COMPOSITIONS AND METHODS FOR MEMBRANE PROTEIN DELIVERY

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Serial No. 62/631,747 filed February 17, 2018, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 12, 2019, is named V2050-7013WO_SL.txt and is 14,911 bytes in size.

BACKGROUND

[0003] Cell–cell fusion is required in biological processes as diverse as fertilization, development, immune response, and tumorigenesis.

SUMMARY

[0004] The present disclosure provides technologies relating to fusosomes and their use to deliver membrane proteins to target cells. In some embodiments, a fusosome comprises a lipid bilayer, a lumen surrounded by the lipid bilayer, a fusogen, and a cargo that includes a membrane protein payload agent, In some embodiments, such cargo may be or comprise a membrane protein itself; in some embodiments, such cargo may be or comprise a nucleic acid that encodes (or is complementary to a nucleic acid that encodes) a membrane protein.

[0005] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and

(d) a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell) that comprises or encodes one or more of:

i) a chimeric antigen receptor;

ii) an integrin membrane protein payload, e.g., chosen from Table 5;
iii) an ion channel protein chosen from Table 6;
iv) a pore forming protein, e.g., chosen from Tables 7 and 8;
v) a Toll-Like Receptor, e.g., chosen from Table 9;
vi) an interleukin receptor payload, e.g., chosen from Table 10;
vii) a cell adhesion protein chosen from Tables 11-12;
viii) a transport protein chosen from Table 15;
ix) a signal sequence that is heterologous relative to the naturally-occurring membrane protein; or
x) a signal sequence listed in Table 4;

wherein optionally the fusosome does not comprise a nucleocapsid protein or a viral matrix protein.

[0006] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;
(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;
(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and
(d) a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell) that comprises or encodes a T cell receptor;

wherein optionally the fusosome does not comprise a nucleocapsid protein or a viral matrix protein.

[0007] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;
(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;
(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and
(d) a membrane protein payload agent that is exogenous or overexpressed relative to the source cell; and

wherein one or more of:

i) the fusosome comprises or is comprised by a cytobiologic;

ii) the fusogen is present at a copy number of at least 1,000 copies, e.g., as measured by an assay of Example 29;

iii) the fusosome comprises a therapeutic agent at a copy number of at least 1,000 copies, e.g., as measured by an assay of Example 43;

iv) the fusosome comprises a lipid wherein one or more of CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG is within 75% of the corresponding lipid level in the source cell;

v) the fusosome comprises a proteomic composition similar to that of the source cell, e.g., using an assay of Example 42;

vi) the fusosome is capable of signal transduction, e.g., transmitting an extracellular signal, e.g., AKT phosphorylation in response to insulin, or glucose (e.g., labeled glucose, e.g., 2-NBDG) uptake in response to insulin, e.g., by at least 10% more than a negative control, e.g., an otherwise similar fusosome in the absence of insulin, e.g., using an assay of Example 63;

vii) the fusosome targets a tissue, e.g., liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, or eye, when administered to a subject, e.g., a mouse, e.g., wherein at least 0.1%, or 10%, of the fusosomes in a population of administered fusosomes are present in the target tissue after 24 hours, e.g., by an assay of Example 87 or 100; or

viii) the source cell is selected from a neutrophil, a granulocyte, a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell, a myeloblast, a myoblast, a hepatocyte, or a neuron e.g., retinal neuronal cell.

[0008] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and

(d) a membrane protein payload agent that:

i) comprises DNA that encodes a membrane protein; or

ii) comprises RNA, e.g., mRNA, that encodes a membrane protein that is exogenous or overexpressed relative to the source cell,

wherein optionally the fusosome does not comprise a nucleocapsid protein or a viral matrix protein.

[0009] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a non-viral, e.g., mammalian, fusogen that is exogenous or overexpressed relative to the source cell, wherein the mammalian fusogen is not Alzheimer's beta-amyloid peptide or fertilin; and

(d) a membrane protein payload agent which is exogenous or overexpressed relative to the source cell,

wherein optionally the fusosome does not comprise a nucleocapsid protein or a viral matrix protein.

[00010] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and

(d) a membrane protein payload agent that is exogenous or overexpressed relative to the source cell;

wherein the fusosome comprises an enucleated cell, and

wherein optionally the fusosome does not comprise a nucleocapsid protein or a viral matrix protein.

[00011] In some aspects, the disclosure provides a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and

(d) a membrane protein payload agent that is exogenous or overexpressed relative to the source cell;

and wherein one or more of:

i) the fusosome comprises or is comprised by a cytobiologic;

ii) the fusosome comprises an enucleated cell;

iii) the fusosome comprises an inactivated nucleus;

iv) the fusosome fuses at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, e.g., in an assay of Example 54;

v) the fusosome fuses at a higher rate with a target cell than non-target fusosomes, e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., in an assay of Example 54;

vi) the fusosome fuses with target cells at a rate such that the membrane protein payload agent in the fusosome is delivered to at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of target cells after 24, 48, or 72 hours, e.g., in an assay of Example 54;

vii) the fusogen is present at a copy number, per fusosome, of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example

29;

viii) the fusosome comprises the membrane protein payload agent at a copy number, per fusosome, of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 43;

ix) the ratio of the copy number of the fusogen to the copy number of the membrane protein payload agent is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000;

x) the fusosome comprises a lipid composition substantially similar to that of the source cell or wherein one or more of CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG is within 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, or 75% of the corresponding lipid level in the source cell;

xi) the fusosome comprises a proteomic composition similar to that of the source cell, e.g., using an assay of Example 42;

xii) the fusosome comprises a ratio of lipids to proteins that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 49;

xiii) the fusosome comprises a ratio of proteins to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 50;

xiv) the fusosome comprises a ratio of lipids to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 51;

xv) the fusosome has a half-life in a subject, e.g., in a mouse, that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the half

life of a reference cell, e.g., the source cell, e.g., by an assay of Example 75;

xvi) the fusosome transports glucose (e.g., labeled glucose, e.g., 2-NBDG) across a membrane, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of glucose, e.g., as measured using an assay of Example 64;

xvii) the fusosome comprises esterase activity in the lumen that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of that of the esterase activity in a reference cell, e.g., the source cell or a mouse embryonic fibroblast, e.g., using an assay of Example 66;

xviii) the fusosome comprises a metabolic activity (e.g., citrate synthase activity) level that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the metabolic activity (e.g., citrate synthase activity) in a reference cell, e.g., the source cell, e.g., as described in Example 68;

xix) the fusosome comprises a respiration level (e.g., oxygen consumption rate) that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the respiration level (e.g., oxygen consumption rate) in a reference cell, e.g., the source cell, e.g., as described in Example 69;

xx) the fusosome comprises an Annexin-V staining level of at most 18,000, 17,000, 16,000, 15,000, 14,000, 13,000, 12,000, 11,000, or 10,000 MFI, e.g., using an assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of an otherwise similar fusosome treated with menadione in the assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of a macrophage treated with menadione in the assay of Example 70,

xxi) the fusosome has a miRNA content level of at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., by an assay of Example 39;

xxii) the fusosome has a soluble : non-soluble protein ratio that is within 1%, 2%, 3%,

