicular steatosis, ballooning degeneration, hepatocyte necrosis, fibrosis, occasional Mallory bodies, and infiltration of inflammatory cells (American Gastroenterological Association, Technical review on nonalcoholic fatty liver disease.

Gastroenterology 123:1705-1725 (2002)). Although NAFLD and NASH are often asymptomatic, elevated concentrations of serum alanine aminotransferase (ALT), a biochemical marker of liver injury, are indicative of NAFLD, but cannot distinguish between NAFLD and NASH (American Gastroenterological Association, Medical position statement: Nonalcoholic fatty liver disease. Gastroenterology 123:1702-1704 (2002)). Serum concentrations of aspartate aminotransferase (AST) may be higher than ALT, especially in the presence of hepatic cirrhosis, and serum alkaline phosphatase (ALP) concentrations may also be elevated (American Gastroenterological Association, Medical position statement: Nonalcoholic fatty liver disease. Gastroenterology 123:1702-1704 (2002)). However, measures of hepatic functional capacity do not become abnormal until cirrhosis has developed and liver failure is imminent (American Gastroenterological Association, Medical position statement: Nonalcoholic fatty liver disease. Gastroenterology 123:1702-1704 (2002)). In obese T2DM, progressive hepatomegaly due to NAFLD occurs frequently and may be accompanied by right upper quadrant discomfort. NAFLD can cause progressive fibrosis leading to cirrhosis and its complications, including portal hypertension and liver failure (American Gastroenterological Association, Medical position statement: Nonalcoholic fatty liver disease. Gastroenterology 123:1702-1704 (2002)). In addition, NASH is associated with decreased insulin-mediated suppression of lipolysis and the resulting elevation in serum free fatty acid concentrations that contribute to impaired pancreatic .beta.-cell function and increased cardiovascular morbidity and mortality (Yki-Jarvinen et al., Curr. Molec. Med. 5:287-295 (2005); American Gastroenterological Association, Medical

position statement: Nonalcoholic fatty liver disease. Gastroenterology 123:1702-1704 (2002); Raz et al., Diabetes/Metab. Res. Rev. 21:3-14 (2005)).

[0323] Accordingly, in one aspect, there is provided a method for treating a disease in a subject. The subject is in need of treatment for the disease. In one embodiment, the disease is diabetes, overweight, obesity, Alzheimer's disease, short bowel syndrome, fatty liver disease, dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.

[0324] In one embodiment, the subject is need of treatment is obese. In one embodiment, the subject has diabetes. Diabetes can include type I, type II, gestational or pre-diabetes as well as HIV or steroid induced diabetes. The method of treatment includes administration to the subject of a engineered polypeptide as described herein in an amount effective to treatment the disease. Particularly useful for these diseases are compounds described herein having glucose lowering activity (e.g. HD1 fragments or analogs linked to an HD2), having reduction of body weight or reduction of food intake activity, lowering of HbA1c, delaying of gastric emptying, lowering of plasma glucagon, and/or intestinal motility benefit.

[0325] In one embodiment, the disease or disorder is diabetes, overweight, obesity, short bowel syndrome, NASH or Parkinson's disease. In one embodiment, the disease is type I diabetes, type II diabetes or prediabetes. In one embodiment, the disease is type II diabetes.

[0326] In one embodiment, the disease is dyslipidemia or hyperlipidemia.

[0327] In one embodiment, the disease or disorder can be diabetes, overweight, obesity, dyslipidemia, Alzheimer's disease, fatty liver disease, SBS, hyperlipidemia, Parkinson's disease or cardiovascular disease or other diseases described herein. The engineered polypeptide may include an exendin or fragment or analog thereof. Accordingly, the engineered polypeptide can have one of the following structures: HD1-HD2 or HD1-L1-HD2. In some embodiments, the exendin is Exendin-4. In some embodiments, the exendin fragment is a fragment of Exendin-4.

In some embodiments, the exendin analog has at least 70%, for example 70%, 75%, 80%, 85%, 90%, 95% or even higher, identity with Exendin-4. Particularly useful for these diseases are compounds described herein having glucose lowering activity (e.g. Exendin-4 or its fragments or analogs linked to a humanized chimeric seal leptin), having reduction of body weight or reduction of food intake activity, a lowering of HbA1c, delaying of gastric emptying, lowering of plasma glucagon, or intestinal motility benefit.

[0328] Additional diseases and disorders which can be treated by the compounds and methods described herein include steroid-induced diabetes, HIV treatment-induced diabetes, latent

autoimmune diabetes in adults (LADA), hypoglycemia unawareness (HU), restrictive lung disease including sarcoidosis, and metabolic syndrome X. In some embodiments, the exendin domain is Exendin-4. In some embodiments, the exendin domain is a fragment of Exendin-4. In some embodiments, the exendin domain is an analog having at least 70%, e.g., 70%, 75%, 80%, 85%, 90%, 95% or even higher, identity with Exendin-4.

V. Assays

[0329] Methods for production and assay of engineered polypeptides described herein are generally available to the skilled artisan. Further, specific methods are described herein as well as in the patent publications and other references cited herein, which are incorporated by reference for this additional purpose.

[0330] **Amylin Receptor Binding Assays.** The amylin receptor binding assay is a ligand binding assay measuring the potency of test compounds, e.g., polypeptides disclosed herein, in displacing ^{125}I -amylin (rat) from human amylin receptor 3 (AMY3) ectopically expressed in a cell line, e.g., a Codex ACTONE™ cell line. This cell line can be generated using ACTONE™ HEK293-CNG-hCalcR cell line (CB-80200-258) stably expressing human RAMP3 (NCBI protein database CAA04474) to produce the human AMY3 receptor.

[0331] Crude membranes from AMY3 cell cultures can be prepared by homogenization in ice cold 20 mM HEPES containing protease inhibitors (Roche Cat#11873580001). The crude membranes can be incubated with 20 pM ^{125}I - amylin (Perkin Elmer Cat#NEX4480) (2000 Ci/mmol) and increasing concentration of test peptide. Incubation can be carried out in 20 mM HEPES with 5 mM MgCl_2 and 1 mM CaCl_2 for 60 minutes at ambient temperature in 96-well polystyrene plates (Costar Cat#3797). Incubations can be terminated by rapid filtration through UniFilter® 96 plates GF/B (Perkin Elmer, Cat#6005199), pre-soaked for at least 30 minutes in 0.5% polyethylenimine. The Unifilter® plates can be washed several times using ice cold PBS using a MicroMate 96 Cell Harvester (Perkin Elmer). Unifilter plates can then be dried, scintillant added, MICROSCINT™ 20(Perkin Elmer Cat#6013621) and CPM determined by reading on a Perkin Elmer/Wallac TriLux multiwell scintillation counter capable of reading radiolabeled iodine.

[0332] The potency (IC_{50}) of test peptide is determined by the analysis of a concentration-response curve using non-linear regression analysis fitted to a 4-parameter curve. Binding affinities can be calculated using GraphPad Prism® software (GraphPad Software, Inc., San Diego, CA).

[0333] **Calcitonin Receptor Binding Assays.** The calcitonin receptor binding assay is a ligand binding assay measuring the potency of test compounds in displacing ^{125}I -calcitonin (human) from HEK293 cells stably expressing the rat C1a calcitonin receptor. The methodology is the same as for AMY3, wherein ^{125}I -calcitonin (Perkin-Elmer NEX4220) can be used at a concentration of 50 pM.

[0334] **CGRP Receptor Binding Assays.** The calcitonin gene-related peptide (CGRP) receptor binding assay is a ligand assay measuring the potency of test compounds in displacing ^{125}I -CGRP(human) from SKNMC cells, as known in the art, endogenously expressing the human CGRP receptor. The methodology is the same as for AMY3, wherein ^{125}I -CGRP (Perkin-Elmer NEX3540) can be used at a concentration of 50 pM.

[0335] **Adrenomedulin Receptor Binding Assays.** The adrenomedulin receptor binding assay is a ligand binding assay measuring the potency of test compounds in displacing ^{125}I -adrenomedulin (rat) from RAT2 cells, as known in the art, endogenously expressing the rat adrenomedulin receptor. The methodology is the same as for AMY3, wherein ^{125}I -adrenomedulin (Perkin-Elmer NEX4270) can be used at a concentration of 25 pM.

[0336] **GLP-1 Receptor Binding Assays.** The GLP-1 receptor binding assay is a ligand binding assay measuring the potency of test compounds in displacing ^{125}I -GLP-1(human) from 6-23 (clone 6) cells, as known in the art, endogenously expressing the rat GLP-1 receptor. The methodology is the same as for AMY3, wherein ^{125}I -GLP-1 (Perkin-Elmer NEX3080) can be used at a concentration of 60 pM.

[0337] **Leptin Receptor Binding Assays.** The leptin receptor binding assay is a ligand binding assay measuring the potency of test compounds in displacing ^{125}I -leptin (murine) from 32D-OBCEA cells, as known in the art, stably expressing the human Leptin receptor. The methodology is the same as for AMY3, wherein ^{125}I -Leptin(Perkin-Elmer NEX3400) can be used at a concentration of 100 pM, and wherein the incubation time can be increased from 60 minutes to 180 minutes.

[0338] **Amylin Functional Assays.** The amylin functional assay can be used to measure increases in cyclic-AMP (cAMP) in the Codex ACTONETMHEK293-CNG-hCalcR cell line (CB-80200-258) stably transfected with human RAMP3 (NCBI protein database CAA04474) to produce the human AMY3 receptor. Accumulation of cAMP can be measured following 30 minute incubation with test compounds. Efficacy of peptides is determined relative to cell treatment with 10uM forskolin (a constitutive activator of adenylate cyclase). Potency (EC_{50}) of

compounds can be determined by the analysis of a concentration-response curve using non-linear regression analysis fitted to a 4-parameter model, using e.g., GraphPad Prism® software.

[0339] Peptides can be diluted in assay buffer(HBSS, 01%BSA) and incubated with cells in the presence of 250 uM IBMX(Calbiochem 410957). The cAMP can be measured using the cAMP Dynamic 2 assay (Cisbio) as per the manufacturer's instructions. cAMP can be detected by a decrease in time-resolved fluorescence energy transfer (TR-FRET) using an GeniousPro plate reader (Tecan).

[0340] **Calcitonin Functional Assays.** The calcitonin functional assay can be used to measure increases in cyclic-AMP (cAMP) in HEK293 cells stably expressing the rat C1a calcitonin receptor. Accumulation of cAMP can be measured following 30 minute incubation with test compounds. Efficacy of peptides is determined relative to cell treatment with 10uM forskolin (a constitutive activator of adenylate cyclase), and potency (EC_{50}) of peptides is determined by the analysis of a concentration-response curve using non-linear regression analysis fitted to a 4-parameter model.

[0341] Peptides can be diluted in assay buffer(HBSS, 01%BSA) and incubated with cells in the presence of 250 uM IBMX(Calbiochem 410957). The cAMP can be measured using the cAMP Dynamic 2 assay(Cisbio) as per the manufacturer's instructions. cAMP can be detected by a decrease in time-resolved fluorescence energy transfer (TR-FRET) using an GeniousPro plate reader (Tecan).

[0342] **GLP-1 Functional Assays.** The GLP-1 functional assay can be used to measure increases in cyclic-AMP (cAMP) in 6-23(clone 6) cells endogenously expressing the rat GLP-1 receptor. Accumulation of cAMP can be measured following 30 minute incubation with test compounds. Efficacy of peptides can be determined relative to cell treatment with 10uM forskolin (a constitutive activator of adenylate cyclase), and potency (EC_{50}) of peptides can be determined by the analysis of a concentration-response curve using non-linear regression analysis fitted to a 4-parameter model.

[0343] Peptides can be diluted in assay buffer(HBSS, 01%BSA) and incubated with cells in the presence of 250 uM IBMX(Calbiochem 410957). The cAMP can be measured using the cAMP Dynamic 2 assay(Cisbio) as per the manufacturer's instructions. cAMP can be detected by a decrease in time-resolved fluorescence energy transfer (TR-FRET) using an GeniousPro plate reader (Tecan).

[0344] **Leptin Functional Assays.** The leptin functional assay can be used to measure accumulation of phosphorylated STAT5 (Signal Transducer and Activator of Transcription 5) following 30 minute peptide treatment of 32D-OBECA cells at 37°. The measurement of pSTAT5 in the cell lysates can be determined using the Perkin Elmer AlphaScreen® SureFire® pSTAT5 assay kit in a 384-well format (PROXIPLATE™ 384 Plus). Efficacy of peptides can be determined relative to the maximal signal in cell lysates from cells treated with Human leptin (100nM). The EC₅₀ of the peptides can be determined by the analysis of a concentration-response curve using non-linear regression analysis fitted to a 4-parameter model.

[0345] ***In vivo* assays for activity, duration of action and pharmacokinetics.** *In vivo*

assays for activity and duration of action and pharmacokinetics can be done using known methods. For example, duration can be performed using an oral glucose tolerance test (OGTT) in which the drug is administered to the subject at a desired time point before the glucose is administered orally (to measure drug duration of action; OGTT DOA) and glucose blood levels are measured (e.g. readily done in mice). Activity and duration can also be measured using an intravenous glucose tolerance test (IVGTT) in which the drug is administered to the subject at a desired time point before the glucose is administered IV (IVGTT DOA) and blood glucose levels are measured (e.g. can readily be done in rats).

