

TAT₁₁-EGFP to the cytosol (see Figure 3 of Salomone et al., 2012), the CM₁₈-TAT₁₁ peptide (alone or conjugated to Atto-633) was not shown to act as a shuttle agent that can increase delivery of a polypeptide cargo *from an extracellular space to inside of the cell* – i.e., *across the plasma membrane*. In fact, Salomone et al., 2012 compared co-treatment (simultaneous treatment of TAT₁₁-EGFP and CM₁₈-TAT₁₁-Atto-633) versus time-shifted treatment (i.e., incubation of cells with TAT₁₁-EGFP alone, fluorescence imaging, and then incubation of the same cells with the CM₁₈-TAT₁₁-Atto-633 peptide alone, and again fluorescence imaging), and the authors reported that “*both yielded the same delivery results*” (see page 295 of Salomone et al., 2012, last sentence of first paragraph under the heading “2.9 Cargo delivery assays”). In other words, Salomone et al., 2012 described that the peptide CM₁₈-TAT₁₁ (alone or conjugated to Atto-633) had no effect on delivery of a polypeptide cargo from an extracellular space to inside of the cell (i.e., protein transduction). Thus, there remains a need for improved shuttle agents capable of increasing the transduction efficiency of polypeptide cargos, and delivering the cargos to the cytosol and/or nucleus of target eukaryotic cells.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY

A plurality of different peptides was screened with the goal of identifying polypeptide-based shuttle agents that can deliver independent polypeptide cargos intracellularly to the cytosol/nucleus of eukaryotic cells. On one hand, these large-scale screening efforts led to the surprising discovery that certain domain-based peptide shuttle agents increase the transduction efficiency of polypeptide cargos in eukaryotic cells, by increasing the number and/or proportion of cells that ultimately internalize the polypeptide cargos, and also enable the internalized cargos to gain access to the cytosol/nuclear compartment (thus avoiding or reducing cargo endosomal entrapment). These domain-base shuttle agents comprise an endosome leakage domain (ELD) operably linked to a cell penetrating domain (CPD), and optionally one or more histidine-rich domains. On the other hand, the above screening efforts also revealed some peptides having no or low polypeptide cargo transduction activity, excessive toxicity, and/or other undesirable properties (e.g., poor solubility and/or stability). These empirical data (both positive and negative) were used herein to identify physiochemical properties of successful, less successful, and failed peptides in order to arrive at a set of design parameters that enable the rational design and/or identification of peptides having protein transduction activity.

In one aspect, the present disclosure refers to 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
- (6) the peptide has a predicted net charge of at least +6 at physiological pH, and

wherein at least six of parameters (4), (5) and (7) 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;
- (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 does not comprise the cell penetrating domain of any one of SEQ ID NOs: 16-27 or 65,
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.

In another aspect, the present disclosure refers to 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 disclosed herein, 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.

In another aspect, the present disclosure refers to 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 disclosed herein, and a suitable container, and 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.

In another aspect, the present disclosure refers to an *in vitro* 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, and 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.

In another aspect, the present disclosure refers to 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 disclosed herein, and the 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

Accordingly, the present description relates to methods for delivering polypeptide cargos from an extracellular space to the cytosol and/or nucleus of a target eukaryotic cell by contacting the cell with the polypeptide cargo in the presence of a peptide shuttle agent as described herein, at a concentration sufficient to increase the polypeptide cargo's transduction efficiency, as compared to in the absence of the shuttle agent. More particularly, the present description relates to parameters that may be used in the rational design of such synthetic peptide shuttle agents, peptide shuttle agents that satisfy one or more of these design parameters, as well as methods and compositions relating to the use of the synthetic peptide shuttle agents for delivery of a variety of polypeptide cargos from an extracellular space to the cytosol and/or nucleus of target eukaryotic cells. The present description also relates to machine-learning or computer-assisted approaches that may be used to generate peptide variants that respect one or more of the design parameters described herein.

The present description also relates to co-transducing a polypeptide cargo of interest and a marker protein as a means to identify and/or enrich transduced cells. It was surprisingly discovered that a strikingly high proportion of target eukaryotic cells that were successfully transduced with a polypeptide cargo of interest, were also successfully transduced with a marker protein. Conversely, a strikingly high proportion of cells that were not transduced with the polypeptide cargo of interest, were also not transduced with the marker protein. Isolating cells positive for the marker protein (e.g., via FACS) resulted in a significant increase in the proportion of cells that were successfully transduced with the polypeptide cargo of interest, and the correlation was found to be concentration dependent in that cell populations exhibiting the highest fluorescence of the marker protein also tended to exhibit the highest proportion of transduction with the polypeptide cargo of interest. It was also discovered that cells that were unsuccessfully transduced following a first round of transduction with a polypeptide cargo of interest, may be isolated and re-transduced with the polypeptide cargo of interest in subsequent rounds of transduction. Thus, in some examples, the present description relates to methods comprising co-transduction of a polypeptide cargo of interest with a marker protein, wherein the marker protein may be used to isolate or enrich cells transduced with a polypeptide cargo of interest. In some embodiments, the present description also relates to methods comprising repeated successive transduction experiments performed on, for example, cells that were not successfully transduced with a marker protein following a first or previous transduction reaction. Such methods present attractive approaches for increasing transduction efficiency in valuable cell populations (e.g., patient-derived cells for cell therapy), and/or in cell populations that are inherently more difficult to transduce.

General Definitions

Headings, and other identifiers, e.g., (a), (b), (i), (ii), etc., are presented merely for ease of reading the specification and claims. The use of headings or other identifiers in the specification or claims does not necessarily require the steps or elements be performed in alphabetical or numerical order or the order in which they are presented.

The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one” but it is also consistent with the meaning of “one or more”, “at least one”, and “one or more than one”.

The term “**about**” is used to indicate that a value includes the standard deviation of error for the device or method being employed in order to determine the value. In general, the terminology “about” is meant to designate a possible variation of up to 10%. Therefore, a variation of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% of a value is included in the term “about”. Unless indicated otherwise, use of the term “about” before a range applies to both ends of the range.

As used in this specification and claim(s), the words “**comprising**” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

As used herein, “**protein**” or “**polypeptide**” or “**peptide**” means any peptide-linked chain of amino acids, which may or may not comprise any type of modification (e.g., chemical or post-translational modifications such as acetylation, phosphorylation, glycosylation, sulfatation, sumoylation, prenylation, ubiquitination, etc.). For further clarity, protein/polypeptide/peptide

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
- (6) the peptide has a predicted net charge of at least +6 at physiological pH, and

wherein at least six of parameters (4), (5) and (7) 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;
- (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 does not comprise the cell penetrating domain of any one of SEQ ID NOs: 16-27 or 65,

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), (5) and (7) 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 +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 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.

- 5 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, and 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.
- 10 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.
- 15 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.
- 20 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.
- 25 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.
- 30 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.
- 35 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.
- 40 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, *Neisseria meningitidis* 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. An *in vitro* 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, and 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.

21. The *in vitro* 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 *in vitro* 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.
- 5 23. The *in vitro* 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.
- 10 24. The *in vitro* 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.
- 15 25. 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 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.
- 20 26. The composition of claim 25, wherein the polypeptide cargo is as defined in any one of claims 10 to 17.