4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., within 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of that of the source cell, e.g., by an assay of Example 47;

xxiii) the fusosome has an LPS level less than 5%, 1%, 0.5%, 0.01%, 0.005%, 0.0001%, 0.00001% or less of the LPS content of the source cell, e.g., as measured by mass spectrometry as described in Example 48;

xxiv) the fusosome and/or compositions or preparations thereof, are capable of signal transduction, e.g., transmitting an extracellular signal, e.g., AKT phosphorylation in response to insulin, or glucose (e.g., labeled glucose, e.g., 2-NBDG) uptake in response to insulin, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of insulin, e.g., using an assay of Example 63;

xxv) the fusosome targets a tissue, e.g., liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, or eye, when administered to a subject, e.g., a mammal, e.g., an experimental mammal (e.g., a mouse), a domesticated animal (e.g., a pet or farm animal), or a human, wherein at least 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fusosomes in a population of administered fusosomes are present in the target tissue after 24, 48, or 72 hours, e.g., by an assay of Example 87 or 100;

xxvi) the fusosome has juxtacrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71;

xxvii) the fusosome has paracrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% greater than the level of paracrine signaling induced by a reference cell, e.g., the source cell or a macrophage, e.g., by an assay of Example 72;

xxviii) the fusosome polymerizes actin at a level within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% compared to the level of polymerized actin in a reference cell, e.g., the source cell or a C2C12 cell, e.g., by the assay of Example 73;

xxix) the fusosome has a membrane potential within about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the membrane potential of a reference cell, e.g., the source cell or a C2C12 cell, e.g., by an assay of Example 74, or wherein the fusosome has a membrane potential of about -20 to -150mV, -20 to -50mV, -50 to -100mV, or -100 to -150mV;

xxx) the fusosome and/or compositions or preparations thereof, are capable of extravasation from blood vessels, e.g., at a rate at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% the rate of extravasation of a cell of the same type as the source cell, e.g., using an assay of Example 57, e.g., wherein the source cell is a neutrophil, lymphocyte, B cell, macrophage, or NK cell;

xxxi) the fusosome and/or compositions or preparations thereof, are capable of crossing a cell membrane, e.g., an endothelial cell membrane or the blood brain barrier, e.g., at a rate at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% that of a cell of the same type as the source cell;

xxxii) the fusosome and/or compositions or preparations thereof, are capable of secreting a protein, e.g., at a rate at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than a reference cell, e.g., a mouse embryonic fibroblast or the source cell, e.g., using an assay of Example 62;

xxxiii) the fusosome meets a pharmaceutical or good manufacturing practices (GMP) standard;

xxxiv) the fusosome was made according to good manufacturing practices (GMP);

xxxv) a pharmaceutical preparation comprising a plurality of fusosomes as described herein has a pathogen level below a predetermined reference value, e.g., is substantially free of pathogens;

xxxvi) a pharmaceutical preparation comprising a plurality of fusosomes as described herein has a contaminant level below a predetermined reference value, e.g., is

substantially free of contaminants;

xxxvii) a pharmaceutical preparation comprising a plurality of fusosomes as described herein has low immunogenicity, e.g., as described herein;

xxxviii) the source cell is selected from a neutrophil, a granulocyte, a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell, a myeloblast, a myoblast, a hepatocyte, or a neuron e.g., retinal neuronal cell; or

xxxix) the source cell is other than a 293 cell, HEK cell, human endothelial cell, or a human epithelial cell, monocyte, macrophage, dendritic cell, or stem cell.

[00012] In some embodiments, a membrane protein relevant to the present disclosure is an integral membrane protein; in some embodiments, a membrane protein is a peripheral membrane protein. In other embodiments, a membrane protein is temporarily associated with a membrane. In some embodiments, a membrane protein is a protein that is associated with, and/or wholly or partially spans (e.g., as a transmembrane protein) a target cell's membrane. In some embodiments, a membrane protein is an integral monotopic protein (i.e., associated with only one side of a membrane). In some embodiments, a membrane protein is or becomes associated with (e.g., is partly or wholly present on) an outer surface of a target cell's membrane. In some embodiments, a membrane protein is or becomes associated with (e.g., is partly or wholly present on) an inner surface of a target cell's membrane.

[00013] In some embodiments, a membrane protein relevant to the present disclosure is a therapeutic membrane protein. In some embodiments, a membrane protein relevant to the present disclosure is or comprises a receptor (e.g., a cell surface receptor and/or a transmembrane receptor), a cell surface ligand, a membrane transport protein (e.g., an active or passive transport protein such as, for example, an ion channel protein, a pore-forming protein [e.g., a toxin protein], etc), a membrane enzyme, and/or a cell adhesion protein).

[00014] In some embodiments, a membrane protein relevant to the present disclosure comprises a sequence of a naturally-occurring membrane protein. In some embodiments, a membrane protein relevant to the present disclosure is or comprises a variant or modified version of a naturally-occurring membrane protein. In some embodiments, a membrane protein relevant

to the present disclosure is or comprises an engineered membrane protein. In some embodiments, a membrane protein relevant to the present disclosure is or comprises a fusion protein.

[00015] In some embodiments, the present disclosure provides and/or utilizes fusosome preparations in which a membrane protein payload agent is partially or wholly disposed in a fusosome lumen. In some embodiments, the present disclosure provides fusosome preparations in which a membrane protein payload agent is associated with (e.g., partially or wholly located within) a fusosome's lipid bilayer. In some embodiments, the relevant membrane protein is associated with and/or partially or wholly displayed on the fusosome's external surface.

[00016] The present disclosure provides, in some aspects, a fusosome comprising:

(a) a lipid bilayer,

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer,

(d) a membrane protein payload agent, e.g., a membrane protein exogenous to the source cell,

wherein the fusosome is derived from a source cell; and

wherein the fusosome has partial or complete nuclear inactivation (e.g. lacks an intact nucleus as found in the source cell, nuclear removal/enucleation, non-functional nucleus, etc.).