[0346] For example, test compound can be injected subcutaneously at t=0 immediately following a baseline sample into NIH/Swiss female mice. Blood samples are taken at desired time periods such as t= 2, 4, and 8 hours during day 1 and then daily through day 5 or through to day 7 or longer. Blood glucose is measured with a OneTouch® Ultra® (LifeScan, Inc., a Johnson & Johnson Company, Milpitas, CA). For a duration of activity (DOA) determination, such as for glucose control activity of a drug, an OGTT or IVGTT can be performed at the desired point after drug administration. Body weight can also be measured, as well as food intake, or other pharmacological or pharmacokinetic parameter. For example, female NIH/Swiss mice (8-20 weeks old) are group housed with a 12:12 hour light:dark cycle with lights on at 0600. Water and a standard pelleted mouse chow diet is available ad libitum, except as noted. The morning of the experiment, animals are divided into experimental groups and fasted starting at approximately 0630 hrs. In a typical study, n=2 cages with 3 mice/cage. At time=0 min, a blood glucose sample is taken and immediately followed by an intraperitoneal injection of vehicle or compound in an amount ranging from about 1 nmol/kg to 25 nmol/kg. Blood glucose can be measured at 30, 60, 120, 180, and 240 min and daily for a week or longer after the single dose. In a variation of the experiment, doses are provided daily or even weekly over a longer period such as 14 or 28 days. Percent pre-treatment is calculated by dividing the blood glucose

at the measured time point, e.g. 60 minutes or 1 day, by the blood glucose at time=0 min. Significant treatment effects can be identified by ANOVA ($p<0.05$). Where a significant difference exists, test means are compared to the control mean using Dunnett's test (Prism® v. 4.01, GraphPad Software Inc., San Diego, CA). Blood glucose can be measured with a OneTouch® Ultra® (LifeScan, Inc., a Johnson & Johnson Company, Milpitas, CA). * $p<0.05$ vs. vehicle control; ANOVA, Dunnett's test. Other parameters can also be measured.

[0347] In vivo assay for food intake inhibition: The engineered polypeptides may be tested for their duration and extent of appetite suppression and for their duration and extent of effect on body weight loss in various known methods. For example, the polypeptides may be tested for appetite suppression in the mouse food intake assay and for their effect on body weight gain in diet-induced obesity (DIO) mice. An experimental protocol for such assays are described below.

[0348] For example, female NIH/Swiss mice (8-24 weeks old) are group housed with a 12:12 hour light:dark cycle with lights on at 0600. Water and a standard pelleted mouse chow diet are available ad libitum, except as noted. Animals are fasted starting at approximately 1500 hrs, 1 day prior to experiment. The morning of the experiment, animals are divided into experimental groups. In a typical study, $n=4$ cages with 3 mice/cage. At time=0 min, all animals are given an intraperitoneal injection of vehicle or test compound, typically in an amount ranging from about 2 nmol/kg to 75 nmol/kg, and immediately given a pre-weighed amount (10-15g) of standard chow. Food is removed and weighed at various times, typically 30, 60, and 120 minutes or longer, such as daily, to determine the amount of food consumed (Morley, Flood *et al.*, 1994, *Am. J. Physiol.* **267**: R178-R184). Food intake is calculated by subtracting the weight of the food remaining at the e.g., 30 or 60 minute time point, from the weight of the food provided initially at time=0. Significant treatment effects are identified by ANOVA ($p<0.05$). Where a significant difference exists, test means are compared to the control mean using Dunnett's test (Prism® v. 2.01, GraphPad Software Inc., San Diego, CA). Body weight can also be measured.

[0349] Body Weight, fat redistribution, and lean body mass Assays: Assays for body weight and related effects can also be performed as follows. Diet-induced obesity (DIO) in the Sprague-Dawley rat is a valuable model for the study of obesity and regulation of energy homeostasis. These rats were developed from a line of (CrI:CD®(SD)BR) rats that are prone to become obese on a diet relatively high in fat and energy. See, for example, Levin, 1994, *Am. J. Physiol.* **267**:R527-R535, Levin et al., 1997, *Am. J. Physiol.* **273**:R725-R730. DIO male rats are obtained from Charles River Laboratories, Inc. (Wilmington, MA). The rats are housed individually in shoebox cages at 22 °C in a 12/12-hour light dark cycle. Rats are maintained ad-

libitum on a moderately high fat diet (32% kcal from fat; Research Diets D1226B). The animals typically achieve a mean body weight of about 500 g. Levin DIO rats are habituated to caging environment for 7 days. During the 3 nights of habituation, animals receive a single intraperitoneal (IP) injection of vehicle. On test day, rats are administered a single IP injection of compound or vehicle (e.g. 10% DMSO) at the onset of the dark cycle. Food intake is measured by an automated food intake measuring system (BioDAQ, Research Diets) at 5 sec intervals throughout the course of the study. Body weight is recorded nightly.

[0350] Body composition can be measured prior to and after drug treatment using NMR (Echo Medical Systems, Houston, TX). For body composition measurements, rats are briefly placed (~1 min) in a well-ventilated plexiglass tube that was then inserted into a specialized rodent NMR machine. This enabled the calculation of changes in actual grams of fat and dry lean tissue (e.g., grams of body fat after treatment – grams of body fat at baseline = change in grams of body fat) and changes in % body composition for fat and dry lean tissue (e.g., % body fat after treatment - % body fat at baseline = change in % body fat). All data are represented as mean $\pm$ SEM. Analysis of variance (ANOVA) and post-hoc tests are used to test for group difference. A P-value <0.05 is considered significant. Statistical analysis and graphing are performed using PRISM® 4 for Windows (GraphPad Software, Inc., San Diego, CA). Graphs and results are typically presented as vehicle-corrected changes in percent body weight, body fat and changes in body protein

VI. Pharmaceutical Compositions

[0351] In one aspect, there is provided a pharmaceutical composition including an engineered polypeptide described herein in combination with a pharmaceutically acceptable excipient (e.g., carrier). The term “pharmaceutically acceptable carrier,” as used herein refers to pharmaceutical excipients, for example, pharmaceutically, physiologically, acceptable organic or inorganic carrier substances suitable for enteral or parenteral application that do not deleteriously react with the active agent. Suitable pharmaceutically acceptable carriers include water, salt solutions (e.g., Ringer's solution and the like), alcohols, oils, gelatins, and carbohydrates such as lactose, amylose or starch, fatty acid esters, hydroxymethylcellulose, and polyvinyl pyrrolidone. Such preparations can be sterilized and, if desired, mixed with auxiliary agents such as lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and the like that do not deleteriously react with the compounds of the invention.

[0352] In a further aspect, there is provided a pharmaceutical composition which includes a engineered polypeptide as described herein in combination with a pharmaceutically acceptable

excipient. In some embodiments, the pharmaceutical composition is a long lasting pharmaceutical composition. The term “long lasting” in the context of administration of a pharmaceutical composition refers to duration of action. Accordingly, a long lasting pharmaceutical composition may be administered at intervals of, for example, 1 hr, 2 hr, 4 hr, 8
5 hr, 12 hr, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 1 month or even longer. In one embodiment, administration is twice a day (i.e., “twice daily”).

[0353] In one embodiment, the pharmaceutical composition is a parenteral pharmaceutical composition. In one embodiment, the pharmaceutical composition is a sustained release or long lasting pharmaceutical composition. In one embodiment, the pharmaceutical composition is
10 formulated as a twice daily pharmaceutical composition. In one embodiment, the pharmaceutical composition is formulated as a once daily pharmaceutical composition. In one embodiment, the pharmaceutical composition is formulated as a once weekly pharmaceutical composition.

[0354] In one embodiment, the pharmaceutical composition is useful for treating a disease in a subject. In one embodiment, the disease is diabetes, overweight, obesity, Alzheimer’s disease, fatty liver disease, short bowel syndrome, dyslipidemia, coronary artery disease, stroke,
15 hyperlipidemia, NASH or Parkinson's disease. In one embodiment, the disease is diabetes, overweight, obesity, short bowel syndrome, or Parkinson's disease. In one embodiment, the disease is type I diabetes, type II diabetes or prediabetes.

[0355] In one embodiment, the pharmaceutical composition includes an engineered polypeptide as set forth in Table 1 (SEQ ID NOS:184-375). In one embodiment, the
20 pharmaceutical composition is an engineered polypeptide as set forth in Table 1 (SEQ ID NOS:184-375).

[0356] In one embodiment, the pharmaceutical composition includes an engineered polypeptide with sequence (SEQ ID NO:261).

25 A. Formulations

[0357] The engineered polypeptides described herein can be administered alone or can be co-administered to a subject. Co-administration is meant to include simultaneous or sequential administration of the compounds individually or in combination (more than one compound). For example, it has been found that obesity can be beneficially treated with a combination therapy
30 including leptin (e.g., metreleptin) and an amylin (e.g., pramlintide). See e.g., U.S. Published Appl. No. 2008/0207512. Accordingly, an engineered polypeptide described herein useful for treatment of e.g., obesity and overweight, can be administered alone to achieve such treatment or

co-administered with either a leptin or leptin agonist, e.g. metreleptin, and/or an amylin or amylin agonist, e.g. pramlintide.

[0358] In some embodiments, the formulations and methods described herein further provide that the engineered polypeptide is co-administered with one or more anti-diabetic agents, such as anti-hyperglycemia agents, e.g. insulin (including regular, short acting, long-acting, and basal insulins), amylin, pramlintide, metformin and thiazolidinediones (including rosiglitazone and pioglitazone).

[0359] In some embodiments, the formulations and methods described herein further provide that the engineered polypeptide is co-administered with one or more cholesterol and/or triglyceride lowering agents. Exemplary agents include HMG CoA reductase inhibitors (e.g., atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, simvastatin); bile acid sequestrants (e.g., colestyramine, colestipol); fibrates (e.g., fenofibrate, clofibrate, gemfibrozil); ezetimibe, nicotinic acid, probucol, a lovastatin/niacin combination; an atorvastatin/amlodipine combination; and a simvastatin/ezetimibe combination.

[0360] The present disclosure provides the composition for use as a medicament, i.e. for use in therapy, since the exendin compound is a therapeutically active compound, and surprisingly retains activity when fused to a humanized chimeric seal leptin or analog or fragment thereof. Compositions including an engineered polypeptide, either liquid or dry form, and optionally at least one pharmaceutically acceptable carrier and/or excipient are also specifically contemplated herein.

[0361] Co-administration can be achieved by separately administering the exendin, exendin agonist, or exendin analog agonist engineered polypeptide with the second agent, or by administering a single pharmaceutical formulation including the exendin, exendin agonist, or exendin analog agonist engineered polypeptide and the second agent. Appropriate dosage regimens for the second agents are generally known in the art.

[0362] The preparations can also be co-administered, when desired, with other active substances (e.g. to reduce metabolic degradation) as known in the art or other therapeutically active agents. An exendin engineered polypeptide described herein can be administered with other active anti-diabetes or anti-obesity agents, such as leptin or leptin agonists and amylin or amylin agonist compounds, e.g. the amylin, including daivalintide and their analogs.

[0363] **Amylins.** Amylin is a peptide hormone synthesized by pancreatic β -cells that is co-secreted with insulin in response to nutrient intake. The sequence of amylin is highly preserved across mammalian species, with structural similarities to calcitonin gene-related peptide (CGRP),

the calcitonins, the intermedins, and adrenomedulin, as known in the art. The glucoregulatory actions of amylin complement those of insulin by regulating the rate of glucose appearance in the circulation via suppression of nutrient-stimulated glucagon secretion and slowing gastric emptying. In insulin-treated patients with diabetes, pramlintide, a synthetic and equipotent
 5 analogue of human amylin, reduces postprandial glucose excursions by suppressing inappropriately elevated postprandial glucagon secretion and slowing gastric emptying. The sequences of rat amylin, human amylin and pramlintide follow:

KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY (SEQ ID NO:376);

KCNTATCATQRLANFLVHSSNNFSGAILSTNVGSNTY (SEQ ID NO:377);

10 KCNTATCATQRLANFLVHSSNNFGPILPPTNVGSNTY (SEQ ID NO:378).

[0364] Divalintide. Divalintide, also known as “AC-2307” is a potent amylin agonist useful in the treatment of a variety of disease indications. *See* WO 2006/083254 and WO 2007/114838, each of which is incorporated by reference herein in its entirety and for all purposes. Divalintide is a chimeric peptide, having an N-terminal loop region of amylin or calcitonin and analogs
 15 thereof, an alpha-helical region of at least a portion of an alpha-helical region of calcitonin or analogs thereof or an alpha-helical region having a portion of an amylin alpha-helical region and a calcitonin alpha-helical region or analog thereof, and a C-terminal tail region of amylin or calcitonin. The sequences of human calcitonin, salmon calcitonin and divalintide follow:

CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP (SEQ ID NO:379);

20 CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP (SEQ ID NO:380);

KCNTATCVLGRLSQELHRLQTYPRTNTGSNTY (SEQ ID NO:381).

[0365] Without wishing to be bound by any theory, it is believed that amylin and divalintide, and fragment and analogs thereof, can require C-terminal amidation to elicit a full biological response. It is understood that amylin compounds such as those described herein
 25 which include amylin and/or divalintide, and fragment and analogs thereof, can be amidated at the C-terminal.

[0366] “Amylin agonist compounds” include native amylin peptides, amylin analog peptides, and other compounds (e.g., small molecules) that have amylin agonist activity. The “amylin agonist compounds” can be derived from natural sources, can be synthetic, or can be derived
 30 from recombinant DNA techniques. Amylin agonist compounds have amylin agonist receptor binding activity and may include amino acids (e.g., natural, unnatural, or a combination thereof), peptide mimetics, chemical moieties, and the like. The skilled artisan will recognize amylin agonist compounds using amylin receptor binding assays or by measuring amylin agonist activity in soleus muscle assays. In one embodiment, amylin agonist compounds will have an

IC₅₀ of about 200 nM or less, about 100 nM or less, or about 50 nM or less, in an amylin receptor binding assay, such as that described herein, in US Patent No. 5,686,411, and US Publication No. 2008/0176804, the disclosures of which are incorporated by reference herein in their entireties and for all purposes. In one embodiment, amylin agonist compounds will have an EC₅₀ of about 20 nM or less, about nM 15 or less, about nM 10 or less, or about nM 5 or less in a soleus muscle assay, such as that described herein and in US Patent No. 5,686,411. In one embodiment, the amylin agonist compound has at least 90% or 100% sequence identity to ^{25,28,29}Pro-human-amylin. In one embodiment, the amylin agonist compound is a peptide chimera of amylin (e.g., human amylin, rat amylin, and the like) and calcitonin (e.g., human calcitonin, salmon calcitonin, and the like). Suitable and exemplary amylin agonist compounds are also described in US Publication No. 2008/0274952, the disclosure of which is incorporated by reference herein in its entirety and for all purposes.

