

CLAIMS

1. A synthetic peptide shuttle agent when used in delivering a polypeptide cargo from an extracellular space to the cytosol of a target eukaryotic cell *ex vivo* or *in vitro*, wherein said shuttle agent is:

- (1) a peptide having an overall length of between 20 and 150 amino acids comprising
- (2) an amphipathic alpha-helical motif having
- (3) a positively-charged hydrophilic outer face, and a hydrophobic outer face,

wherein at least seven of the following parameters (4) to (15) are respected:

- (4) the hydrophobic outer face comprises a highly hydrophobic core consisting of spatially adjacent L, I, F, V, W, and/or M amino acids representing 12 to 50% of the amino acids of the peptide, based on an open cylindrical representation of the alpha-helix having 3.6 residues per turn;
- (5) the peptide has a hydrophobic moment (μ) of 3.5 to 11;
- (6) the peptide has a predicted net charge of at least +4 at physiological pH;
- (7) the peptide has an isoelectric point (pI) of 8 to 13;
- (8) the peptide is composed of 35% to 65% of any combination of the amino acids: A, C, G, I, L, M, F, P, W, Y, and V;
- (9) the peptide is composed of 0% to 30% of any combination of the amino acids: N, Q, S, and T;
- (10) the peptide is composed of 35% to 85% of any combination of the amino acids: A, L, K, or R;
- (11) the peptide is composed of 15% to 45% of any combination of the amino acids: A and L, provided there being at least 5% of L in the peptide;
- (12) the peptide is composed of 20% to 45% of any combination of the amino acids: K and R;
- (13) the peptide is composed of 0% to 10% of any combination of the amino acids: D and E;
- (14) the difference between the percentage of A and L residues in the peptide (% A + L), and the percentage of K and R residues in the peptide (% K + R), is less than or equal to 10%; and
- (15) the peptide is composed of 10% to 45% of any combination of the amino acids: Q, Y, W, P, I, S, G, V, F, E, D, C, M, N, T and H,

wherein the shuttle agent is used at a concentration sufficient to increase the transduction efficiency of the polypeptide cargo as compared to in the absence of said shuttle agent, and

wherein the shuttle agent and the polypeptide cargo comprise independent polypeptide backbones or are not covalently bound.

2. The synthetic peptide shuttle agent when used according to claim 1, wherein the shuttle agent respects at least eight, at least nine, at least ten, at least eleven, or respects all of parameters (4) to (15).

3. The synthetic peptide shuttle agent when used according to claim 1 or 2, wherein:

- (i) said shuttle agent is a peptide having a minimum length of 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids, and a maximum length of 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 60, 65, 70, 80, 90, 100, 110, 120, 130, or 140 amino acids;
- (ii) said amphipathic alpha-helical motif has a hydrophobic moment (μ) between a lower limit of 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and an upper limit of 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, or 11.0;
- (iii) said amphipathic alpha-helical motif comprises a positively-charged hydrophilic outer face comprising: (a) at least two, three, or four adjacent positively-charged K and/or R residues upon helical wheel projection; and/or (b) a

segment of six adjacent residues comprising three to five K and/or R residues upon helical wheel projection, based on an alpha helix having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn;

- (iv) said amphipathic alpha-helical motif comprises a hydrophobic outer face comprising: (a) at least two adjacent L residues upon helical wheel projection; and/or (b) a segment of ten adjacent residues comprising at least five hydrophobic residues selected from: L, I, F, V, W, and M, upon helical wheel projection, based on an alpha helix having angle of rotation between consecutive amino acids of 100 degrees and/or an alpha-helix having 3.6 residues per turn;
- (v) said hydrophobic outer face comprises a highly hydrophobic core consisting of spatially adjacent L, I, F, V, W, and/or M amino acids representing from 12.5%, 13%, 13.5%, 14%, 14.5%, 15%, 15.5%, 16%, 16.5%, 17%, 17.5%, 18%, 18.5%, 19%, 19.5%, or 20%, to 25%, 30%, 35%, 40%, or 45% of the amino acids of the peptide;
- (vi) said peptide has a hydrophobic moment (μ) between a lower limit of 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and an upper limit of 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, or 10.5;
- (vii) said peptide has a predicted net charge of between +4, +5, +6, +7, +8, +9, to +10, +11, +12, +13, +14, or +15;
- (viii) said peptide has a predicted pI of 10-13; or
- (ix) any combination of (i) to (viii).