[00017] The present disclosure provides, in some aspects, a fusosome comprising:

(a) a lipid bilayer,

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the target cell, e.g., wherein the fusogen is disposed in the lipid bilayer (e.g., wherein the fusogen is endogenous or exogenous to the source cell), and

(d) a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell) that:

xi) comprises or encodes a chimeric antigen receptor;

xii) comprises or encodes an integrin membrane protein payload, e.g., chosen from Table 5;

xiii) comprises or encodes an ion channel protein chosen from Table 6;

xiv) comprises or encodes a pore forming protein, e.g., chosen from Tables 7 and 8;
xv) comprises or encodes a Toll-Like Receptor, e.g., chosen from Table 9;
xvi) comprises or encodes an interleukin receptor payload, e.g., chosen from Table 10;
xvii) comprises or encodes a cell adhesion protein chosen from Tables 11-12;
xviii) comprises or encodes a transport protein chosen from Table 15;
xix) comprises or encodes a signal sequence that is heterologous relative to the naturally-occurring membrane protein;
xx) comprises or encodes a signal sequence listed in Table 4;

wherein the fusosome does not comprise viral capsid or viral envelope proteins.

[00018] The present disclosure provides, in some aspects, a fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;
(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;
(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and
(d) a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell) that comprises or encodes one or more of:
i) a lipid-anchored protein;
ii) an extracellular protein that binds a transmembrane protein;
iii) an extracellular protein that lacks a transmembrane domain;
iv) a protein that partially spans a membrane (e.g., a membrane of the target cell or the fusosome) and does not completely span the membrane (e.g., the protein comprises an in-plane membrane helix, or the protein comprises a hydrophobic loop that does not completely span the membrane); or
v) the protein does not comprise a transmembrane domain, wherein the protein interacts with a membrane surface, e.g., through electropstatic or ionic interactions;

wherein the fusosome does not comprise a viral structural protein, e.g., a viral capsid protein or a viral envelope protein.

[00019] In some embodiments, one or more of the following is present:

xl) the fusosome comprises or is comprised by a cytobiologic;
xli) the fusosome comprises an enucleated cell;
xlii) the fusosome comprises an inactivated nucleus;
xliii) the fusosome fuses at a higher rate with a target cell than with a non-target cell,

e.g., by at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, e.g., in an assay of Example 54;

xliv) the fusosome fuses at a higher rate with a target cell than non-target fusosomes, e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., in an assay of Example 54;

xlv) the fusosome fuses with target cells at a rate such that the membrane protein payload agent in the fusosome is delivered to at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of target cells after 24, 48, or 72 hours, e.g., in an assay of Example 54;

xlvi) the fusogen is present at a copy number, per fusosome, of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 29;

xlvii) the fusosome comprises the membrane protein payload agent at a copy number, per fusosome, of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 43;

xlviii) the ratio of the copy number of the fusogen to the copy number of the membrane protein payload agent is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000;

xlix) the fusosome comprises a lipid composition substantially similar to that of the source cell or wherein one or more of CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG is within 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, or 75% of the corresponding lipid level in the source cell;

l) the fusosome comprises a proteomic composition similar to that of the source cell, e.g., using an assay of Example 42;

li) the fusosome comprises a ratio of lipids to proteins that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 49;

lii) the fusosome comprises a ratio of proteins to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 50;

liii) the fusosome comprises a ratio of lipids to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 51;

liv) the fusosome has a half-life in a subject, e.g., in a mouse, that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the half life of a reference cell, e.g., the source cell, e.g., by an assay of Example 75;

lv) the fusosome transports glucose (e.g., labeled glucose, e.g., 2-NBDG) across a membrane, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of glucose, e.g., as measured using an assay of Example 64;

lvi) the fusosome comprises esterase activity in the lumen that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of that of the esterase activity in a reference cell, e.g., the source cell or a mouse embryonic fibroblast, e.g., using an assay of Example 66;

lvii) the fusosome comprises a metabolic activity (e.g., citrate synthase activity) level that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the metabolic activity (e.g., citrate synthase activity) in a reference cell, e.g., the source cell, e.g., as described in Example 68;

lviii) the fusosome comprises a respiration level (e.g., oxygen consumption rate) that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the respiration level (e.g., oxygen consumption rate) in a reference cell,

e.g., the source cell, e.g., as described in Example 69;

lix) the fusosome comprises an Annexin-V staining level of at most 18,000, 17,000, 16,000, 15,000, 14,000, 13,000, 12,000, 11,000, or 10,000 MFI, e.g., using an assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of an otherwise similar fusosome treated with menadione in the assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of a macrophage treated with menadione in the assay of Example 70,

lx) the fusosome has a miRNA content level of at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., by an assay of Example 39;

lxi) the fusosome has a soluble : non-soluble protein ratio that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., within 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of that of the source cell, e.g., by an assay of Example 47;

lxii) the fusosome has an LPS level less than 5%, 1%, 0.5%, 0.01%, 0.005%, 0.0001%, 0.00001% or less of the LPS content of the source cell, e.g., as measured by mass spectrometry as described in Example 48;

lxiii) the fusosome and/or compositions or preparations thereof, are capable of signal transduction, e.g., transmitting an extracellular signal, e.g., AKT phosphorylation in response to insulin, or glucose (e.g., labeled glucose, e.g., 2-NBDG) uptake in response to insulin, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of insulin, e.g., using an assay of Example 63;

lxiv) the fusosome targets a tissue, e.g., liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, or eye, when administered to a subject, e.g., a mammal, e.g., an experimental

mammal (e.g., a mouse), a domesticated animal (e.g., a pet or farm animal), or a human, wherein at least 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fusosomes in a population of administered fusosomes are present in the target tissue after 24, 48, or 72 hours, e.g., by an assay of Example 87 or 100;

lxv) the fusosome has juxtacrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71;

lxvi) the fusosome has paracrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% greater than the level of paracrine signaling induced by a reference cell, e.g., the source cell or a macrophage, e.g., by an assay of Example 72;

lxvii) the fusosome polymerizes actin at a level within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% compared to the level of polymerized actin in a reference cell, e.g., the source cell or a C2C12 cell, e.g., by the assay of Example 73;

lxviii) the fusosome has a membrane potential within about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the membrane potential of a reference cell, e.g., the source cell or a C2C12 cell, e.g., by an assay of Example 74, or wherein the fusosome has a membrane potential of about -20 to -150mV, -20 to -50mV, -50 to -100mV, or -100 to -150mV;

lxix) the fusosome and/or compositions or preparations thereof, are capable of extravasation from blood vessels, e.g., at a rate at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% the rate of extravasation of the source cell, e.g., using an assay of Example 57, e.g., wherein the source cell is a neutrophil, lymphocyte, B cell, macrophage, or NK cell;

lxx) the fusosome and/or compositions or preparations thereof, are capable of crossing a cell membrane, e.g., an endothelial cell membrane or the blood brain barrier;

lxxi) the fusosome and/or compositions or preparations thereof, are capable of secreting

a protein, e.g., at a rate at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than areference cell, e.g., a mouse embryonic fibroblast or the source cell, e.g., using an assay of Example 62;

lxxii) the fusosome meets a pharmaceutical or good manufacturing practices (GMP) standard;

lxxiii) the fusosome was made according to good manufacturing practices (GMP);

lxxiv) a pharmaceutical preparation comprising a plurality of fusosomes as described herein has a pathogen level below a predetermined reference value, e.g., is substantially free of pathogens;

lxxv) a pharmaceutical preparation comprising a plurality of fusosomes as described herein has a contaminant level below a predetermined reference value, e.g., is substantially free of contaminants;

lxxvi) a pharmaceutical preparation comprising a plurality of fusosomes as described herein has low immunogenicity, e.g., as described herein;

lxxvii) the source cell is selected from a neutrophil, a granulocyte, a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell, a myeloblast, a myoblast, a hepatocyte, or a neuron e.g., retinal neuronal cell; or

lxxviii) the source cell is other than a 293 cell, HEK cell, human endothelial cell, or a human epithelial cell, monocyte, macrophage, dendritic cell, or stem cell.