[0367] When co-administered with another active agent, the compounds can be administered simultaneously or sequentially, together or separately formulated. Since the engineered compounds herein are inherently long-acting, they are suitable for once daily, once weekly or longer administration. Accordingly, the other agent may be administered either in one or multiple doses, e.g. once daily, twice daily, three times daily, once weekly, as needed, during the period of dosing for the exendin engineered polypeptide, e.g. once weekly.

[0368] Single and multiple-use formulations of other agents such as amylin compounds have been reported. For example, pramlintide has been formulated for and successfully administered for once, twice and three times daily administration for treating diabetes and for treating obesity.

[0369] These pharmaceutical compounds may be formulated with pharmaceutically acceptable carriers or diluents as well as any other known adjuvants and excipients in accordance with conventional techniques such as those disclosed in *Remington's Pharmaceutical Sciences* by E. W. Martin. See also Wang et al. (1988) *J. of Parenteral Sci. and Tech.*, Technical Report No. 10, Supp. 42:2 S.

[0370] In general, the engineered polypeptides may be formulated into a stable, safe pharmaceutical composition for administration to a patient. Pharmaceutical formulations contemplated for use in the methods of the invention may include approximately 0.01 to 1.0% (w/v), in certain cases 0.05 to 1.0%, of the engineered polypeptide, approximately 0.02 to 0.5% (w/v) of an acetate, phosphate, citrate or glutamate buffer allowing a pH of the final composition of from about 3.0 to about 7.0; approximately 1.0 to 10% (w/v) of a carbohydrate or polyhydric alcohol tonicifier and, optionally, approximately 0.005 to 1.0% (w/v) of a preservative selected

from the group of m-cresol, benzyl alcohol, methyl, ethyl, propyl and butyl parabens and phenol. Such a preservative is generally included if the formulated peptide is to be included in a multiple use product.

[0371] In particular embodiments, a pharmaceutical formulation of the present engineered polypeptides may contain a range of concentrations of the compound(s), e.g., between about 0.01% to about 98% w/w, or between about 1 to about 98% w/w, or preferably between 80% and 90% w/w, or preferably between about 0.01% to about 50% w/w, or more preferably between about 10% to about 25% w/w in these embodiments. A sufficient amount of water for injection may be used to obtain the desired concentration of solution.

[0372] Additional tonicifying agents such as sodium chloride, as well as other known excipients, may also be present, if desired. In some cases, such excipients are useful in maintenance of the overall tonicity of the compound. An excipient may be included in the presently described formulations at various concentrations. For example, an excipient may be included in the concentration range from about 0.02% to about 20% w/w, preferably between about 0.02% and 0.5% w/w, about 0.02% to about 10% w/v, or about 1% to about 20% w/w. In addition, similar to the present formulations themselves, an excipient may be included in solid (including powdered), liquid, semi-solid or gel form.

[0373] The pharmaceutical formulations may be composed in various forms, e.g., solid, liquid, semisolid or liquid. The term "solid", as used herein, is meant to encompass all normal uses of this term including, for example, powders and lyophilized formulations. The presently described formulations may be lyophilized.

[0374] The terms buffer, buffer solution and buffered solution, when used with reference to hydrogen-ion concentration or pH, refer to the ability of a system, particularly an aqueous solution, to resist a change of pH on adding acid or alkali, or on dilution with a solvent.

Characteristic of buffered solutions, which undergo small changes of pH on addition of acid or base, is the presence either of a weak acid and a salt of the weak acid, or a weak base and a salt of the weak base. An example of the former system is acetic acid and sodium acetate. The change of pH is slight as long as the amount of hydronium or hydroxyl ion added does not exceed the capacity of the buffer system to neutralize it.

[0375] As described herein, a variety of liquid vehicles are suitable for use in the formulations of engineered polypeptides, for example, water or an aqueous/organic solvent mixture or suspension.

[0376] The stability of a engineered polypeptide formulation for use as described herein is enhanced by maintaining the pH of the formulation in a range determined by methods known in the art. In certain embodiments, the pH of the formulation is maintained in the range of about 3.5 to 5.0, or about 3.5 to 6.5, in some embodiments from about 3.7 to 4.3, or about 3.8 to 4.2.

5 In some embodiments, pH may be about 4.0, about 5.0, about 6.0, about 7.0, about 8.0, about 9.0, or even higher. In some embodiments, pH may be in the physiological range, pH 6-8, preferably pH 7-7.6.

[0377] In certain embodiments, the buffer with the engineered polypeptide is an acetate buffer (preferably at a final formulation concentration of from about 1-5 to about 60 mM), phosphate
10 buffer (preferably at a final formulation concentration of from about 1-5 to about to about 30 mM) or glutamate buffer (preferably at a final formulation concentration of from about 1-5 to about to about 60 mM). In some embodiments, the buffer is acetate (preferably at a final formulation concentration of from about 5 to about 30 mM).

[0378] A stabilizer may be included in the formulations but is not necessarily needed. If
15 included, however, a stabilizer useful in the practice of the present invention is a carbohydrate or a polyhydric alcohol. A suitable stabilizer useful in the practice of the present invention is approximately 1.0 to 10% (w/v) of a carbohydrate or polyhydric alcohol. The polyhydric alcohols and carbohydrates share the same feature in their backbones, i.e., --CHOH--CHOH--, which is responsible for stabilizing the proteins. The polyhydric alcohols include such
20 compounds as sorbitol, mannitol, glycerol, and polyethylene glycols (PEGs). These compounds are straight-chain molecules. The carbohydrates, such as mannose, ribose, sucrose, fructose, trehalose, maltose, inositol, and lactose, on the other hand, are cyclic molecules that may contain a keto or aldehyde group. These two classes of compounds have been demonstrated to be effective in stabilizing protein against denaturation caused by elevated temperature and by
25 freeze-thaw or freeze-drying processes. Suitable carbohydrates include: galactose, arabinose, lactose or any other carbohydrate which does not have an adverse affect on a diabetic patient, i.e., the carbohydrate is not metabolized to form unacceptably large concentrations of glucose in the blood. Such carbohydrates are well known in the art as suitable for diabetics. Sucrose and fructose are suitable for use with the compound in non-diabetic applications (e.g. treating
30 obesity).

[0379] In certain embodiments, if a stabilizer is included, the compound is stabilized with a polyhydric alcohol such as sorbitol, mannitol, inositol, glycerol, xylitol, and polypropylene/ethylene glycol copolymer, as well as various polyethylene glycols (PEG) of molecular weight 200, 400, 1450, 3350, 4000, 6000, 8000 and even higher). Mannitol is the

preferred polyhydric alcohol in some embodiments. Another useful feature of the lyophilized formulations of the present invention is the maintenance of the tonicity of the lyophilized formulations described herein with the same formulation component that serves to maintain their stability. In some embodiments, mannitol is the preferred polyhydric alcohol used for this purpose.

[0380] The United States Pharmacopeia (USP) states that anti-microbial agents in bacteriostatic or fungistatic concentrations must be added to preparations contained in multiple dose containers. They must be present in adequate concentration at the time of use to prevent the multiplication of microorganisms inadvertently introduced into the preparation while withdrawing a portion of the contents with a hypodermic needle and syringe, or using other invasive means for delivery, such as pen injectors. Antimicrobial agents should be evaluated to ensure compatibility with all other components of the formula, and their activity should be evaluated in the total formula to ensure that a particular agent that is effective in one formulation is not ineffective in another. It is not uncommon to find that a particular antimicrobial agent will be effective in one formulation but not effective in another formulation.

[0381] A preservative is, in the common pharmaceutical sense, a substance that prevents or inhibits microbial growth and may be added to pharmaceutical formulations for this purpose to avoid consequent spoilage of the formulation by microorganisms. While the amount of the preservative is not great, it may nevertheless affect the overall stability of the peptide.

[0382] While the preservative for use in the pharmaceutical compositions can range from 0.005 to 1.0% (w/v), in some embodiments range for each preservative, alone or in combination with others, is: benzyl alcohol (0.1-1.0%), or m-cresol (0.1-0.6%), or phenol (0.1-0.8%) or combination of methyl (0.05-0.25%) and ethyl or propyl or butyl (0.005%-0.03%) parabens. The parabens are lower alkyl esters of para-hydroxybenzoic acid. A detailed description of each preservative is set forth in *Remington's Pharmaceutical Sciences (Id.)*

[0383] Engineered polypeptides may not have a tendency to adsorb onto the glass in a glass container when in a liquid form, therefore, a surfactant may not be required to further stabilize the pharmaceutical formulation. However, with regard to compounds which do have such a tendency when in liquid form, a surfactant should be used in their formulation. These formulations may then be lyophilized. Surfactants frequently cause denaturation of protein, both of hydrophobic disruption and by salt bridge separation. Relatively low concentrations of surfactant may exert a potent denaturing activity, because of the strong interactions between surfactant moieties and the reactive sites on proteins. However, judicious use of this interaction

can stabilize proteins against interfacial or surface denaturation. Surfactants which could further stabilize the engineered polypeptide may optionally be present in the range of about 0.001 to 0.3% (w/v) of the total formulation and include polysorbate 80 (i.e., polyoxyethylene(20) sorbitan monooleate), CHAPS® (i.e., 3-[(3-cholamidopropyl) dimethylammonio]1-propanesulfonate), Brij® (e.g., Brij® 35, which is (polyoxyethylene (23) lauryl ether), poloxamer, or another non-ionic surfactant.

[0384] It may also be desirable to add sodium chloride or other salt to adjust the tonicity of the pharmaceutical formulation, depending on the tonicifier selected. However, this is optional and depends on the particular formulation selected. Parenteral formulations preferably may be isotonic or substantially isotonic.

[0385] A preferred vehicle for parenteral products is water. Water of suitable quality for parenteral administration can be prepared either by distillation or by reverse osmosis. Water for injection is the preferred aqueous vehicle for use in the pharmaceutical formulations.

[0386] It is possible that other ingredients may be present in the pharmaceutical formulations.

Such additional ingredients may include, e.g., wetting agents, emulsifiers, oils, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous vehicles, proteins (e.g., human serum albumin, gelatin or proteins) and a zwitterion (e.g., an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Additionally, polymer solutions, or mixtures with polymers provide the opportunity for controlled release of the peptide. Such additional ingredients, of course, should not adversely affect the overall stability of the pharmaceutical formulation of the present invention.

[0387] Containers are also an integral part of the formulation of an injection and may be considered a component, for there is no container that is totally inert, or does not in some way affect the liquid it contains, particularly if the liquid is aqueous. Therefore, the selection of a container for a particular injection must be based on a consideration of the composition of the container, as well as of the solution, and the treatment to which it will be subjected. Adsorption of the peptide to the glass surface of the vial can also be minimized, if necessary, by use of borosilicate glass, for example, Wheaton Type I borosilicate glass #33 (Wheaton Type I-33) or its equivalent (Wheaton Glass Co.). Other vendors of similar borosilicate glass vials and cartridges acceptable for manufacture include Kimbel Glass Co., West Co., Bunder Glas GMBH and Form a Vitrum. The biological and chemical properties of the compound may be stabilized by formulation and lyophilization in a Wheaton Type I-33 borosilicate serum vial to a final

concentration of 0.1 mg/ml and 10 mg/ml of the compound in the presence of 5% mannitol, and 0.02% Tween 80.

[0388] For formulations to be delivered by injection, in order to permit introduction of a needle from a hypodermic syringe into a multiple-dose vial and provide for resealing as soon as the needle is withdrawn, the open end of each vial is preferably sealed with a rubber stopper closure held in place by an aluminum band.

[0389] Stoppers for glass vials, such as, West 4416/50, 4416/50 (Teflon faced) and 4406/40, Abbott 5139 or any equivalent stopper can be used as the closure for pharmaceutical for injection. For formulations including peptidic anti-obesity agents, these stoppers are compatible with the peptide as well as the other components of the formulation. The inventors have also discovered that these stoppers pass the stopper integrity test when tested using patient use patterns, e.g., the stopper can withstand at least about 100 injections. Alternatively, the peptide can be lyophilized in to vials, syringes or cartridges for subsequent reconstitution. Liquid formulations of the present invention can be filled into one or two chambered cartridges, or one or two chamber syringes.

[0390] Each of the components of the pharmaceutical formulation described above is known in the art and is described in *Pharmaceutical Dosage Forms: Parenteral Medications*, Vol. 1, 2nd ed., Avis et al. Ed., Mercel Dekker, New York, N.Y. 1992, which is incorporated by reference in its entirety herein.

[0391] The manufacturing process for the above liquid formulations generally involves compounding, sterile filtration and filling steps. The compounding procedure involves dissolution of ingredients in a specific order (preservative followed by stabilizer/tonicity agents, buffers and peptide) or dissolving at the same time.

[0392] Alternative formulations, e.g., non-parenteral, may not require sterilization. However, if sterilization is desired or necessary, any suitable sterilization process can be used in developing the peptide pharmaceutical formulation of the present invention. Typical sterilization processes include filtration, steam (moist heat), dry heat, gases (e.g., ethylene oxide, formaldehyde, chlorine dioxide, propylene oxide, beta-propiolactone, ozone, chloropicrin, peracetic acid methyl bromide and the like), exposure to a radiation source, and aseptic handling. Filtration is the preferred method of sterilization for liquid formulations of the present invention. The sterile filtration involves filtration through 0.45 um and 0.22 um (1 or 2) which may be connected in series. After filtration, the solution is filled into appropriate vials or containers.

[0393] In certain embodiments, the engineered polypeptides described herein are administered peripherally to the subjects. In some embodiments, the liquid pharmaceutical formulations of the present invention are intended for parenteral administration. Suitable routes of administration include intramuscular, intravenous, subcutaneous, intradermal, intraarticular, intrathecal and the like. In some embodiments, the subcutaneous route of administration is preferred.

[0394] In addition, parenteral controlled release delivery can be achieved by forming polymeric microcapsules, matrices, solutions, implants and devices and administering them parenterally or by surgical means. Examples of controlled release formulations are described in U.S. Pat. Nos. 6,368,630, 6,379,704, and 5,766,627, which are incorporated herein by reference. These dosage forms may have a lower bioavailability due to entrapment of some of the peptide in the polymer matrix or device. *See e.g.*, U.S. Pat. Nos. 6,379,704, 6,379,703, and 6,296,842, each of which is incorporated herein by reference in its entirety and for all purposes.