4. The synthetic peptide shuttle agent when used according to any one of claims 1 to 3, wherein said shuttle agent respects at least one, at least two, at least three, at least four, at least five, at least six, or all of the following parameters:

- (8) the peptide is composed of 36% to 64%, 37% to 63%, 38% to 62%, 39% to 61%, or 40% to 60% of any combination of the amino acids: A, C, G, I, L, M, F, P, W, Y, and V;
- (9) the peptide is composed of 1% to 29%, 2% to 28%, 3% to 27%, 4% to 26%, 5% to 25%, 6% to 24%, 7% to 23%, 8% to 22%, 9% to 21%, or 10% to 20% of any combination of the amino acids: N, Q, S, and T;
- (10) the peptide is composed of 36% to 80%, 37% to 75%, 38% to 70%, 39% to 65%, or 40% to 60% of any combination of the amino acids: A, L, K, or R;
- (11) the peptide is composed of 15% to 40%, 20% to 40%, 20 to 35%, or 20 to 30% of any combination of the amino acids: A and L;
- (12) the peptide is composed of 20% to 40%, 20 to 35%, or 20 to 30% of any combination of the amino acids: K and R;
- (13) the peptide is composed of 5 to 10% of any combination of the amino acids: D and E;
- (14) the difference between the percentage of A and L residues in the peptide (% A+ L), and the percentage of K and R residues in the peptide (K + R), is less than or equal to 9%, 8%, 7%, 6%, or 5%; and
- (15) the peptide is composed of 15 to 40%, 20% to 35%, or 20% to 30% of any combination of the amino acids: Q, Y, W, P, I, S, G, V, F, E, D, C, M, N, T, and H.

5. The synthetic peptide shuttle agent when used according to any one of claims 1 to 4, wherein said peptide comprises a histidine-rich domain positioned towards the N terminus and/or towards the C terminus of the shuttle agent, wherein said histidine-rich domain is a stretch of at least 3, at least 4, at least 5, or at least 6 amino acids comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% histidine residues; and/or comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, or at least 9 consecutive histidine residues.

6. The synthetic peptide shuttle agent when used according to any one of claims 1 to 5, wherein said peptide comprises a flexible linker domain rich in serine and/or glycine residues.

7. The synthetic peptide shuttle agent when used according to any one of claims 1 to 6, wherein said peptide comprises or consists of the amino acid sequence of:

- (a) [X1]-[X2]-[linker]-[X3]-[X4] (Formula 1);
- (b) [X1]-[X2]-[linker]-[X4]-[X3] (Formula 2);
- (c) [X2]-[X1]-[linker]-[X3]-[X4] (Formula 3);
- (d) [X2]-[X1]-[linker]-[X4]-[X3] (Formula 4);
- (e) [X3]-[X4]-[linker]-[X1]-[X2] (Formula 5);
- (f) [X3]-[X4]-[linker]-[X2]-[X1] (Formula 6);
- (g) [X4]-[X3]-[linker]-[X1]-[X2] (Formula 7); or
- (h) [X4]-[X3]-[linker]-[X2]-[X1] (Formula 8),

wherein:

[X1] is selected from: 2[Φ]-1[+]-2[Φ]-1[ζ]-1[+]-; 2[Φ]-1[+]-2[Φ]-2[+]-; 1[+]-1[Φ]-1[+]-2[Φ]-1[ζ]-1[+]-; and 1[+]-1[Φ]-1[+]-2[Φ]-2[+]-;

[X2] is selected from: -2[Φ]-1[+]-2[Φ]-2[ζ]-; -2[Φ]-1[+]-2[Φ]-2[+]-; -2[Φ]-1[+]-2[Φ]-1[+]-1[ζ]-; -2[Φ]-1[+]-2[Φ]-1[ζ]-1[+]-; -2[Φ]-2[+]-1[Φ]-2[+]-; -2[Φ]-2[+]-1[Φ]-2[ζ]-; -2[Φ]-2[+]-1[Φ]-1[+]-1[ζ]-; and -2[Φ]-2[+]-1[Φ]-1[ζ]-1[+]-;

[X3] is selected from: -4[+]-A-; -3[+]-G-A-; -3[+]-A-A-; -2[+]-1[Φ]-1[+]-A-; -2[+]-1[Φ]-G-A-; -2[+]-1[Φ]-A-A-; or -2[+]-A-1[+]-A-; -2[+]-A-G-A-; -2[+]-A-A-A-; -1[Φ]-3[+]-A-; -1[Φ]-2[+]-G-A-; -1[Φ]-2[+]-A-A-; -1[Φ]-1[+]-1[Φ]-1[+]-A-; -1[Φ]-1[+]-1[Φ]-G-A-; -1[Φ]-1[+]-1[Φ]-A-A-; -1[Φ]-1[+]-A-1[+]-A-; -1[Φ]-1[+]-A-G-A-; -1[Φ]-1[+]-A-A-A-; -A-1[+]-A-1[+]-A-; -A-1[+]-A-G-A-; and -A-1[+]-A-A-A-;