[00020] In some embodiments, one or more of the following is present:

i) the fusosome transports glucose (e.g., labeled glucose, e.g., 2-NBDG) across a membrane, e.g., by at least 10% more than a negative control, e.g., an otherwise similar fusosome in the absence of glucose, e.g., as measured using an assay of Example 64;

ii) the fusosome comprises esterase activity in the lumen that is within 90% of that of the esterase activity in a reference cell, e.g., the source cell or a mouse embryonic fibroblast, e.g., using an assay of Example 66;

iii) the fusosome comprises a metabolic activity level that is within 90% of the metabolic activity (e.g., citrate synthase activity) in a reference cell, e.g., the source cell, e.g., as

described in Example 68;

iv) the fusosome comprises a respiration level (e.g., oxygen consumption rate) that is within 90% of the respiration level in a reference cell, e.g., the source cell, e.g., as described in Example 69;

v) the fusosome comprises an Annexin-V staining level of at most 18,000, 17,000, 16,000, 15,000, 14,000, 13,000, 12,000, 11,000, or 10,000 MFI, e.g., using an assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of an otherwise similar fusosome treated with menadione in the assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of a macrophage treated with menadione in the assay of Example 70;

vi) the fusosome has an LPS level less than 5% of the lipid content of fusosomes, e.g., as measured by an assay of Example 48;

vii) the fusosome has juxtacrine-signaling level of at least 5% greater than the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71;

viii) the fusosome has paracrine-signaling level of at least 5% greater than the level of paracrine signaling induced by a reference cell, e.g., the source cell or a macrophage, e.g., by an assay of Example 72;

ix) the fusosome polymerizes actin at a level within 5% compared to the level of polymerized actin in a reference cell, e.g., the source cell or a C2C12 cell, e.g., by the assay of Example 73; or

x) the fusosome and/or compositions or preparations thereof, are capable of secreting a protein, e.g., at a rate at least 5% greater than a reference cell, e.g., a mouse embryonic fibroblast, e.g., using an assay of Example 62.

[00021] In some embodiments, a provided fusosome further comprises an organelle, e.g., a therapeutically effective number of organelles, disposed in the lumen.

[00022] Alternatively or additionally, in some embodiments, one or more of the following is present:

i) the source cell is selected from an endothelial cell, a macrophage, a neutrophil, a granulocyte, a leukocyte, a stem cell (e.g., a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell), a myeloblast, a myoblast, a hepatocyte, or a neuron e.g., retinal neuronal cell;

ii) the fusosome comprises an organelle selected from a Golgi apparatus, lysosome, endoplasmic reticulum, vacuole, endosome, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, cnidocyst, peroxisome, proteasome, vesicle, stress granule, and a combination thereof;

iii) the fusosome has a diameter of greater than 5 μm, 10 μm, 20 μm, 50 μm, or 100 μm;

iv) a preparation comprising a plurality of the fusosomes has a density of other than between 1.08 g/mL and 1.12 g/mL, e.g., the preparation has a density of >1.12 g/mL, e.g., 1.25 g/mL +/- 0.1, 1.25 g/mL +/- 0.05, e.g., as measured by an assay of Example 33;

v) the fusosome is not substantially captured by the scavenger system in circulation or by Kupffer cells in the sinus of the liver in an experimental mammal or in a human;

vi) the source cell is other than a 293 cell;

vii) the source cell is not transformed or immortalized;

viii) the source cell is transformed, or immortalized using a method other than adenovirus-mediated immortalization, e.g., immortalized by spontaneous mutation, or telomerase expression;

ix) the fusogen is other than VSVG, a SNARE protein, or a secretory granule protein;

x) the fusosome does not comprise Cre or GFP, e.g., EGFP;

xi) the fusosome further comprises a protein that is exogenous relative to the source cell) other than Cre or GFP, e.g., EGFP;

xii) the fusosome further comprises a nucleic acid (e.g., RNA, e.g., mRNA, miRNA, or siRNA) that is exogenous relative to the source cell) or a protein (e.g., an antibody) that is exogenous to the source cell, e.g., in the lumen;

xiii) the fusosome does not comprise mitochondria, or is substantially free of mitochondria; or

xiv) the fusosome further comprises a nucleic acid (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, an miRNA, or an siRNA) or protein (e.g., an antibody), wherein the nucleic acid or protein is exogenous to the source cell.

[00023] Alternatively or additionally, in some embodiments, one or more of the following is true:

i) the membrane protein payload agent is a membrane protein, or a nucleic acid (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, etc.) encoding or complementary to one that encodes, a membrane protein, e.g., a chimeric antigen receptor (CAR);

ii) the membrane protein is or comprises a receptor, such as an antigen receptor, which in some embodiments may be a natural receptor or an engineered receptor e.g., a CAR;

iii) the membrane protein is or comprises an integrin;

iv) the membrane protein is or comprises a T cell receptor;

v) the membrane protein is or comprises a membrane transport protein such as an ion channel protein or a pore-forming protein (e.g., a hemolysin or colicin);

vi) the membrane protein is or comprises a toll-like receptor;

vii) the membrane protein is or comprises an interleukin receptor;

viii) the membrane protein is or comprises a membrane enzyme;

ix) the membrane protein is or comprises a cell adhesion protein (e.g., cadherin protein, selectin protein, mucin protein, etc.).

[00024] The present disclosure provides, in some aspects, a fusosome comprising:

(a) a lipid bilayer,

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer,

(c) a fusogen that is exogenous relative to the source cell or an overexpressed fusogen, e.g., wherein the fusogen is disposed in the lipid bilayer,

(d) a membrane protein payload agent, and

(e) a functional nucleus,

wherein the fusosome is derived from a source cell.