[0395] The compounds may be provided in dosage unit form containing an amount of the engineered polypeptide that will be effective in one or multiple doses.

[0396] As will be recognized by those in the field, an effective amount of the engineered polypeptide will vary with many factors including the age and weight of the subject, the subject's physical condition, the condition to be treated, and other factors known in the art. An effective amount of the engineered polypeptides will also vary with the particular combination administered. As described herein, administration of the engineered polypeptides in combination may allow for a reduced amount of any of the administered engineered polypeptides to be an effective amount.

B. Effective Dosages

[0397] Pharmaceutical compositions provided herein include compositions wherein the active ingredient is contained in a therapeutically effective amount, i.e., in an amount effective to achieve its intended purpose. The actual amount effective for a particular application will depend, *inter alia*, on the condition being treated. For example, when administered in methods to treat diabetes, such compositions will contain an amount of active ingredient effective to achieve the desired result (e.g. decreasing fasting blood glucose in a subject). When administered in methods to treat obesity, such compositions will contain an amount of active ingredient effective to achieve the desired result (e.g. decrease the body mass).

[0398] The dosage and frequency (single or multiple doses) of compound administered can vary depending upon a variety of factors, including route of administration; size, age, sex, health, body weight, body mass index, and diet of the recipient; nature and extent of symptoms of the

disease being treated (e.g., the disease responsive to compounds described herein; fasting blood glucose); presence of other diseases or other health-related problems; kind of concurrent treatment; and complications from any disease or treatment regimen. Other therapeutic regimens or agents can be used in conjunction with the methods and compounds of the invention.

5 **[0399]** Therapeutically effective amounts for use in humans may be determined from animal models. For example, a dose for humans can be formulated to achieve a concentration that has been found to be effective in animals. The dosage in humans can be adjusted by monitoring one or more physiological parameters, including but not limited to blood sugar and body mass, and adjusting the dosage upwards or downwards, as described above and known in the art.

10 **[0400]** Dosages may be varied depending upon the requirements of the patient and the compound being employed. The dose administered to a patient, in the context of the present invention, should be sufficient to affect a beneficial therapeutic response in the patient over time. The size of the dose also will be determined by the existence, nature, and extent of any adverse side effects. Generally, treatment is initiated with smaller dosages, which are less than the
15 optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under circumstances is reached. In one embodiment of the invention, the dosage range is 0.001% to 10% w/v. In another embodiment, the dosage range is 0.1% to 5% w/v.

20 **[0401]** However, typical doses may contain from a lower limit of about 1 ug, 5 ug, 10 ug, 50 ug, 100 ug to 150ug per day to an upper limit of about 50 ug, to 100 ug, to 150 ug, to 200 ug or even to 5 mg of the pharmaceutical compound. The doses may be delivered in discrete unit doses at the desired interval, e.g. daily or weekly.

25 **[0402]** Dosage amounts and intervals can be adjusted individually to provide levels of the administered compound effective for the particular clinical indication being treated. This will provide a therapeutic regimen that is commensurate with the severity of the individual's disease state.

30 **[0403]** Utilizing the teachings provided herein, an effective prophylactic or therapeutic treatment regimen can be planned that does not cause substantial toxicity and yet is entirely effective to treat the clinical symptoms demonstrated by the particular patient. This planning should involve the careful choice of active compound by considering factors such as compound potency, relative bioavailability, patient body weight, presence and severity of adverse side effects, preferred mode of administration, and the toxicity profile of the selected agent.

C. Toxicity

[0404] The ratio between toxicity and therapeutic effect for a particular compound is its therapeutic index and can be expressed as the ratio between LD₅₀ (the amount of compound lethal in 50% of the population) and ED₅₀ (the amount of compound effective in 50% of the population). Compounds that exhibit high therapeutic indices are preferred. Therapeutic index data obtained from cell culture assays and/or animal studies can be used in formulating a range of dosages for use in humans. The dosage of such compounds preferably lies within a range of plasma concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. See, e.g. Fingl *et al.*, *In: THE PHARMACOLOGICAL BASIS OF THERAPEUTICS*, Ch.1, p.1, 1975. The exact formulation, route of administration, and dosage can be chosen by the individual physician in view of the patient's condition and the particular method in which the compound is used.

VII. Examples

15 *Example 1: Cloning and Expression Studies*

[0405] Codon optimized nucleotide sequences for proteins were generated by overlap PCR and subcloned into a modified pET32 vector (i.e., EK cleavage site replaced with TEV cleavage site) at Kpn1 and Xho1 restriction sites, as known in the art. Sequence verified vector DNA was then transformed to BL21 cells (Novagen), and induced @ 30C O/N in Magic Media autoinducing media (Invitrogen).

[0406] **Exemplary construct 1.** The DNA sequence following encodes the engineered polypeptide having [¹⁴L]Ex-4 (SEQ ID NO:1) linked to a humanized seal leptin (SEQ ID NO:106) through a TGGGSASG linker:

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gatatacatatgagcgataaaattattcacctgactgacgacagtttgacacggatgtactcaaagcggacggggcg
25 atcctcgtcgatttctgggcagagtgggtgcggtccgtgcaaatgatcgccccgattctggatgaaatcgctgacgaa
tatcagggcaaaactgaccgttgcaaaactgaacatcgatcaaaacccctggcactgcgccgaaatatggcatccgtgg
tatcccgactctgctgctgttcaaaaacgggtgaagtggcggcaaccaaagtgggtgcactgtctaaaggtcagttgaa
agagttcctcgacgctaacctggccggttctggttctggccatatgcaccatcatcatcattcttctggtctggtgcc
acgcggttctggtatgaaagaaaccgctgctgtaattcgaacgccagcacatggacagcccagatctgggtacc
30 gagaatctgtacttctcagcatggcgaaggcagttcacttctgatctgagcaaacagctggaagaagaagcagttcgt
ctgttcattgagtggtgtaaaaaacggcgggtccaagctctggcgtccgccaccgagcaccggtggtggtggttctgc
tagcgggtccgatccagaaagtgcaggatgacaccaaactctgatcaaaaccatcgtcaccgtattaacgacatctc
ccctccgcagggcgctcctcccgctcctcgtgtagcgggtctggacttcacccgcgtgtccagtcctgctgacct

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gagcggatggaccagatcctggccacttaccaacaaatcctgacctccctgcaatctcgtaacgtaatccagattct
 aacgacctggaaaacctgcgtgatctgctgcacgtcctggcggtttccaaaagctgtccgggtgccggtgctcggtg
 tctgataccatcaaaggctctgggtaacgttctgcgcgccagcgtgcactctaccgaggtttagcgctgtcccgtctga
 aagcggctctgcaggacatgctgcgccagctggatcgtaacccgggctgctgactcgagg (SEQ ID
 5 NO:382).

[0407] Translation of the construct provided the peptide sequence following, which contains a TEV protease site suitable for subsequent processing:

MSDKIIHLTDDSFDTDLKADGAILVDFWAEWCGPCKMIAPILDEIADEY
 QGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQL
 10 KEFLDANLAGSGSGHMHSHHSSGLVPRGSGMKETAAAKFERQHMDS
 PDLGTENLYFQHGEFTSDLSKQLEEEAVRLFIEWLKNGGPSSGAPPPST
 GGGGSASGPIQKVQDDTKTLIKTIVTRINDISPPQGVSSRPRVAGLDFIPRV
 QSVRTLSGMDQILATYQQILTSLSRNVIQISNDLENLRDLLHVLAFSKSC
 PVPRARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNPG
 15 C (SEQ ID NO:383) (MW 38010.34).

[0408] Cleavage by TEV protease afforded the polypeptide following:

HGEFTSDLSKQLEEEAVRLFIEWLKNGGPSSGAPPPSTGGGGSASGPIQ
 KVQDDTKTLIKTIVTRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSGMDQ
 ILATYQQILTSLSRNVIQISNDLENLRDLLHVLAFSKSCPVRARGSDTIK
 20 GLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNPGC (SEQ ID
 NO:384) (MW 20747.72).

[0409] Exemplary construct 2. The DNA sequence following encodes the engineered polypeptide which includes Ex-4(1-28) (SEQ ID NO:5) linked to a humanized seal leptin (SEQ ID NO:100) through a TGGGGSAS linker:

gatatacatatgagcgataaaattattcacctgactgacgacagtttgacacggatgtactcaaagcggacggggcg
 atcctcgtcgatttctgggcagagtgggtgcgggtccgtgcaaatgatcgccccgattctggatgaaatcgctgacgaa
 tatcaggggcaaaactgaccgttgcaaaactgaacatcgatcaaaacctggcactgcgccgaaatatggcatccgtgg
 tatcccgactctgctgctgttcaaaaacgggtgaagtggcggaaccaaagtggtgactgtctaaaggtcagttgaa
 agagttcctcgacgtaacctggccggttctggttctggccatatgcaccatcatcatcattcttctggtctggtgcc
 30 acgcgggttctggtatgaaagaaacggctgctgtaattcgaacgccagcacatggacagcccagatctgggtacc
 gagaatctgtacttctcagcatggcgaaggcacgttcacttctgatctgagcaaacagctggaagaagaagcagttcgt
 ctgttcattgagtggtgctgaaaaacggcggtccaagctctggcgtccgccaccgagcaccgggtggtggtggttctgc
 tagcgggtccgatccagaaagtgcaggatgacacaaaactctgatcaaaaccatcgtcaccgtattaacgacatctc
 cctccgcaggggcgtctctcccgctcctcgtgtagcgggtctggacttcacccgcgtgtccagtcctgctgacct

gagcggatggaccagatcctggccacttaccaacaaatcctgacctccctgcaatctcgtaacgtaatccagatttct
 aacgacctggaaaacctgcgtgatctgctgcacgtcctggcggtttccaaaagctgtccgggtgccggtgctcgtggt
 tctgataccatcaaaggtctgggtaacgttctgcgcgccagcgtgcactctaccgaggtttagcgctgtcccgtctga
 aagcggctctgcaggacatgctgcgccagctggatcgtaacccgggctgctgactcgagg (SEQ ID
 NO:385).

[0410] Translation of the construct provided the peptide sequence following, which contains a TEV protease site suitable for subsequent processing:

MSDKIIHLTDDSFDTDLKADGAILVDFWAEWCGPCKMIAPILDEIADEY
 QGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQL
 KEFLDANLAGSGSGHMHSHHSSGLVPRGSGMKETAAAKFERQHMD
 PDLGTENLYFQHGEFTSDLSKQLEEEAVRLFIEWLKNTGGGGSASPIQR
 VQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL
 SGMDQILATYQQILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCP
 VPRARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:386)
 (MW 37103.46).

[0411] Cleavage by TEV protease afforded the polypeptide following:

HGEFTSDLSKQLEEEAVRLFIEWLKNTGGGGSASPIQRVQDDTKTLIKT
 IITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTL
 SGMDQILATYQQILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCP
 VPRARGSDTIKGLGNVLRASVHS
 TEVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:387) (MW 19840.84).

[0412] Exemplary construct 3. The DNA sequence following encodes the engineered polypeptide which includes of [¹⁴Leu]Exendin-4 (SEQ ID NO:1) linked to a humanized seal leptin (SEQ ID NO:100) through a TGGGGSAS linker:

gatatacatatgagcgataaaattattcacctgactgacgacagtttgacacggatgtactcaaagcggacggggcg
 atcctcgtcgatttctgggcagagtgggtgcggtccgtgcaaaatgatcgccccgattctggatgaaatcgctgacgaa
 tatcagggcaaaactgaccgttgcaaaactgaacatcgatcaaaacctggcactgcgcgaaatatggcatccgtgg
 tatcccgactctgctgctgttcaaaaacgggtgaagtggcggaaccaaagtgggtgcactgtctaaaggtcagttgaa
 agagttcctcgacgctaacctggccggttctggttctggccatatgcacatcatcatcatcattcttctggtctggtgcc
 acgcggttctggtatgaaagaaaccgctgctgctaaattcgaacgccagcacatggacagcccagatctgggtacc
 gagaatctgtactttcagcatggcgaaggcacgttcacttctgatctgagcaaacagctggaagaagaagcagttcgt
 ctgttcattgagtggtgtaaaaacggcggtccaagctctggcgctccgccaccgagcaccggtggtggtggttctgc
 tagcgggtccgatccagaaagtgcaggatgacaccaaactctgatcaaaaccatcgtaaccggtattacgacatctc
 ccctccgcagggcgctcctcccgctcctgtagcgggtctggacttcacccgcgtgtccagtcctgctgacacct
 gagcggatggaccagatcctggccacttaccaacaaatcctgacctccctgcaatctcgtaacgtaatccagatttct

aacgacctggaaaacctgcgtgatctgctgcacgtcctggcggtttccaaaagctgtccggtgccgcgtgctcgtggt
tctgataaccatcaaaggctctgggtaacgttctgcgcgccagcgtgcactctaccgaggtttagcgtgtcccgtctga
aagcggctctgcaggacatgctgcgccagctggatcgtaacccgggctgctgactcgagg (SEQ ID
NO:388).

- 5 [0413] Translation of the construct provided the peptide sequence following, which contains a TEV protease site suitable for subsequent processing:

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEY
QGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQL
KEFLDANLAGSGSGHMHSSGLVPRGSGMKETAAAKFERQHMSD
10 PDLGTENLYFQHGEFTFTSDLSKQLEEEAVRLFIEWLKNGGPSSGAPPPST
GGGGSASPIQRVQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQS
VRTLSGMDQILATYQQILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPV
PRARGSDTIKGLGNVLRASVHSTEVVALSRLKAALQDMLRQLDRNPGC
(SEQ ID NO:389) (MW 37995.33).

- 15 [0414] Cleavage by TEV protease afforded the polypeptide following:

HGEFTFTSDLSKQLEEEAVRLFIEWLKNGGPSSGAPPPSTGGGGSASPIQR
VQDDTKTLIKTIITRINDISPPQGVSSRPRVAGLDFIPRVQSVRTLSDGMDQIL
ATYQQILTSLQSRNVIQISNDLENLRDLLHVLAFSKSCPVPRARGSDTIKGL
20 GNVLRASVHSTEVVALSRLKAALQDMLRQLDRNPGC (SEQ ID NO:390)
(MW 20732.71).