[X4] is selected from: -1[ζ]-2A-1[+]-A-; -1[ζ]-2A-2[+]-; -1[+]-2A-1[+]-A-; -1[ζ]-2A-1[+]-1[ζ]-A-1[+]-; -1[ζ]-A-1[ζ]-A-1[+]-; -2[+]-A-2[+]-; -2[+]-A-1[+]-A-; -2[+]-A-1[+]-1[ζ]-A-1[+]-; -2[+]-1[ζ]-A-1[+]-; -1[+]-1[ζ]-A-1[+]-A-; -1[+]-1[ζ]-A-2[+]-; -1[+]-1[ζ]-A-1[+]-1[ζ]-A-1[+]-; -1[+]-2[ζ]-A-1[+]-; -1[+]-2[ζ]-2[+]-; -1[+]-2[ζ]-1[+]-A-; -1[+]-2[ζ]-1[+]-1[ζ]-A-1[+]-; -1[+]-2[ζ]-1[ζ]-A-1[+]-; -3[ζ]-2[+]-; -3[ζ]-1[+]-A-; -3[ζ]-1[+]-1[ζ]-A-1[+]-; -1[ζ]-2A-1[+]-A-; -1[ζ]-2A-2[+]-; -1[ζ]-2A-1[+]-1[ζ]-A-1[+]-; -2[+]-A-1[+]-A-; -2[+]-1[ζ]-1[+]-A-; -1[+]-1[ζ]-A-1[+]-A-; -1[+]-2A-1[+]-1[ζ]-A-1[+]-; and -1[ζ]-A-1[ζ]-A-1[+]-; and

[linker] is selected from: -Gn-; -Sn-; -(GnSn)n-; -(GnSn)nGn-; -(GnSn)nSn-; -(GnSn)nGn(GnSn)n-; and -(GnSn)nSn(GnSn)n-;

wherein:

[Φ] is an amino acid which is: Leu, Phe, Trp, Ile, Met, Tyr, or Val;

[+] is an amino acid which is: Lys or Arg;

[ζ] is an amino acid which is: Gln, Asn, Thr, or Ser;

A is the amino acid Ala;

G is the amino acid Gly;

S is the amino acid Ser; and

n is an integer from 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 1 to 4, or 1 to 3.

8. A composition when used in delivering a polypeptide cargo from an extracellular space to the cytosol of a target eukaryotic cell *ex vivo* or *in vitro*, the composition comprising the shuttle agent as defined in any one of claims 1 to 7, and the polypeptide cargo to be delivered, wherein the shuttle agent is present in the composition at a concentration sufficient to increase

the transduction efficiency of the polypeptide cargo as compared to in the absence of said shuttle agent, and wherein the shuttle agent and the polypeptide cargo comprise independent polypeptide backbones or are not covalently bound.

9. A kit when used for delivering a polypeptide cargo from an extracellular space to the cytosol of a target eukaryotic cell *ex vivo* or *in vitro*, said kit comprising the shuttle agent as defined in any one of claims 1 to 7, and a suitable container.

10. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7, the composition when used according to claim 8, or the kit when used according to any one of claim 9, wherein said polypeptide cargo lacks a cell penetrating domain.

11. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7, the composition when used according to claim 8, or the kit when used according to any one of claim 9, wherein said polypeptide cargo comprises a cell penetrating domain.

12. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7, 10 or 11, the composition when used according to claim 8, 10 or 11, or the kit when used according to any one of claims 9 to 11, wherein said polypeptide cargo comprises a subcellular targeting domain.

13. The synthetic peptide shuttle agent when used according to claim 12, the composition when used according to claim 12, or the kit when used according to claim 12, wherein said subcellular targeting domain is:

- (a) a nuclear localization signal (NLS);
- (b) a nucleolar signal sequence;
- (c) a mitochondrial signal sequence; or
- (d) a peroxisome signal sequence.

14. The synthetic peptide shuttle agent when used according to claim 13, the composition when used according to claim 13, or the kit when used according to claim 13, wherein:

- (a) said NLS is from: E1a, T-Ag, c-myc, T-Ag, op-T-NLS, Vp3, nucleoplasmin, histone 2B, Xenopus N1, PARP, PDX-1, QKI-5, HCDA, H2B, v-Rel, Amida, RanBP3, Pho4p, LEF-1, TCF-1, BDV-P, TR2, SOX9, or Max;
- (b) said nucleolar signal sequence is from BIRC5 or RECQL4;
- (c) said mitochondrial signal sequence is from Tim9 or Yeast cytochrome c oxidase subunit IV; or
- (d) said peroxisome signal sequence is from PTS1.

15. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7 or 10 to 14, the composition when used according to any one of claims 8 or 10 to 14, or the kit when used according to any one of claims 9 to 14, wherein said polypeptide cargo is complexed with a DNA and/or RNA molecule.

16. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7 or 10 to 15, the composition when used according to any one of claims 8 or 10 to 15, or the kit when used according to any one of claims 9 to 15, wherein said polypeptide cargo is a transcription factor, a nuclease, a cytokine, a hormone, a growth factor, an antibody, a peptide cargo, an enzyme, an enzyme inhibitor, or any combination thereof.

17. The synthetic peptide shuttle agent when used according to claim 16, the composition when used according to claim 16, or the kit when used according to claim 16, wherein:
- (a) said transcription factor is: HOXB4, NUP98-HOXA9, Oct3/4, Sox2, Sox9, Klf4, c-Myc, MyoD, Pdx1, Ngn3, MafA, Blimp-1, Eomes, T-bet, FOXO3A, NF-YA, SALL4, ISL1, FoxA1, Nanog, Esrrb, Lin28, HIF1-alpha, Hlf, Runx1t1, Pbx1, Lmo2, Zfp37, Prdm5, Bcl-6, or any combination thereof;
 - (b) said nuclease is a catalytically active or catalytically dead: RNA-guided endonuclease, CRISPR endonuclease, type I CRISPR endonuclease, type II CRISPR endonuclease, type III CRISPR endonuclease, type IV CRISPR endonuclease, type V CRISPR endonuclease, type VI CRISPR endonuclease, CRISPR associated protein 9 (Cas9), Cpf1, CasY, CasX, zinc-finger nuclease (ZFNs), Transcription activator-like effector nucleases (TALENs), homing endonuclease, meganuclease, DNA-guided nuclease, *Natronobacterium gregoryi* Argonaute (NgAgo), or any combination thereof;
 - (c) said antibody recognizes an intracellular antigen; and/or
 - (d) said peptide cargo recognizes an intracellular molecule.
18. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7 or 10 to 17, the composition when used according to any one of claims 8 or 10 to 17, or the kit when used according to any one of claims 9 to 17, wherein said use in delivering a polypeptide cargo from an extracellular space to the cytosol of a target eukaryotic cell *ex vivo* or *in vitro* is in the context of cell therapy, genome editing, adoptive cell transfer, and/or regenerative medicine.
19. The synthetic peptide shuttle agent when used according to any one of claims 1 to 7 or 10 to 18, the composition when used according to any one of claims 8 or 10 to 18, or the kit when used according to any one of claims 9 to 18, wherein said target eukaryotic cell is an animal cell, a mammalian cell, a human cell, a stem cell, a primary cell, an immune cell, a T cell, an NK cell, or a dendritic cell.
20. A method for enriching eukaryotic cells transduced with a polypeptide cargo of interest, said method comprising:
- (a) co-transducing a target eukaryotic cell population with a polypeptide cargo of interest and a marker protein using the synthetic peptide shuttle agent as defined in any one of claims 1 to 7; and
 - (b) isolating or concentrating eukaryotic cells transduced with the marker protein, thereby enriching eukaryotic cells transduced with the polypeptide cargo of interest,
- wherein the intracellular concentration of the transduced marker protein is positively correlated with the intracellular concentration of the transduced polypeptide cargo of interest.
21. The method of claim 20, wherein:
- (i) the marker protein is not covalently bound to the polypeptide cargo of interest or the marker protein is covalently bound to the polypeptide cargo of interest via a cleavable linker; and/or
 - (ii) the marker protein comprises a detectable label, or the marker protein is a fluorescent protein, a fluorescently-labeled protein, a bioluminescent protein, an isotopically-labelled protein, or a magnetically-labeled protein.
22. The method of claim 20 or 21, further comprising repeating steps (a) and (b), one or more times, on the marker protein-negative cell population, on the marker protein-positive cell population, or on both the marker protein-negative and the marker protein-positive cell populations.

23. The method of any one of claims 20 to 22, wherein the eukaryotic cells transduced with the marker protein are isolated or sorted based on their intracellular concentration of the marker protein.
24. The method of any one of claims 20 to 23, wherein the marker protein is a protein that stimulates cell proliferation, a protein that stimulates cell differentiation, a protein that promotes cell survival, an anti-apoptotic protein, or a protein having another biological activity.
25. A protein transduction agent comprising the peptide as defined in any one of claims 1 to 7.
26. The protein transduction agent of claim 25 for use in therapy.
27. A composition comprising the shuttle agent as defined in any one of claims 1 to 7, and a polypeptide cargo to be delivered from an extracellular space to the cytosol of a target eukaryotic cell, wherein the shuttle agent is present in the composition at a concentration sufficient to increase the transduction efficiency of the polypeptide cargo as compared to in the absence of said shuttle agent, and wherein the shuttle agent and the polypeptide cargo comprise independent polypeptide backbones or are not covalently bound.
28. The composition of claim 27 for use in therapy.
29. The composition of claim 27 or 28, wherein the polypeptide cargo is as defined in any one of claims 10 to 17.