[00025] In some embodiments, one or more of the following is present:

i) the source cell is other than a dendritic cell or tumor cell, e.g., the source cell is selected from an endothelial cell, a macrophage, a neutrophil, a granulocyte, a leukocyte, a stem cell (e.g., a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell), a myeloblast, a myoblast, a hepatocyte, or a neuron e.g., retinal neuronal cell;

ii) the fusogen is other than a fusogenic glycoprotein;

iii) the fusogen is a mammalian protein other than fertilin-beta;

iv) the fusosome has low immunogenicity, e.g., as described herein;

v) the fusosome meets a pharmaceutical or good manufacturing practices (GMP) standard;

vi) a pharmaceutical preparation comprising a plurality of the fusosomes was made according to good manufacturing practices (GMP);

vii) a pharmaceutical preparation comprising a plurality of the fusosomes has a pathogen level below a predetermined reference value, e.g., is substantially free of pathogens; or

viii) a pharmaceutical preparation comprising a plurality of the fusosomes has a contaminant level below a predetermined reference value, e.g., is substantially free of contaminants.

[00026] The present disclosure provides, in some aspects, a frozen purified fusosome preparation comprising a plurality of fusosomes comprising a membrane protein payload agent described herein, wherein the preparation is frozen at a temperature that is at or less than, 4, 0, -4, -10, -12, -16, -20, -80, or -160°C.

[00027] The present disclosure provides, in some aspects, a fusosome preparation (e.g., a pharmaceutical preparation) comprising a plurality of fusosomes described herein.

[00028] The disclosure also provides, in some aspects, a fusosome composition comprising a plurality of fusosomes, wherein at least one fusosome comprises:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer;

(d) a membrane protein payload agent, e.g., as described herein.

[00029] The present disclosure provides, in some aspects, a pharmaceutical composition comprising a fusosome composition or preparation described herein and a pharmaceutically acceptable carrier.

[00030] This disclosure provides, in certain aspects, a method of delivering a fusosome composition or preparation comprising a membrane protein payload agent as described herein to a human subject, a target tissue, or a cell, comprising administering to the human subject, or contacting the target tissue or the cell with, a fusosome composition comprising a plurality of fusosomes described herein, a fusosome composition described herein, or a pharmaceutical composition described herein, thereby administering the fusosome composition to the subject.

[00031] This disclosure provides, in certain aspects, a method of delivering a membrane protein payload agent to a subject, a target tissue, or a cell, comprising administering to the subject, or contacting the target tissue or the cell with, a fusosome composition or preparation described herein (e.g., a pharmaceutical composition described herein), wherein the fusosome composition or preparation is administered in an amount and/or time such that the membrane protein payload agent is delivered.

[00032] This disclosure provides, in certain aspects, a method of modulating, e.g., enhancing, a biological function in a subject, a target tissue, or a cell, comprising administering to the subject, or contacting the target tissue or the cell with, a fusosome composition or preparation

comprising a membrane protein payload agent described herein, e.g., a pharmaceutical composition described herein, thereby modulating the biological function in the subject.

[00033] This disclosure provides, in certain aspects, a method of delivering or targeting a membrane protein function to a subject, comprising administering to the subject a fusosome composition or preparation described herein which comprises a membrane protein payload agent, wherein the fusosome composition or preparation is administered in an amount and/or time such that the membrane protein function is delivered or targeted in the subject. In embodiments, the subject has a cancer, an inflammatory disorder, autoimmune disease, a chronic disease, inflammation, damaged organ function, an infectious disease, a degenerative disorder, a genetic disease, or an injury.

[00034] The disclosure provides, in some aspects, a method of manufacturing a fusosome composition, comprising:

a) providing a source cell comprising, e.g., expressing, a fusogen;

b) producing a fusosome from the source cell, wherein the fusosome comprises a lipid bilayer, a lumen, a fusogen and a membrane protein payload agent, thereby making a fusosome; and

c) formulating the fusosome, e.g., as a pharmaceutical composition suitable for administration to a subject.

[00035] In embodiments, one or more of the following is present:

i) the source cell is other than a 293 cell, HEK cell, human endothelial cell, or a human epithelial cell;

ii) the fusogen is other than a viral protein;

iii) a preparation comprising a plurality of the fusosomes has a density of other than between 1.08 g/mL and 1.12 g/mL;

iv) a preparation comprising a plurality of the fusosomes has a density of 1.25 g/mL +/- 0.05, e.g., as measured by an assay of Example 33;

v) the fusosome is not substantially captured by the scavenger system in circulation or by Kupffer cells in the sinus of the liver;

vi) the fusosome is not substantially captured by the reticulo-endothelial

system (RES) in a subject, e.g., by an assay of Example 76;

vii) when a plurality of fusosomes are administered to a subject, less than 1%, 2%, 3%, 4%, 5% , 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the plurality are captured by the RES after 24, 48, or 72 hours, e.g., by an assay of Example 76;

viii) the fusosome has a diameter of greater than 5 μm, 6 μm, 7 μm, 8 μm, 10 μm, 20 μm, 50 μm, 100 μm, 150 μm, or 200 μm.

ix) the fusosome comprises a cytobiologic;

x) the fusosome comprises an enucleated cell; or

xi) the fusosome comprises an inactivated nucleus.

[00036] In some aspects, the present disclosure provides a method of manufacturing a fusosome composition, comprising:

a) providing a plurality of fusosomes described herein or a fusosome composition described herein; and

b) formulating the fusosomes, e.g., as a pharmaceutical composition suitable for administration to a subject.

[00037] In some aspects, the present disclosure provides a method of manufacturing a fusosome composition, comprising:

a) providing, e.g., producing, a plurality of fusosomes or a fusosome preparation described herein; and

b) assaying a sample of the plurality (e.g., of the preparation) to determine whether one or more (e.g., 2, 3, or more) standards are met. In embodiments, the standard(s) are chosen from:

i) fusosomes in the sample fuse at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, e.g., in an assay of Example 54;

ii) fusosomes in the sample fuse at a higher rate with a target cell than other fusosomes, e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., in an assay of Example 54;

iii) fusosomes in the sample fuse with target cells at a rate such that a membrane protein

payload agent in the fusosome is delivered to at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of target cells after 24, 48, or 72 hours, e.g., in an assay of Example 54;

iv) the fusogen is present at a copy number, per fusosome (e.g., on average in the sample), of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 29;

v) the membrane protein payload agent is detectable in fusosomes of the sample (e.g., on average in the sample) at a copy number of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 43;

vi) the ratio of the copy number of the fusogen to the copy number of the membrane protein payload agent is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000;

vii) fusosomes of the sample are characterized by a lipid composition substantially similar to that of the source cell or wherein one or more of CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG is within 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or 75% of the corresponding lipid level in the source cell;

viii) fusosomes of the sample are characterized by a proteomic composition similar to that of the source cell, e.g., using an assay of Example 42;

ix) fusosomes of the sample are characterized by a ratio of lipids to proteins that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 49;

x) fusosomes of the sample are characterized by a ratio of proteins to nucleic acids