Example 2: Biological and Pharmaceutical Properties

- [0415] As set forth in the table following, humanized chimeric polypeptides, including humanized chimeric seal leptins, described herein have comparable, and some even superior, properties compared with metreleptin (SEQ ID NO:44). These properties include biological
25 properties such as leptin binding activity, leptin functional activity, and food intake in mice, and pharmaceutical properties such as solubility in neutral pH.

- [0416] Leptin binding can be assessed by measuring displacement of ¹²⁵I-recombinant-Leptin (murine) from the surface membrane expressing chimeric Leptin (Hu) – EPO (Mu) receptor presented by the 32D OBECA cell line. *See e.g., J Biol Chem* 1998; **273**(29):18365-18373).
30 Purified cell membranes can be prepared by homogenization from harvested confluent cell cultures of 32D OBECA cells. Membranes can be incubated with ¹²⁵I-rec-Murine-Leptin and increasing concentrations of test compound for 3 hours at ambient temperature in 96-well polystyrene plates. Bound and unbound ligand fractions can then be separated by rapid filtration

onto 96-well GF/B plates pre-blocked for at least 60' in 0.5% PEI (polyethyleneimine). Glass fiber plates can then be dried, scintillant added, and CPM determined by reading on a multiwell scintillation counter capable of reading radiolabeled iodine.

[0417] For a leptin functional assay, increased levels of phosphorylated STAT5 (Signal Transducer and Activator of Transcription 5) can be measured following treatment of 32D-Keptin cells ectopically expressing chimeric Hu-Leptin/Mu-EPO receptor with a test compound. The 32D-Keptin cells (identical to 32D-OBECA cells but maintained in culture with leptin) can be leptin weaned overnight and then treated with test compounds in 96-well plates for 30 minutes at 37°C followed by cell extraction. The pSTAT5 levels in the cell lysates can be determined using the Perkin Elmer AlphaScreen® SureFire® pSTAT5 assay kit in a 384-well format (PROXIPLATE™ 384 Plus). The efficacy of test compounds can be determined relative to the maximal signal in cell lysates from cells treated with Human leptin.

[0418] Food intake activity in mice was tested with the following assay: C57BL6 female mice and their food were weighed daily 3 hours prior to lights out. Immediately after weighing, on days 0, 1, 2 and 3 mice were injected SC with leptin compound or mutant in 1xPBS. Points represent mean $\pm$ sd of n=9 cages (3 mice/cage). The results reported under "Mouse Food Intake" in Table 3 correspond to the vehicle corrected, change in % body weight measured after Day 4.

[0419] Solubility was measured with the following assay: proteins were concentrated at 4C, spun to remove precipitates, then allowed to equilibrate at room temperature overnight. They were filtered to remove precipitates and then the concentration was determined by measuring absorbance at OD₂₈₀ and using the theoretical molar extinction coefficient.

Table 2. Biological and pharmaceutical properties of chimeric polypeptides

SEQ ID NO:	IC ₅₀ (Obeca cell binding) nM	EC ₅₀ (Obeca Stat5 functional assay) nM	Mouse Food Intake	Solubility in neutral pH PBS mg/ml*
44	0.2nM-0.8nM	0.02	4.053	5
40	1.996	0.019	6.7	35
73	ND	0.095	3.1	ND
91	2.412	0.588	0	17
77	0.555	0.028	10.8	21
83	2.017	0.043	8.4	17
75	3.082	0.054	6.5	17

SEQ ID NO:	IC ₅₀ (Obeca cell binding) nM	EC ₅₀ (Obeca Stat5 functional assay) nM	Mouse Food Intake	Solubility in neutral pH PBS mg/ml*
79	3.542	0.032	6.4	20
85	0.527	0.029	10.3	18
81	0.479	0.042	~0-2%	20
93	0.788	0.0625	~4%	15
101	0.036	0.039	4.9	14
105	0.105	0.034	4	13
89	0.214	0.022	8.3	5
87	0.119	0.038	3.8	25
95	ND	0.044	ND	19

ND = not determined

*These numbers represent a lower limit to the solubility of each compound.

[0420] In view of the table, it is observed that metreleptin (SEQ ID NO:44) is soluble at about 5 mg/mL at neutral pH in phosphate buffered saline (PBS). In contrast, seal leptin (SEQ ID NO:40) has significantly greater solubility relative to metreleptin. Indeed, each of the tested humanized chimeric seal leptins has at least equivalent solubility to metreleptin, and many have much greater solubility under the test conditions. As judged by IC₅₀ binding data above, some of the humanized chimeric seal leptins bind with greater affinity than does metreleptin. Seal leptin *per se* (SEQ ID NO:40) binds in the Obeca cell binding assay with reduced affinity relative to metreleptin. However, some of the tested humanized chimeric seal leptin bind with greater affinity relative to metreleptin. Moreover, most of the tested humanized chimeric seal leptins demonstrate similar functional properties in the Obeca Stat5 functional assay.

Example 3: Stability of Humanized Chimeric Seal Leptins

[0421] As set forth in Table 3 following, chimeric polypeptides described herein have comparable, and some even superior, physical stability compared with A100 (SEQ ID NO:44). The compounds were formulated in the following buffer: 10mM glutamic acid, 2% glycine, 1% sucrose, 0.01% Tween 20, pH 4.25 and stored at 37°C. Samples were pulled at T = 0, 2, 5, 7, and 14 days and tested by visual analysis, reverse phase high performance liquid chromatography (HPLC), UV spectrometry, and dynamic light scattering (DLS). As shown in Table 4, the chimeric polypeptides have comparable or superior purity and potency, compared with A100 (SEQ ID NO:44).

Table 3.

SEQ ID NO:	Conc.	Potency remaining	Purity Loss	Corrected Potency	Visual	Size Change
40	1mg/ml	96.3%	1.8%	94.6%	Clear	1.0
77	1mg/ml	92.2%	1.9%	90.4%	Clear	2.0
101	1mg/ml	88.8%	2.7%	86.1%	Clear	1.7
83	1mg/ml	96.3%	14.9%	81.4%	Clear	1.9
105	1mg/ml	94.5%	13.0%	81.5%	Clear	1.0
44	1mg/ml	98.5%	10.7%	87.8%	Clear	1.0

Example 4: Functional and Binding Assays

[0422] Functional assays for GLP-1 and leptin function were conducted for compounds

5 disclosed herein. Methods were as described above, or as known in the art.

[0423] With reference to Table 4 following, GLP-1, [¹⁴Leu]Exendin-4, [¹⁴Leu]Exendin-4(1-28) demonstrated sub-nanomolar EC₅₀ in the GLP-1 functional assay. The engineered polypeptide (SEQ ID NO:261) demonstrated sub-micromolar functional activity in this assay. Moreover, this compound demonstrated functional activity in the leptin assay as a level similar to the activity of

10 metreleptin, or to the humanized chimeric seal leptin with structure of SEQ ID NO:100.

[0424] Accordingly, engineered polypeptides can provide sub-micromolar GLP-1 functional activity and sub-nanomolar leptin functional activity.

Table 4.

Compound (SEQ ID NO:)	GLP-1 Functional (cAMP) (nM)		Leptin Functional (STAT5) (nM)	
	EC ₅₀ (nM)	Normalized, EC ₅₀ (nm)	EC ₅₀ (nM)	Normalized, EC ₅₀ (nm)
GLP-1 (10)	0.053	0.01		
SEQ ID NO:1	0.019	0.004		
SEQ ID NO:11	0.113	0.021		
SEQ ID NO:261	122.4	23.24	0.049	0.027
A100 (44)			0.072	0.040
SEQ ID NO:100			0.044, 0.039	0.024, 0.022

15 **[0425]** Binding to the GLP-1 receptor, using methods described herein or known in the art, was conducted for GLP-1, [¹⁴L]Exendin-4, [¹⁴L]Exendin-4(1-28), and the engineered polypeptide with sequence SEQ ID NO:261. As shown in Table 5 following, GLP-1 and the exendin analogs demonstrate sub-nanomolar binding at the GLP-1 receptor, and the engineered polypeptide demonstrates an affinity within an order of magnitude of GLP-1.

Table 5.

Compound (SEQ ID NO:)	GLP-1 Binding (nM)
GLP-1 (10)	0.143
SEQ ID NO:1	0.125
SEQ ID NO:11	0.181
SEQ ID NO:261	1.125

[0426] As judged by the current studies, humanized chimeric seal leptins have unexpected superiority over seal leptin *per se* with respect to binding and activity at the human leptin receptor. Moreover, humanized chimeric seal leptins demonstrate significantly greater solubility relative to human leptin, and the binding of the engineered polypeptide to the GLP-1 receptor is reduced by less than a factor of 10 relative to GLP-1. Thus, without wishing to be bound by any theory, it is believed that these unexpected advantages of humanized chimeric seal leptins accrue to the exendin agonist engineered polypeptides contemplated herein. Moreover, as known in the art, the levels of leptin required for leptin therapy are high relative to the levels required for GLP-1 agonists in GLP-1 agonist therapy, e.g., exendins as described herein. Accordingly, the apparent decrease in GLP-1 functional activity of the engineered polypeptide as disclosed for SEQ ID NO:261 above relative to GLP-1 and the exendins, can be viewed as a mechanism for balancing multiple activities, i.e., GLP-1 and leptin functional activity, in a single engineered polypeptide for therapeutic use.

VIII. Embodiments

[0427] Embodiment 1. An engineered polypeptide comprising: a first peptide hormone domain (HD1) comprising an exendin domain sequence; and a second peptide hormone domain (HD2) comprising a humanized chimeric seal leptin sequence; wherein said HD1 is covalently bonded to said HD2 through a bond, or through a linker L1.

[0428] Embodiment 2. The engineered polypeptide according to embodiment 1, wherein said humanized chimeric seal leptin sequence has the sequence of SEQ ID NO:40, wherein 5% to 55% of SEQ ID NO:40 is substituted with a corresponding human leptin sequence.

[0429] Embodiment 3. The engineered polypeptide according to embodiment 1, wherein said humanized chimeric seal leptin sequence has the sequence of SEQ ID NO:41, wherein 5% to 55% of SEQ ID NO:41 is substituted with a corresponding human leptin sequence.

[0430] Embodiment 4. The engineered polypeptide according to embodiment 1, said engineered polypeptide having greater binding at a human leptin receptor relative to seal leptin binding.

[0431] Embodiment 5. The engineered polypeptide according to embodiment 1, said engineered polypeptide having greater solubility relative to human leptin solubility.

[0432] Embodiment 6. The engineered polypeptide according to any one of embodiments 1 to 5, said linker L1 covalently linking said HD1 and said HD2.

[0433] Embodiment 7. The engineered polypeptide according to any one of embodiments 1 to 6, wherein said engineered polypeptide comprises said HD1 as an N-terminal moiety and said HD2 as a C terminal moiety.

[0434] Embodiment 8. The engineered polypeptide according to embodiment 7, having the structure HD1 HD2.

[0435] Embodiment 9. The engineered polypeptide according to embodiment 7, having the structure HD1 L1 HD2.

[0436] Embodiment 10. The engineered polypeptide according to any one of embodiments 1 to 9, wherein said HD1 sequence consists of an exendin domain sequence.

[0437] Embodiment 11. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence has at least 65% identity with an Exendin-4 sequence (SEQ ID NO:3).

[0438] Embodiment 12. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence is an Exendin-4 sequence (SEQ ID NO:3).

[0439] Embodiment 13. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence is an [¹⁴Leu]Exendin-4 sequence (SEQ ID NO: 1).

[0440] Embodiment 14. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence has at least 90% identity with an Exendin-4(1-32) sequence (SEQ ID NO: 9).

[0441] Embodiment 15. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence is a [14Leu]Exendin-4(1-32) sequence (SEQ ID NO:15).

[0442] Embodiment 16. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence is the sequence of Exendin-4(1-28) (SEQ ID NO:5), Exendin-4(1-

29) (SEQ ID NO:6), Exendin-4(1-30) (SEQ ID NO:7), Exendin-4(1-31) (SEQ ID NO:8) or Exendin-4(1-32) (SEQ ID NO: 9).

[0443] Embodiment 17. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence comprises a sequence selected from the group consisting of (SEQ ID NO:3), (SEQ ID NO:1), (SEQ ID NO:4), and SEQ ID NOS:32-39.

[0444] Embodiment 18. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence has at least 70% identity with an Exendin-4 sequence (SEQ ID NO:3) or to a sequence selected from the group consisting of sequences (SEQ ID NO:3), (SEQ ID NO:1), (SEQ ID NO:4), and SEQ ID NOS:32-39.

[0445] Embodiment 19. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence comprises from 1 to 5 amino acid modifications relative to an Exendin-4 sequence (SEQ ID NO:3), said 1 to 5 amino acid modifications each independently selected from an insertion, deletion, addition or substitution.

[0446] Embodiment 20. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence comprises from 1 to 5 amino acid modifications relative to Exendin-4(1-32) sequence (SEQ ID NO:9), said 1 to 5 amino acid modifications each independently selected from an insertion, deletion, addition or substitution.

[0447] Embodiment 21. The engineered polypeptide according to embodiment 10, wherein said exendin domain sequence comprises from 1 to 5 amino acid modifications relative to Exendin-4(1-28) sequence (SEQ ID NO:5), said 1 to 5 amino acid modifications each independently selected from an insertion, deletion, addition or substitution.

[0448] Embodiment 22. The engineered polypeptide according to any one of embodiments 1 to 21, wherein said HD2 sequence comprises an analog of said humanized chimeric seal leptin sequence or an active fragment of an analog of said humanized chimeric seal leptin sequence.

[0449] Embodiment 23. The engineered polypeptide according to embodiment 22, wherein said HD2 has at least 50% identity with an amino acid sequence selected from the group consisting of: SEQ ID NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102,

SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

[0450] Embodiment 24. The engineered polypeptide according to embodiment 22, wherein said HD2 has at least 90% identity with an amino acid sequence selected from the group

consisting of: SEQ ID NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

[0451] Embodiment 25. The engineered polypeptide according to embodiment 22, wherein said HD2 has the amino acid sequence of SEQ ID NO:77.

[0452] Embodiment 26. The engineered polypeptide according to embodiment 22, wherein said HD2 has an amino acid sequence selected from the group consisting of: SEQ ID NO:77, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

[0453] Embodiment 27. The engineered polypeptide according to embodiment 22, wherein said HD2 sequence comprises a seal leptin sequence (SEQ ID NO:40); wherein at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with at least one contiguous region of from 8 to 30 amino acids of a human leptin sequence (SEQ ID NO:44); wherein said seal leptin sequence comprises a seal leptin Helix 1 sequence, a seal leptin Helix 2 sequence, a seal leptin Helix 3 sequence, a seal leptin Helix 4 sequence, a seal leptin AB Loop sequence, and a seal leptin Loop 3-4 sequence; and wherein said human leptin sequence comprises a human leptin Helix 1 sequence, a human leptin Helix 2 sequence, a human leptin Helix 3 sequence, a human leptin Helix 4 sequence, a human leptin AB Loop sequence, and a human leptin Loop 3-4 sequence

[0454] Embodiment 28. The engineered polypeptide according to embodiment 27, wherein said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence corresponds to said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence.

5 [0455] Embodiment 29. The engineered polypeptide according to any one of embodiments 27 to 28, further comprising from 1 to 5 amino acid modifications not encompassed within said at least one contiguous region of 8 to 30 amino acids of said seal leptin sequence, said 1 to 5 amino acid modifications each independently selected from an insertion, deletion, addition or substitution.

10 [0456] Embodiment 30. The engineered polypeptide according to embodiment 29, wherein at least one of said 1 to 5 amino acid modification not encompassed within said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence is substitution of cysteine at a position corresponding to position 30 of said seal leptin sequence.

[0457] Embodiment 31. The engineered polypeptide according to any one of embodiments
15 27 to 30, wherein said seal leptin Helix 1 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 1 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 1 sequence is replaced by said human leptin Helix 1 sequence.

20 [0458] Embodiment 32. The engineered polypeptide according to any one of embodiments 27 to 30, wherein said seal leptin Helix 2 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 2 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 2 sequence is replaced by said human
25 leptin Helix 2 sequence.

[0459] Embodiment 33. The engineered polypeptide according to any one of embodiments 27 to 30, wherein said seal leptin Helix 3 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 3
30 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 3 sequence is replaced by said human leptin Helix 3 sequence.

[0460] Embodiment 34. The engineered polypeptide according to any one of embodiments 27 to 30, wherein said seal leptin Helix 4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 4 sequence is replaced by said human leptin Helix 4 sequence.

[0461] Embodiment 35. The engineered polypeptide according to any one of embodiments 27 to 30, wherein said seal leptin AB Loop sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin AB Loop sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin AB Loop sequence is replaced by said human leptin AB Loop sequence.

[0462] Embodiment 36. The engineered polypeptide according to any one of embodiments 27 to 30, wherein said seal leptin Loop 3-4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Loop 3-4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Loop 3-4 sequence is replaced by said human leptin Loop 3-4 sequence.

[0463] Embodiment 37. The engineered polypeptide according to any one of embodiments 27 to 30, wherein two contiguous regions of from 8 to 30 amino acids of said seal leptin sequence have been replaced with two contiguous regions of from 8 to 30 amino acids of a human leptin sequence (SEQ ID NO:44).

[0464] Embodiment 38. The engineered polypeptide according to any one of embodiments 27 to 30, wherein a first contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with a first contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein a second contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with a second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0465] Embodiment 39. The engineered polypeptide according to embodiment 38, wherein said seal leptin Helix 1 sequence comprises said first contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 1 sequence comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin Helix 3 sequence comprises said second contiguous region of from 8 to 30 amino acids of

said seal leptin sequence, and wherein said human leptin Helix 3 sequence comprises said second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0466] Embodiment 40. The engineered polypeptide according to embodiment 38, wherein said seal leptin Helix 3 sequence comprises said first contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 3 sequence comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin AB Loop sequence comprises said second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and wherein said human leptin AB Loop sequence comprises said second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0467] Embodiment 41. The engineered polypeptide according to embodiment 38, wherein said seal leptin Helix 3 sequence comprises said first contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 3 sequence comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and wherein said human leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0468] Embodiment 42. The engineered polypeptide according to embodiment 38, wherein said seal leptin Helix 4 sequence comprises said first contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 4 sequence comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin AB Loop sequence comprises said second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and wherein said human leptin AB Loop sequence comprises said second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0469] Embodiment 43. The engineered polypeptide according to embodiment 38, wherein said seal leptin AB Loop sequence comprises said first contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin AB Loop sequence comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, and wherein said human leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0470] Embodiment 44. The engineered polypeptide according to any one of embodiments 27 to 30, wherein a first contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with a first contiguous region of from 8 to 30 amino acids of said

human leptin sequence, wherein a second contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with a second contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein a third contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with a third contiguous region of from 8 to 30 amino acids of said human leptin sequence.

[0471] Embodiment 45. The engineered polypeptide according to embodiment 44, wherein said seal leptin AB Loop sequence comprises said first contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin AB Loop sequence comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin Helix 3 sequence comprises said third contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 3 sequence comprises said third contiguous region of 8 to 30 amino acids of said human leptin sequence.

[0472] Embodiment 46. The engineered polypeptide according to any one of embodiments 1 to 45, wherein said linker L1 is a peptide linker comprising from 1 to 30 amino acids.

[0473] Embodiment 47. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises amino acids selected from the 20 naturally occurring amino acids.

[0474] Embodiment 48. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises a non-natural amino acid incorporated by chemical synthesis, post-translational chemical modification or by in vivo incorporation by recombinant expression in a host cell.

[0475] Embodiment 49. The engineered polypeptide according to embodiment 46, wherein said linker L1 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine.

[0476] Embodiment 50. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises a majority of amino acids that are sterically unhindered.

[0477] Embodiment 51. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises polyglycine, polyalanine, poly(Gly-Ala) or poly(Gly-Ser).

[0478] Embodiment 52. The engineered polypeptide according to embodiment 51, wherein said linker L1 comprises the sequence (Gly)₃, (Gly)₄ (SEQ ID NO:139), or (Gly)₅ (SEQ ID NO:140).

[0479] Embodiment 53. The engineered polypeptide according to embodiment 51, wherein said linker L1 comprises the sequence GGG, GGS, GGGG (SEQ ID NO:139), or GGGS (SEQ ID NO:141).

[0480] Embodiment 54. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises the sequence (Gly)₃Lys(Gly)₄ (SEQ ID NO:114); (Gly)₃AsnGlySer(Gly)₂ (SEQ ID NO:115); (Gly)₃Cys(Gly)₄ (SEQ ID NO:116); or GlyProAsnGlyGly (SEQ ID NO:117).

[0481] Embodiment 55. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises combinations of Gly and Ala.

[0482] Embodiment 56. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises combination of Gly and Ser.

[0483] Embodiment 57. The engineered polypeptide according to embodiment 46, wherein said linker L1 is a glycine rich peptide.

[0484] Embodiment 58. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises an N-terminal dipeptide, said N-terminal dipeptide comprising amino acids residues T, A, S or G.

[0485] Embodiment 59. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises an N-terminal TG dipeptide.

[0486] Embodiment 60. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises a C-terminal dipeptide, said C-terminal dipeptide comprising amino acids residues T, A, S or G.

[0487] Embodiment 61. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises a C-terminal AS dipeptide.

[0488] Embodiment 62. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises an N-terminal TG dipeptide and a C-terminal AS dipeptide.

[0489] Embodiment 63. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises a sequence selected from the group consisting of TG-(GGG)₁ (SEQ ID NO:142), TG-(GGGG)₁ (SEQ ID NO:143), TG-(GGGGG)₁ (SEQ ID NO:144), TG-(GGG)₂

(SEQ ID NO:145), TG (GGGGGGG)1 (SEQ ID NO:146), TG-(GGGG)2 (SEQ ID NO:147), TG-(GGG)3 (SEQ ID NO:148), (GGG)1-AS (SEQ ID NO:149), (GGGG)1-AS (SEQ ID NO:150), (GGGGG)1-AS (SEQ ID NO:151), (GGG)2-AS (SEQ ID NO:152), (GGGGGGG)1-AS (SEQ ID NO:153), (GGGG)2-AS (SEQ ID NO:154), (GGG)3-AS (SEQ ID NO:155), TG-(GGG)1-AS (SEQ ID NO:156), TG-(GGGG)1-AS (SEQ ID NO:157), TG-(GGGGG)1-AS (SEQ ID NO:158), TG-(GGG)2-AS (SEQ ID NO:159), TG-(GGGGGGG)1-AS (SEQ ID NO:160), TG-(GGGG)2-AS (SEQ ID NO:161), and TG-(GGG)3-AS (SEQ ID NO:162).

[0490] Embodiment 64. The engineered polypeptide according to embodiment 46, wherein said linker L1 comprises a sequence selected from the group consisting of TG-(GGS)1 (SEQ ID NO:163), TG-(GGGS)1 (SEQ ID NO:164), TG-(GGGGG)1 (SEQ ID NO:165), TG-(GGS)2 (SEQ ID NO:166), TG (GGGGGGG)1 (SEQ ID NO:167), TG-(GGGS)2 (SEQ ID NO:168), TG-(GGS)3 (SEQ ID NO:169), (GGS)1-AS (SEQ ID NO:170), (GGGS)1-AS (SEQ ID NO:171), (GGGGG)1-AS (SEQ ID NO:172), (GGS)2-AS (SEQ ID NO:173), (GGGGGGG)1-AS (SEQ ID NO:174), (GGGS)2-AS (SEQ ID NO:175), (GGS)3-AS (SEQ ID NO:176), TG-(GGS)1-AS (SEQ ID NO:177), TG-(GGGS)1-AS (SEQ ID NO:178), TG-(GGGGG)1-AS (SEQ ID NO:179), TG-(GGS)2-AS (SEQ ID NO:180), TG-(GGGGGGG)1-AS (SEQ ID NO:181), TG-(GGGS)2-AS (SEQ ID NO:182), and TG-(GGS)3-AS (SEQ ID NO:183).

[0491] Embodiment 65. The engineered polypeptide according to embodiment 46, wherein said linker L1 consists of a sequence selected from the group consisting of TG-(GGG)1 (SEQ ID NO:142), TG-(GGGG)1 (SEQ ID NO:143), TG-(GGGGG)1 (SEQ ID NO:144), TG-(GGG)2 (SEQ ID NO:145), TG (GGGGGGG)1 (SEQ ID NO:146), TG-(GGGG)2 (SEQ ID NO:147), TG-(GGG)3 (SEQ ID NO:148), (GGG)1-AS (SEQ ID NO:149), (GGGG)1-AS (SEQ ID NO:150), (GGGGG)1-AS (SEQ ID NO:151), (GGG)2-AS (SEQ ID NO:152), (GGGGGGG)1-AS (SEQ ID NO:153), (GGGG)2-AS (SEQ ID NO:154), (GGG)3-AS (SEQ ID NO:155), TG-(GGG)1-AS (SEQ ID NO:156), TG-(GGGG)1-AS (SEQ ID NO:157), TG-(GGGGG)1-AS (SEQ ID NO:158), TG-(GGG)2-AS (SEQ ID NO:159), TG-(GGGGGGG)1-AS (SEQ ID NO:160), TG-(GGGG)2-AS (SEQ ID NO:161), and TG-(GGG)3-AS (SEQ ID NO:162).

[0492] Embodiment 66. The engineered polypeptide according to embodiment 46, wherein said linker L1 consists of a sequence selected from the group consisting of TG-(GGS)1 (SEQ ID NO:163), TG-(GGGS)1 (SEQ ID NO:164), TG-(GGGGG)1 (SEQ ID NO:165), TG-(GGS)2 (SEQ ID NO:166), TG (GGGGGGG)1 (SEQ ID NO:167), TG-(GGGS)2 (SEQ ID NO:168), TG-(GGS)3 (SEQ ID NO:169), (GGS)1-AS (SEQ ID NO:170), (GGGS)1-AS (SEQ ID NO:171), (GGGGG)1-AS (SEQ ID NO:172), (GGS)2-AS (SEQ ID NO:173), (GGGGGGG)1-AS (SEQ ID NO:174), (GGGS)2-AS (SEQ ID NO:175), (GGS)3-AS (SEQ ID NO:176), TG-(GGS)1-AS

(SEQ ID NO:177), TG-(GGGS)1-AS (SEQ ID NO:178), TG-(GGGGS)1-AS (SEQ ID NO:179), TG-(GGS)2-AS (SEQ ID NO:180), TG-(GGGGGS)1-AS (SEQ ID NO:181), TG-(GGGS)2-AS (SEQ ID NO:182), and TG-(GGS)3-AS (SEQ ID NO:183).

- 5 [0493] Embodiment 67. The engineered polypeptide of embodiment 1 comprising a sequence as set forth in any one SEQ ID NOS:184-375.
- [0494] Embodiment 68. The engineered polypeptide of embodiment 1 consisting of a sequence as set forth in any one SEQ ID NOS:184-375.
- [0495] Embodiment 69. The engineered polypeptide of embodiment 1 comprising the sequence of SEQ ID NO:261.
- 10 [0496] Embodiment 70. The engineered polypeptide of embodiment 1 consisting of the sequence of SEQ ID NO:261.
- [0497] Embodiment 71. A method for treating a disease in a subject, comprising administering a engineered polypeptide according to any one of embodiments 1 to 70 to a subject in need thereof in an amount effective to treat said disease.
- 15 [0498] Embodiment 72. The method according to embodiment 71, wherein said disease is diabetes, overweight, obesity, Alzheimer's disease, short bowel syndrome, fatty liver disease, dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.
- [0499] Embodiment 73. The method according to embodiment 72, wherein said disease is diabetes, overweight, obesity, short bowel syndrome, NASH or Parkinson's disease.
- 20 [0500] Embodiment 74. The method according to embodiment 73, wherein said disease is type I diabetes, type II diabetes or prediabetes.
- [0501] Embodiment 75. The method according to embodiment 72, wherein said disease is type II diabetes.
- [0502] Embodiment 76. The method according to embodiment 72, wherein said disease is
25 dyslipidemia or hyperlipidemia.
- [0503] Embodiment 77. The method according to embodiment 72, wherein the subject in need of such treatment is obese.
- [0504] Embodiment 78. A pharmaceutical composition comprising an engineered polypeptide according to any one of embodiments 1 to 70 and a pharmaceutically acceptable
30 excipient.