(e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 50;

xi) fusosomes of the sample are characterized by a ratio of lipids to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 51;

xii) fusosomes of the sample are characterized by a half-life in a subject, e.g., in a an experimental animal such as a mouse, that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the half life of a reference cell, e.g., the source cell, e.g., by an assay of Example 75;

xiii) fusosomes of the sample are characterized in that they transport glucose (e.g., labeled glucose, e.g., 2-NBDG) across a membrane, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., fusosomes of an otherwise similar sample in the absence of glucose, e.g., as measured using an assay of Example 64;

xiv) fusosomes of the sample are characterized by esterase activity in the lumen that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of that of the esterase activity in a reference cell, e.g., the source cell or a mouse embryonic fibroblast, e.g., using an assay of Example 66;

xv) fusosomes of the sample are characterized by a metabolic activity (e.g., citrate synthase activity) level that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the metabolic activity, e.g., citrate synthase activity, in a reference cell, e.g., the source cell, e.g., as described in Example 68;

xvi) fusosomes of the sample are characterized by a respiration level (e.g., oxygen consumption rate) that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the respiration level in a reference cell, e.g., the source cell, e.g., as described in Example 69;

xvii) fusosomes of the sample are characterized by an Annexin-V staining level of at most 18,000, 17,000, 16,000, 15,000, 14,000, 13,000, 12,000, 11,000, or 10,000 MFI, e.g., using an assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the

Annexin-V staining level of an otherwise similar fusosome treated with menadione in the assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of a macrophage treated with menadione in the assay of Example 70,

xviii) fusosomes of the sample are characterized by a miRNA content level of at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., by an assay of Example 39;

xix) the fusosome has a soluble : non-soluble protein ratio is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., within 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of that of the source cell, e.g., by an assay of Example 47;

xx) fusosomes of the sample are characterized by an LPS level less than 5%, 1%, 0.5%, 0.01%, 0.005%, 0.0001%, 0.00001% or less of the LPS content of the source cell or a reference cell, e.g., as measured by an assay of Example 48;

xxi) fusosomes of the sample are capable of signal transduction, e.g., transmitting an extracellular signal, e.g., AKT phosphorylation in response to insulin, or glucose (e.g., labeled glucose, e.g., 2-NBDG) uptake in response to insulin, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of insulin, e.g., using an assay of Example 63;

xxii) fusosomes of the sample are characterized by a juxtacrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71;

xxiii) fusosomes of the sample are characterized by a paracrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% greater than the level of paracrine signaling induced by a reference cell, e.g., the

source cell or a macrophage, e.g., by an assay of Example 72;

xxiv) fusosomes of the sample are characterized in that they polymerize actin at a level within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% compared to the level of polymerized actin in a reference cell, e.g., the source cell or a C2C12 cell, e.g., by the assay of Example 73;

xxv) fusosomes of the sample are characterized by a membrane potential within about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the membrane potential of a reference cell, e.g., the source cell or a C2C12 cell, e.g., by an assay of Example 74, or wherein the fusosome has a membrane potential of about -20 to -150mV, -20 to -50mV, -50 to -100mV, or -100 to -150mV;

xxvi) fusosomes of the sample are capable of secreting a protein, e.g., at a rate at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than the a reference cell, e.g., a mouse embryonic fibroblast, e.g., using an assay of Example 62; or

xxvii) fusosomes of the sample are characterized by low immunogenicity, e.g., as described herein; and

c) (optionally) approving the plurality of fusosomes or fusosome composition for release if one or more of the standards is met or (optionally) formulating the plurality of fusosomes or the fusosome preparation as a drug product if the one or more standards is met.

[00038] The present disclosure also provides, in some aspects, a method of manufacturing a fusosome composition, comprising:

a) providing, e.g., producing, a plurality of fusosomes described herein or a fusosome composition or preparation described herein; and

b) assaying a sample of the plurality or preparation to determine the presence or level of one or more of the following factors:

i) an immunogenic molecule, e.g., an immunogenic protein, e.g., as described herein;

ii) a pathogen, e.g., a bacterium or virus; or

iii) a contaminant (e.g., a nuclear structure or component such as nuclear DNA); and

c) (optionally) approving the plurality of fusosomes or fusosome preparation for release if one or more of the factors is deviates significantly (e.g., by more than a specified amount) from a reference value or (optionally) formulating the plurality of fusosomes or the fusosome preparation as a drug product if the one or more factors does not significantly deviate (e.g., does not deviate by more than the specified about) from the reference value.

[00039] The present disclosure also provides, in some aspects, a method of delivering a membrane protein payload agent to a subject, for example comprising:

a) administering to the subject first fusogen, under conditions that allow for disposition of the first fusogen in one or more target cells in the subject, wherein one or more of:

i) administering the first fusogen comprises administering a nucleic acid encoding the first fusogen, under conditions that allow for expression of the first fusogen in the one or more target cells, or

ii) the first fusogen does not comprise a coiled-coil motif, and

b) administering to the human subject a fusosome composition or preparation as described herein, comprising a plurality of fusosomes comprising a second fusogen and a membrane protein payload agent, wherein the second fusogen is compatible with the first fusogen, wherein the plurality of fusosomes further comprise a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell),

thereby delivering the membrane protein payload agent to the subject.

[00040] The present disclosure also provides, in some aspects, a method of modulating, e.g., enhancing, a biological function in a subject, comprising:

a) administering to the subject first fusogen, under conditions that allow for disposition of the first fusogen in one or more target cells in the subject, wherein one or more of:

i) administering the first fusogen comprises administering a nucleic acid encoding the first fusogen, under conditions that allow for expression of the first fusogen in the one or more target cells, or

ii) the first fusogen does not comprise a coiled-coil motif, and

b) administering to the human subject a fusosome composition or preparation as described herein, comprising a plurality of fusosomes comprising a second fusogen, wherein the second

fusogen is compatible with the first fusogen, wherein the plurality of fusosomes further comprise a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell),
thereby modulating the biological function in the subject.

[00041] In some aspects, a fusosome comprises a chondrisome and a fusogen.

[00042] In some aspects, a composition comprises a plurality of fusosomes, wherein at least one fusosome comprises a chondrisome and a fusogen.

[00043] In some aspects, provided herein is a method of manufacturing a fusosome composition, comprising:

a) providing a source cell comprising, e.g., expressing, a fusogen;

b) producing a fusosome from the source cell, wherein the fusosome comprises a lipid bilayer, a lumen, a fusogen, and a membrane protein payload agent, thereby making a fusosome; and

c) formulating the fusosome, e.g., as a pharmaceutical composition suitable for administration to a subject, wherein one or more of:

i) the source cell is other than a 293 cell, HEK cell, human endothelial cell, or a human epithelial cell;

ii) the fusogen is other than a viral protein;

iii) the fusosome and/or compositions or preparations thereof has a density of other than between 1.08 g/mL and 1.12 g/mL, e.g.,

iv) the fusosome and/or compositions or preparations thereof has a density of 1.25 g/mL +/- 0.05, e.g., as measured by an assay of Example 33;

v) the fusosome is not captured by the scavenger system in circulation or by Kupffer cells in the sinus of the liver;

vi) the fusosome is not captured by the reticulo-endothelial system (RES) in a subject, e.g., by an assay of Example 76;

vii) when a plurality of fusosomes are administered to a subject, less than 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, of the plurality are not captured by the RES after 24 hours, e.g., by an assay of

Example 76;

viii) the fusosome has a diameter of greater than 5 μm, 6 μm, 7 μm, 8 μm, 10 μm, 20 μm, 50 μm, 100 μm, 150 μm, or 200 μm.

ix) the fusosome comprises a cytobiologic;

x) the fusosome comprises an enucleated cell; or

xi) the fusosome comprises an inactivated nucleus.

[00044] In some aspects, provided herein is a method of manufacturing a fusosome composition, comprising:

i) providing a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein; and

ii) formulating the plurality of fusosomes, fusosome composition, or pharmaceutical composition , e.g., as a fusosome drug product suitable for administration to a subject.

[00045] In some aspects, provided herein is a method of manufacturing a fusosome composition, comprising:

i) providing a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein; and

b) assaying one or more fusosomes from the plurality to determine whether one or more (e.g., 2, 3, or all) of the following standards are met:

i) the fusosome fuses at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 10% e.g., in an assay of Example 54;

ii) the fusosome fuses at a higher rate with a target cell than with other fusosomes, e.g., by at least 50% e.g., in an assay of Example 54;

iii) the fusosome fuses with target cells at a rate such that an agent in the fusosome is delivered to at least 10% of target cells after 24 hours, e.g., in an assay of Example 54;

iv) the fusogen is present at a copy number of at least 1,000 copies, e.g., as measured by an assay of Example 29;

v) the fusosome comprises a protein membrane payload at a copy number of at least 1,000 copies, e.g., as measured by an assay of Example 43;

vi) the ratio of the copy number of the fusogen to the copy number of the protein membrane payload is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000;

vii) the fusosome comprises a lipid composition wherein one or more of CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG is within 75% of the corresponding lipid level in the source cell;

viii) the fusosome comprises a proteomic composition similar to that of the source cell, e.g., using an assay of Example 42;

ix) the fusosome comprises a ratio of lipids to proteins that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 49;

x) the fusosome comprises a ratio of proteins to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 50;

xi) the fusosome comprises a ratio of lipids to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 51;

xii) the fusosome has a half-life in a subject, e.g., in a mouse, that is within 90% of the half-life of a reference cell, e.g., the source cell, e.g., by an assay of Example 75;

xiii) the fusosome transports glucose (e.g., labeled glucose, e.g., 2-NBDG) across a membrane, e.g., by at least 10% more than a negative control, e.g., an otherwise similar fusosome in the absence of glucose, e.g., as measured using an assay of Example 64;

xiv) the fusosome comprises esterase activity in the lumen that is within 90% of that of the esterase activity in a reference cell, e.g., the source cell or a mouse embryonic fibroblast, e.g., using an assay of Example 66;

xv) the fusosome comprises a metabolic activity level that is within 90% of the metabolic activity (e.g., citrate synthase activity) in a reference cell, e.g., the source cell, e.g., as described in Example 68;

xvi) the fusosome comprises a respiration level (e.g., oxygen consumption rate) that is within 90% of the respiration level in a reference cell, e.g., the source cell, e.g., as described in Example 69;

xvii) the fusosome comprises an Annexin-V staining level of at most 18,000, 17,000, 16,000, 15,000, 14,000, 13,000, 12,000, 11,000, or 10,000 MFI, e.g., using an assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of an otherwise similar fusosome treated with menadione in the assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, or 50% lower than the Annexin-V staining level of a macrophage treated with menadione in the assay of Example 70;

xviii) the fusosome has a miRNA content level of at least 1% than that of the source cell, e.g., by an assay of Example 39;

xix) the fusosome has a soluble : non-soluble protein ratio is within 90% of that of the source cell, e.g., by an assay of Example 47;

xx) the fusosome has an LPS level less than 5% of the lipid content of fusosomes, e.g., as measured by an assay of Example 48;

xxi) the fusosome and/or compositions or preparations thereof, are capable of signal transduction, e.g., transmitting an extracellular signal, e.g., AKT phosphorylation in response to insulin, or glucose (e.g., labeled glucose, e.g., 2-NBDG) uptake in response to insulin, e.g., by at least 10% more than a negative control, e.g., an otherwise similar fusosome in the absence of insulin, e.g., using an assay of Example 63;

xxii) the fusosome has juxtacrine-signaling level of at least 5% greater than the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71;

xxiii) the fusosome has paracrine-signaling level of at least 5% greater than the level of paracrine signaling induced by a reference cell, e.g., the source cell or a macrophage, e.g., by an assay of Example 72;

xxiv) the fusosome polymerizes actin at a level within 5% compared to the level of polymerized actin in a reference cell, e.g., the source cell or a C2C12 cell, e.g., by the assay of Example 73;

xxv) the fusosome has a membrane potential within about 5% of the membrane potential of a reference cell, e.g., the source cell or a C2C12 cell, e.g., by an assay of Example 74, or wherein the fusosome has a membrane potential of about -20 to -150mV, -20 to -50mV, -50 to -100mV, or -100 to -150mV;

xxvi) the fusosome and/or compositions or preparations thereof, are capable of secreting a protein, e.g., at a rate at least 5% greater than a reference cell, e.g., a mouse embryonic fibroblast, e.g., using an assay of Example 62; or

xxvii) the fusosome has low immunogenicity, e.g., as described herein; and

c) (optionally) approving the plurality of fusosomes or fusosome composition for release if one or more of the standards is met;

thereby manufacturing a fusosome drug product composition

[00046] In some aspects, provided herein is a method of manufacturing a fusosome composition, comprising:

a) providing a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein; and

b) assaying one or more fusosomes from the plurality to determine the presence or level of one or more of the following factors:

i) an immunogenic molecule, e.g., an immunogenic protein, e.g., as described herein;

ii) a pathogen, e.g., a bacterium or virus; or

iii) a contaminant;

c) (optionally) approving the plurality of fusosomes or fusosome composition for release if one or more of the factors is below a reference value;

thereby manufacturing a fusosome drug product composition.

[00047] In some aspects, provided herein is a method of administering a fusosome composition to a subject, e.g., a human subject, comprising administering to the subject a fusosome composition comprising a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein, thereby administering the fusosome composition to the subject.