[0505] Embodiment 79. The pharmaceutical composition according to embodiment 78, wherein said pharmaceutical composition is a parenteral pharmaceutical composition.

[0506] Embodiment 80. The pharmaceutical composition according to embodiment 78, wherein said pharmaceutical composition is a sustained release or long lasting pharmaceutical composition.

[0507] Embodiment 81. The pharmaceutical composition according to embodiment 78, wherein said pharmaceutical composition is formulated as a twice daily pharmaceutical composition.

[0508] Embodiment 82. The pharmaceutical composition according to embodiment 78, wherein said pharmaceutical composition is formulated as a once daily pharmaceutical composition.

[0509] Embodiment 83. The pharmaceutical composition according to embodiment 78, wherein said pharmaceutical composition is formulated as a once weekly pharmaceutical composition.

[0510] Embodiment 84. The pharmaceutical composition of embodiment 78 for treating a disease in a subject.

[0511] Embodiment 85. The pharmaceutical composition of embodiment 84 wherein said disease is diabetes, overweight, obesity, Alzheimer's disease, fatty liver disease, short bowel syndrome, dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.

[0512] Embodiment 86. The pharmaceutical composition of embodiment 85 wherein said disease is diabetes, overweight, obesity, short bowel syndrome, or Parkinson's disease.

[0513] Embodiment 87. The pharmaceutical composition of embodiment 86, wherein said disease is type I diabetes, type II diabetes or prediabetes.

[0514] Embodiment 88. The pharmaceutical composition of any one of embodiments 78 to 87, wherein said engineered polypeptide comprises a sequence as set forth in any one SEQ ID NOS:184-375.

[0515] Embodiment 89. The pharmaceutical composition of any one of embodiments 78 to 87, wherein said engineered polypeptide consists of a sequence as set forth in any one SEQ ID NOS:184-375.

[0516] Embodiment 90. The pharmaceutical composition of any one of embodiments 78 to 87, wherein said engineered polypeptide comprises (SEQ ID NO: 261).

WHAT IS CLAIMED IS:

- 1 1. An engineered polypeptide comprising:
2 a first peptide hormone domain (HD1) comprising an exendin domain sequence;
3 and
4 a second peptide hormone domain (HD2) comprising a humanized chimeric seal
5 leptin sequence;
6 wherein said HD1 is covalently bonded to said HD2 through a bond, or through a
7 linker L1.
- 1 2. The engineered polypeptide according to claim 1, wherein said humanized
2 chimeric seal leptin sequence has the sequence of SEQ ID NO:40, wherein 5% to 55% of SEQ
3 ID NO:40 is substituted with a corresponding human leptin sequence.
- 1 3. The engineered polypeptide according to claim 1, wherein said humanized
2 chimeric seal leptin sequence has the sequence of SEQ ID NO:41, wherein 5% to 55% of SEQ
3 ID NO:41 is substituted with a corresponding human leptin sequence.
- 1 4. The engineered polypeptide according to claim 1, said engineered
2 polypeptide having greater binding at a human leptin receptor relative to seal leptin binding.
- 1 5. The engineered polypeptide according to claim 1, said engineered
2 polypeptide having greater solubility relative to human leptin solubility.
- 1 6. The engineered polypeptide according to any one of claims 1 to 5, said
2 linker L1 covalently linking said HD1 and said HD2.
- 1 7. The engineered polypeptide according to any one of claims 1 to 6, wherein
2 said engineered polypeptide comprises said HD1 as an N-terminal moiety and said HD2 as a
3 C-terminal moiety.
- 1 8. The engineered polypeptide according to claim 7, having the structure
2 HD1-HD2.
- 1 9. The engineered polypeptide according to claim 7, having the structure
2 HD1-L1-HD2.
- 1 10. The engineered polypeptide according to any one of claims 1 to 9, wherein
2 said HD1 sequence consists of an exendin domain sequence.

1 11. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence has at least 65% identity with an Exendin-4 sequence (SEQ ID NO:3).

1 12. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence is an Exendin-4 sequence (SEQ ID NO:3).

1 13. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence is an [¹⁴Leu]Exendin-4 sequence (SEQ ID NO: 1).

1 14. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence has at least 90% identity with an Exendin-4(1-32) sequence (SEQ ID NO: 9).

1 15. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence is a [¹⁴Leu]Exendin-4(1-32) sequence (SEQ ID NO:15).

1 16. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence is the sequence of Exendin-4(1-28) (SEQ ID NO:5), Exendin-4(1-29) (SEQ ID
3 NO:6), Exendin-4(1-30) (SEQ ID NO:7), Exendin-4(1-31) (SEQ ID NO:8) or Exendin-4(1-32)
4 (SEQ ID NO: 9).

1 17. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence comprises a sequence selected from the group consisting of (SEQ ID NO:3),
3 (SEQ ID NO:1), (SEQ ID NO:4), and SEQ ID NOS:32-39.

1 18. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence has at least 70% identity with an Exendin-4 sequence (SEQ ID NO:3) or to a
3 sequence selected from the group consisting of sequences (SEQ ID NO:3), (SEQ ID NO:1),
4 (SEQ ID NO:4), and SEQ ID NOS:32-39.

1 19. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence comprises from 1 to 5 amino acid modifications relative to an Exendin-4
3 sequence (SEQ ID NO:3), said 1 to 5 amino acid modifications each independently selected from
4 an insertion, deletion, addition or substitution.

1 20. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence comprises from 1 to 5 amino acid modifications relative to Exendin-4(1-32)
3 sequence (SEQ ID NO:9), said 1 to 5 amino acid modifications each independently selected from
4 an insertion, deletion, addition or substitution.

1 21. The engineered polypeptide according to claim 10, wherein said exendin
2 domain sequence comprises from 1 to 5 amino acid modifications relative to Exendin-4(1-28)
3 sequence (SEQ ID NO:5), said 1 to 5 amino acid modifications each independently selected from
4 an insertion, deletion, addition or substitution.

1 22. The engineered polypeptide according to any one of claims 1 to 21,
2 wherein said HD2 sequence comprises an analog of said humanized chimeric seal leptin
3 sequence or an active fragment of an analog of said humanized chimeric seal leptin sequence.

1 23. The engineered polypeptide according to claim 22, wherein said HD2 has
2 at least 50% identity with an amino acid sequence selected from the group consisting of: SEQ ID
3 NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101,
4 SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID
5 NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ
6 ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90,
7 SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID
8 NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103,
9 SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and
10 SEQ ID NO:109.

1 24. The engineered polypeptide according to claim 22, wherein said HD2 has
2 at least 90% identity with an amino acid sequence selected from the group consisting of: SEQ ID
3 NO:40, SEQ ID NO:76, SEQ ID NO:100, SEQ ID NO:42, SEQ ID NO:77, SEQ ID NO:101,
4 SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID
5 NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ
6 ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90,
7 SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID
8 NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103,
9 SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and
10 SEQ ID NO:109.

1 25. The engineered polypeptide according to claim 22, wherein said HD2 has
2 the amino acid sequence of SEQ ID NO:77.

1 26. The engineered polypeptide according to claim 22, wherein said HD2 has
2 an amino acid sequence selected from the group consisting of:

SEQ ID NO:77, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, and SEQ ID NO:109.

27. The engineered polypeptide according to claim 22, wherein said HD2 sequence comprises a seal leptin sequence (SEQ ID NO:40);

wherein at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence has been replaced with at least one contiguous region of from 8 to 30 amino acids of a human leptin sequence (SEQ ID NO:44);

wherein said seal leptin sequence comprises a seal leptin Helix 1 sequence, a seal leptin Helix 2 sequence, a seal leptin Helix 3 sequence, a seal leptin Helix 4 sequence, a seal leptin AB Loop sequence, and a seal leptin Loop 3-4 sequence; and

wherein said human leptin sequence comprises a human leptin Helix 1 sequence, a human leptin Helix 2 sequence, a human leptin Helix 3 sequence, a human leptin Helix 4 sequence, a human leptin AB Loop sequence, and a human leptin Loop 3-4 sequence

28. The engineered polypeptide according to claim 27, wherein said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence corresponds to said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence.

29. The engineered polypeptide according to any one of claims 27 to 28, further comprising from 1 to 5 amino acid modifications not encompassed within said at least one contiguous region of 8 to 30 amino acids of said seal leptin sequence, said 1 to 5 amino acid modifications each independently selected from an insertion, deletion, addition or substitution.

30. The engineered polypeptide according to claim 29, wherein at least one of said 1 to 5 amino acid modification not encompassed within said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence is substitution of cysteine at a position corresponding to position 30 of said seal leptin sequence.

31. The engineered polypeptide according to any one of claims 27 to 30, wherein said seal leptin Helix 1 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 1 sequence

comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 1 sequence is replaced by said human leptin Helix 1 sequence.

32. The engineered polypeptide according to any one of claims 27 to 30, wherein said seal leptin Helix 2 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 2 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 2 sequence is replaced by said human leptin Helix 2 sequence.

33. The engineered polypeptide according to any one of claims 27 to 30, wherein said seal leptin Helix 3 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 3 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 3 sequence is replaced by said human leptin Helix 3 sequence.

34. The engineered polypeptide according to any one of claims 27 to 30, wherein said seal leptin Helix 4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin Helix 4 sequence is replaced by said human leptin Helix 4 sequence.

35. The engineered polypeptide according to any one of claims 27 to 30, wherein said seal leptin AB Loop sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin AB Loop sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin sequence, and wherein said seal leptin AB Loop sequence is replaced by said human leptin AB Loop sequence.

36. The engineered polypeptide according to any one of claims 27 to 30, wherein said seal leptin Loop 3-4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Loop 3-4 sequence comprises said at least one contiguous region of from 8 to 30 amino acids of said human leptin

5 sequence, and wherein said seal leptin Loop 3-4 sequence is replaced by said human leptin Loop
6 3-4 sequence.

1 37. The engineered polypeptide according to any one of claims 27 to 30,
2 wherein two contiguous regions of from 8 to 30 amino acids of said seal leptin sequence have
3 been replaced with two contiguous regions of from 8 to 30 amino acids of a human leptin
4 sequence (SEQ ID NO:44).

1 38. The engineered polypeptide according to any one of claims 27 to 30,
2 wherein a first contiguous region of from 8 to 30 amino acids of said seal leptin sequence has
3 been replaced with a first contiguous region of from 8 to 30 amino acids of said human leptin
4 sequence, and wherein a second contiguous region of from 8 to 30 amino acids of said seal leptin
5 sequence has been replaced with a second contiguous region of from 8 to 30 amino acids of said
6 human leptin sequence.

1 39. The engineered polypeptide according to claim 38, wherein said seal
2 leptin Helix 1 sequence comprises said first contiguous region of from 8 to 30 amino acids of
3 said seal leptin sequence, wherein said human leptin Helix 1 sequence comprises said first
4 contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin
5 Helix 3 sequence comprises said second contiguous region of from 8 to 30 amino acids of said
6 seal leptin sequence, and wherein said human leptin Helix 3 sequence comprises said second
7 contiguous region of from 8 to 30 amino acids of said human leptin sequence.

1 40. The engineered polypeptide according to claim 38, wherein said seal
2 leptin Helix 3 sequence comprises said first contiguous region of from 8 to 30 amino acids of
3 said seal leptin sequence, wherein said human leptin Helix 3 sequence comprises said first
4 contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin
5 AB Loop sequence comprises said second contiguous region of from 8 to 30 amino acids of said
6 seal leptin sequence, and wherein said human leptin AB Loop sequence comprises said second
7 contiguous region of from 8 to 30 amino acids of said human leptin sequence.

1 41. The engineered polypeptide according to claim 38, wherein said seal
2 leptin Helix 3 sequence comprises said first contiguous region of from 8 to 30 amino acids of
3 said seal leptin sequence, wherein said human leptin Helix 3 sequence comprises said first
4 contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin
5 Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said

6 seal leptin sequence, and wherein said human leptin Loop 3-4 sequence comprises said second
7 contiguous region of from 8 to 30 amino acids of said human leptin sequence.

1 42. The engineered polypeptide according to claim 38, wherein said seal
2 leptin Helix 4 sequence comprises said first contiguous region of from 8 to 30 amino acids of
3 said seal leptin sequence, wherein said human leptin Helix 4 sequence comprises said first
4 contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin
5 AB Loop sequence comprises said second contiguous region of from 8 to 30 amino acids of said
6 seal leptin sequence, and wherein said human leptin AB Loop sequence comprises said second
7 contiguous region of from 8 to 30 amino acids of said human leptin sequence.

1 43. The engineered polypeptide according to claim 38, wherein said seal
2 leptin AB Loop sequence comprises said first contiguous region of from 8 to 30 amino acids of
3 said seal leptin sequence, wherein said human leptin AB Loop sequence comprises said first
4 contiguous region of 8 to 30 amino acids of said human leptin sequence, wherein said seal leptin
5 Loop 3-4 sequence comprises said second contiguous region of from 8 to 30 amino acids of said
6 seal leptin sequence, and wherein said human leptin Loop 3-4 sequence comprises said second
7 contiguous region of from 8 to 30 amino acids of said human leptin sequence.

1 44. The engineered polypeptide according to any one of claims 27 to 30,
2 wherein a first contiguous region of from 8 to 30 amino acids of said seal leptin sequence has
3 been replaced with a first contiguous region of from 8 to 30 amino acids of said human leptin
4 sequence, wherein a second contiguous region of from 8 to 30 amino acids of said seal leptin
5 sequence has been replaced with a second contiguous region of from 8 to 30 amino acids of said
6 human leptin sequence, and wherein a third contiguous region of from 8 to 30 amino acids of
7 said seal leptin sequence has been replaced with a third contiguous region of from 8 to 30 amino
8 acids of said human leptin sequence.

1 45. The engineered polypeptide according to claim 44,
2 wherein said seal leptin AB Loop sequence comprises said first contiguous region of from 8 to
3 30 amino acids of said seal leptin sequence, wherein said human leptin AB Loop sequence
4 comprises said first contiguous region of 8 to 30 amino acids of said human leptin sequence,
5 wherein said seal leptin Loop 3-4 sequence comprises said second contiguous region of from 8 to
6 30 amino acids of said seal leptin sequence, wherein said human leptin Loop 3-4 sequence
7 comprises said second contiguous region of from 8 to 30 amino acids of said human leptin
8 sequence, wherein said seal leptin Helix 3 sequence comprises said third contiguous region of

9 from 8 to 30 amino acids of said seal leptin sequence, wherein said human leptin Helix 3
10 sequence comprises said third contiguous region of 8 to 30 amino acids of said human leptin
11 sequence.

1 46. The engineered polypeptide according to any one of claims 1 to 45,
2 wherein said linker L1 is a peptide linker comprising from 1 to 30 amino acids.

1 47. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises amino acids selected from the 20 naturally occurring amino acids.

1 48. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises a non-natural amino acid incorporated by chemical synthesis, post-translational
3 chemical modification or by in vivo incorporation by recombinant expression in a host cell.

1 49. The engineered polypeptide according to claim 46, wherein said linker L1
2 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine.

1 50. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises a majority of amino acids that are sterically unhindered.

1 51. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises polyglycine, polyalanine, poly(Gly-Ala) or poly(Gly-Ser).

1 52. The engineered polypeptide according to claim 51, wherein said linker L1
2 comprises the sequence (Gly)₃, (Gly)₄ (SEQ ID NO:139), or (Gly)₅ (SEQ ID NO:140).

1 53. The engineered polypeptide according to claim 51, wherein said linker L1
2 comprises the sequence GGG, GGS, GGGG (SEQ ID NO:139), or GGGS (SEQ ID NO:141).

1 54. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises the sequence (Gly)₃Lys(Gly)₄ (SEQ ID NO:114); (Gly)₃AsnGlySer(Gly)₂ (SEQ ID
3 NO:115); (Gly)₃Cys(Gly)₄ (SEQ ID NO:116); or GlyProAsnGlyGly (SEQ ID NO:117).

1 55. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises combinations of Gly and Ala.

1 56. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises combination of Gly and Ser.

1 57. The engineered polypeptide according to claim 46, wherein said linker L1
2 is a glycine rich peptide.

1 58. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises an N-terminal dipeptide, said N-terminal dipeptide comprising amino acids residues
3 T, A, S or G.

1 59. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises an N-terminal TG dipeptide.

1 60. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises a C-terminal dipeptide, said C-terminal dipeptide comprising amino acids residues T,
3 A, S or G.

1 61. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises a C-terminal AS dipeptide.

1 62. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises an N-terminal TG dipeptide and a C-terminal AS dipeptide.

1 63. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises a sequence selected from the group consisting of TG-(GGG)₁ (SEQ ID NO:142), TG-
3 (GGGG)₁ (SEQ ID NO:143), TG-(GGGGG)₁ (SEQ ID NO:144), TG-(GGG)₂ (SEQ ID
4 NO:145), TG-(GGGGGGG)₁ (SEQ ID NO:146), TG-(GGGG)₂ (SEQ ID NO:147), TG-(GGG)₃
5 (SEQ ID NO:148), (GGG)₁-AS (SEQ ID NO:149), (GGGG)₁-AS (SEQ ID NO:150),
6 (GGGGG)₁-AS (SEQ ID NO:151), (GGG)₂-AS (SEQ ID NO:152), (GGGGGGG)₁-AS (SEQ ID
7 NO:153), (GGGG)₂-AS (SEQ ID NO:154), (GGG)₃-AS (SEQ ID NO:155), TG-(GGG)₁-AS
8 (SEQ ID NO:156), TG-(GGGG)₁-AS (SEQ ID NO:157), TG-(GGGGG)₁-AS (SEQ ID NO:158),
9 TG-(GGG)₂-AS (SEQ ID NO:159), TG-(GGGGGGG)₁-AS (SEQ ID NO:160), TG-(GGGG)₂-
10 AS (SEQ ID NO:161), and TG-(GGG)₃-AS (SEQ ID NO:162).

1 64. The engineered polypeptide according to claim 46, wherein said linker L1
2 comprises a sequence selected from the group consisting of TG-(GGS)₁ (SEQ ID NO:163), TG-
3 (GGGS)₁ (SEQ ID NO:164), TG-(GGGGS)₁ (SEQ ID NO:165), TG-(GGS)₂ (SEQ ID NO:166),
4 TG-(GGGGGS)₁ (SEQ ID NO:167), TG-(GGGS)₂ (SEQ ID NO:168), TG-(GGS)₃ (SEQ ID
5 NO:169), (GGS)₁-AS (SEQ ID NO:170), (GGGS)₁-AS (SEQ ID NO:171), (GGGGGS)₁-AS (SEQ
6 ID NO:172), (GGS)₂-AS (SEQ ID NO:173), (GGGGGS)₁-AS (SEQ ID NO:174), (GGGS)₂-AS

7 (SEQ ID NO:175), (GGS)₃-AS (SEQ ID NO:176), TG-(GGS)₁-AS (SEQ ID NO:177), TG-
 8 (GGGS)₁-AS (SEQ ID NO:178), TG-(GGGS)₁-AS (SEQ ID NO:179), TG-(GGS)₂-AS (SEQ
 9 ID NO:180), TG-(GGGGGGS)₁-AS (SEQ ID NO:181), TG-(GGGS)₂-AS (SEQ ID NO:182),
 10 and TG-(GGS)₃-AS (SEQ ID NO:183).

1 65. The engineered polypeptide according to claim 46, wherein said linker L1
 2 consists of a sequence selected from the group consisting of TG-(GGG)₁ (SEQ ID NO:142), TG-
 3 (GGGG)₁ (SEQ ID NO:143), TG-(GGGGG)₁ (SEQ ID NO:144), TG-(GGG)₂ (SEQ ID
 4 NO:145), TG-(GGGGGGG)₁ (SEQ ID NO:146), TG-(GGGG)₂ (SEQ ID NO:147), TG-(GGG)₃
 5 (SEQ ID NO:148), (GGG)₁-AS (SEQ ID NO:149), (GGGG)₁-AS (SEQ ID NO:150),
 6 (GGGGG)₁-AS (SEQ ID NO:151), (GGG)₂-AS (SEQ ID NO:152), (GGGGGGG)₁-AS (SEQ ID
 7 NO:153), (GGGG)₂-AS (SEQ ID NO:154), (GGG)₃-AS (SEQ ID NO:155), TG-(GGG)₁-AS
 8 (SEQ ID NO:156), TG-(GGGG)₁-AS (SEQ ID NO:157), TG-(GGGGG)₁-AS (SEQ ID NO:158),
 9 TG-(GGG)₂-AS (SEQ ID NO:159), TG-(GGGGGGG)₁-AS (SEQ ID NO:160), TG-(GGGG)₂-
 10 AS (SEQ ID NO:161), and TG-(GGG)₃-AS (SEQ ID NO:162).

1 66. The engineered polypeptide according to claim 46, wherein said linker L1
 2 consists of a sequence selected from the group consisting of TG-(GGS)₁ (SEQ ID NO:163), TG-
 3 (GGGS)₁ (SEQ ID NO:164), TG-(GGGGS)₁ (SEQ ID NO:165), TG-(GGS)₂ (SEQ ID NO:166),
 4 TG-(GGGGGGS)₁ (SEQ ID NO:167), TG-(GGGS)₂ (SEQ ID NO:168), TG-(GGS)₃ (SEQ ID
 5 NO:169), (GGS)₁-AS (SEQ ID NO:170), (GGGS)₁-AS (SEQ ID NO:171), (GGGGS)₁-AS (SEQ
 6 ID NO:172), (GGS)₂-AS (SEQ ID NO:173), (GGGGGGS)₁-AS (SEQ ID NO:174), (GGGS)₂-AS
 7 (SEQ ID NO:175), (GGS)₃-AS (SEQ ID NO:176), TG-(GGS)₁-AS (SEQ ID NO:177), TG-
 8 (GGGS)₁-AS (SEQ ID NO:178), TG-(GGGGS)₁-AS (SEQ ID NO:179), TG-(GGS)₂-AS (SEQ
 9 ID NO:180), TG-(GGGGGGS)₁-AS (SEQ ID NO:181), TG-(GGGS)₂-AS (SEQ ID NO:182),
 10 and TG-(GGS)₃-AS (SEQ ID NO:183).

1 67. The engineered polypeptide of claim 1 comprising a sequence as set forth
 2 in any one SEQ ID NOS:184-375.

1 68. The engineered polypeptide of claim 1 consisting of a sequence as set
 2 forth in any one SEQ ID NOS:184-375.

1 69. The engineered polypeptide of claim 1 comprising the sequence of SEQ
 2 ID NO:261.

1 70. The engineered polypeptide of claim 1 consisting of the sequence of SEQ
2 ID NO:261.

1 71. A method for treating a disease in a subject, comprising administering a
2 engineered polypeptide according to any one of claims 1 to 70 to a subject in need thereof in an
3 amount effective to treat said disease.

1 72. The method according to claim 71, wherein said disease is diabetes,
2 overweight, obesity, Alzheimer's disease, short bowel syndrome, fatty liver disease,
3 dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.

1 73. The method according to claim 72, wherein said disease is diabetes,
2 overweight, obesity, short bowel syndrome, NASH or Parkinson's disease.

1 74. The method according to claim 73, wherein said disease is type I diabetes,
2 type II diabetes or prediabetes.

1 75. The method according to claim 72, wherein said disease is type II
2 diabetes.

1 76. The method according to claim 72, wherein said disease is dyslipidemia or
2 hyperlipidemia.

1 77. The method according to claim 72, wherein the subject in need of such
2 treatment is obese.

1 78. A pharmaceutical composition comprising an engineered polypeptide
2 according to any one of claims 1 to 70 and a pharmaceutically acceptable excipient.

1 79. The pharmaceutical composition according to claim 78, wherein said
2 pharmaceutical composition is a parenteral pharmaceutical composition.

1 80. The pharmaceutical composition according to claim 78, wherein said
2 pharmaceutical composition is a sustained release or long lasting pharmaceutical composition.

1 81. The pharmaceutical composition according to claim 78, wherein said
2 pharmaceutical composition is formulated as a twice daily pharmaceutical composition.

1 82. The pharmaceutical composition according to claim 78, wherein said
2 pharmaceutical composition is formulated as a once daily pharmaceutical composition.

1 83. The pharmaceutical composition according to claim 78, wherein said
2 pharmaceutical composition is formulated as a once weekly pharmaceutical composition.

1 84. The pharmaceutical composition of claim 78 for treating a disease in a
2 subject.

1 85. The pharmaceutical composition of claim 84 wherein said disease is
2 diabetes, overweight, obesity, Alzheimer's disease, fatty liver disease, short bowel syndrome,
3 dyslipidemia, coronary artery disease, stroke, hyperlipidemia, NASH or Parkinson's disease.

1 86. The pharmaceutical composition of claim 85 wherein said disease is
2 diabetes, overweight, obesity, short bowel syndrome, or Parkinson's disease.

1 87. The pharmaceutical composition of claim 86, wherein said disease is type
2 I diabetes, type II diabetes or prediabetes.

1 88. The pharmaceutical composition of any one of claims 78 to 87, wherein
2 said engineered polypeptide comprises a sequence as set forth in any one SEQ ID NOS:184-375.

1 89. The pharmaceutical composition of any one of claims 78 to 87, wherein
2 said engineered polypeptide consists of a sequence as set forth in any one SEQ ID NOS:184-375.

1 90. The pharmaceutical composition of any one of claims 78 to 87, wherein
2 said engineered polypeptide comprises (SEQ ID NO: 261).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/034152**A. CLASSIFICATION OF SUBJECT MATTER****C07K 19/00(2006.01)i, C12N 15/62(2006.01)i, C12N 15/63(2006.01)i, A61K 38/22(2006.01)i, A61P 3/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 19/00; A61K 39/395; A61P 3/10; C07K 16/18; C07K 14/575; A61K 38/17; C07K 17/00; A61K 38/16; A61K 38/22; C12N 15/62; C12N 15/63; A61P 3/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: engineered polypeptide, extendin, humanized chimeric seal leptin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006-0293232 A1 (LEVY, ODILE ESTHER et al.) 28 December 2006 See claims 1, 3, 6, 13 and 51 ; paragraphs [0051] and [0078].	1-3,6
A		4,5,67-70
Y	GenBank (UniProtKB/Swiss-Prot): Q706D0, 1 May 2005 See the whole document.	1-3,6
A		4,5,67-70
A	US 2010-0292172 A1 (GHOSH, SOUMITRA S. et al.) 18 November 2010 See claims 1 and 6-8; paragraphs [0051], [0103] and [0271].	1-6,67-70
A	US 2009-0214534 A1 (HOLMES, STEVE et al.) 27 August 2009 See claims 1 and 42; paragraph [016].	1-6,67-70
A	US 2011-0200623 A1 (SONG, DAE HAE et al.) 18 August 2011 See claims 1-2 and 20.	1-6,67-70
A	US 2010-0168011 A1 (JENNINGS, JR., ROBERT N. et al.) 1 July 2010 See claims 35 and 39-40; paragraph [0305].	1-6,67-70



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 July 2013 (26.07.2013)

Date of mailing of the international search report

26 July 2013 (26.07.2013)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/034152

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

☐

on paper

☒

in electronic form

b. time of filing or furnishing

☒

contained in the international application as filed

☐

filed together with the international application in electronic form

☐

furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 71-77
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 71-77 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☒ Claims Nos.: 8,9,11-21,23-28,30,39-43,45,47-66,72-77,79-87
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The subject matter of claims 8, 9, 11-21, 23-28, 30, 39-43, 45, 47-66, 72-77 and 79-87 is so unclear and indefinite that no meaningful search could be made because these claims are drafted in reference to multiple dependent claims such as claims 7, 10, 22, 29, 31-38, 44, 46, 71, 78 and 88-90 (See the category Box No. II-3 below).
3. ☒ Claims Nos.: 7,10,22,29,31-38,44,46,71,78,88-90
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

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