[00048] In some aspects, provided herein a method of delivering a protein membrane payload to a subject comprising administering to the subject a fusosome composition comprising a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein, wherein the fusosome composition is administered in an amount and/or time such that the protein membrane payload is delivered

[00049] In some aspects, provided herein is a method of modulating, e.g., enhancing, a biological function in a subject, comprising administering to the subject a fusosome composition comprising a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein thereby modulating the biological function in the subject.

[00050] In some aspects, provided herein is a method of delivering or targeting a function to a subject, comprising administering to the subject a fusosome composition comprising a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein, wherein the fusosome composition is administered in an amount and/or time such that the function in the subject is delivered or targeted.

[00051] In some aspects, provided herein is a method of treating a disease or disorder in a patient comprising administering to the subject a fusosome composition comprising a plurality of fusosomes, a fusosome composition, or a pharmaceutical composition as described herein, wherein the fusosome composition is administered in an amount and/or time such that the disease or disorder is treated.

[00052] In some aspects, provided herein is a method of administering a fusosome

composition to a human subject, comprising

a) administering to the subject a first fusogen, under conditions that allow for disposition of the first fusogen in one or more target cells in the subject, wherein one or more of:

i) administering the first fusogen comprises administering a nucleic acid encoding the first fusogen, under conditions that allow for expression of the first fusogen in the one or more target cells, or

ii) the first fusogen does not comprise a coiled-coil motif, and

b) administering to the human subject a fusosome composition comprising a plurality of fusosomes comprising a second fusogen, wherein the second fusogen is compatible with the first fusogen, wherein the plurality of fusosomes further comprise a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell);

thereby administering the fusosome composition to the subject.

[00053] In some aspects, provided herein is a method of delivering a membrane protein payload agent to a subject, comprising:

a) administering to the subject first fusogen, under conditions that allow for disposition of the first fusogen in one or more target cells in the subject, wherein one or more of:

i) administering the first fusogen comprises administering a nucleic acid encoding the first fusogen, under conditions that allow for expression of the first fusogen in the one or more target cells, or

ii) the first fusogen does not comprise a coiled-coil motif, and

b) administering to the human subject a fusosome composition comprising a plurality of fusosomes comprising a second fusogen and a therapeutic agent, wherein the second fusogen is compatible with the first fusogen, wherein the plurality of fusosomes further comprise a membrane protein payload agent;

thereby delivering the membrane protein payload agent to the subject.

[00054] In some aspects, provided herein is a method of modulating, e.g., enhancing, a biological function in a subject, comprising:

a) administering to the subject first fusogen, under conditions that allow for disposition of

the first fusogen in one or more target cells in the subject, wherein one or more of:

i) administering the first fusogen comprises administering a nucleic acid encoding the first fusogen, under conditions that allow for expression of the first fusogen in the one or more target cells, or

ii) the first fusogen does not comprise a coiled-coil motif, and

b) administering to the human subject a fusosome composition comprising a plurality of fusosomes comprising a second fusogen, wherein the second fusogen is compatible with the first fusogen, wherein the plurality of fusosomes further comprise a membrane protein payload agent;

thereby modulating the biological function in the subject.

[00055] Any of the aspects herein, e.g., the fusosomes, fusosome compositions, preparations and methods above, can be combined with one or more of the embodiments herein, e.g., one or of the embodiments described herein.

[00056] In some embodiments, the biological function is selected from:

a) modulating, e.g., increasing or decreasing, an interaction between two cells;
b) modulating, e.g. increasing or decreasing, an immune response;
c) modulating, e.g. increasing or decreasing, recruitment of cells to a target tissue;
d) decreasing the growth rate of a cancer; or
e) reducing the number of cancerous cells in the subject.

[00057] In some embodiments, a plurality of the fusosomes, when contacted with a target cell population in the presence of an inhibitor of endocytosis, and when contacted with a reference target cell population not treated with the inhibitor of endocytosis, delivers the cargo to at least 30%, 40%, 50%, 60%, 70%, or 80% of the number of cells in the target cell population compared to the reference target cell population.

[00058] In some embodiments, when the plurality of fusosomes are contacted with a cell population comprising target cells and non-target cells, the cargo is present in at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold more target cells than non-target cells. In some embodiments, the fusosomes of the plurality fuse at a higher rate with a target cell than with a non-

target cell by at least at least 50%.

[00059] In some embodiments, the membrane protein payload agent is other than, does not comprise, does not encode, or is not complementary to a sequence that encodes, a connexin, CFTR, thyrotropin receptor, myelin protein zero, melacortin 4, myelin proteolipid protein, low-density lipoprotein receptor, ABC transporter, CD81, mCAT-1, CXCR4, CD4, CCR5, sialic acid-rich proteins, claudins, CD21, T-cell receptors, B cell receptors, TNFR1, CD63, GLUT4, VEGF, or ICAM. In some embodiments, the membrane protein payload agent comprises or encodes a chimeric protein which does not bind a cell surface marker or target cell moiety of a target cell and which does not comprise a fluorescent protein.

[00060] In some embodiments, the membrane protein payload agent comprises a therapeutic protein, e.g., a therapeutic protein described herein. In some embodiments, the membrane protein payload agent comprises a Golgi apparatus protein, a secreted protein, or an endoplasmic reticulum protein, or a combination thereof. In some embodiments, the membrane protein payload agent does not comprise one or more of: a dimer (e.g., a dimer that is exogenous to the source cell), a heterodimer (e.g., a heterodimer that is exogenous to the source cell), or a dimerization domain (e.g., a dimerization domain in a polypeptide that is exogenous to the source cell). In some embodiments, the membrane protein payload agent comprises a nucleic acid (e.g., DNA or RNA) encoding a membrane protein. In some embodiments, the fusogen is a non-viral fusogen, e.g., a mammalian fusogen. In some embodiments, the fusogen (e.g., exogenous or overexpressed fusogen) does not promote vesicle formation from a source cell. In some embodiments, the fusosome comprises an enucleated cell.

[00061] In some embodiments, the membrane protein payload agent comprises or encodes a membrane protein that comprises a transmembrane domain. In some embodiments, the membrane protein payload agent comprises or encodes a lipid-anchored protein. In some embodiments, the membrane protein payload agent comprises or encodes a protein that binds a transmembrane protein. For instance, the protein may be an extracellular protein that binds an extracellular portion of a transmembrane protein, or the protein may be an intracellular protein that binds an intracellular portion of a transmembrane protein. In some embodiments, the membrane protein payload agent comprises or encodes a protein that lacks a transmembrane domain. In some

embodiments, the membrane protein payload agent comprises or encodes a protein that partially spans a membrane (e.g., a membrane of the target cell or the fusosome) and does not completely span the membrane. For instance, in some embodiments, the protein comprises an in-plane membrane helix or the protein comprises a hydrophobic loop that does not completely span the membrane. In some embodiments, the membrane protein payload agent comprises or encodes a protein that does not comprise a transmembrane domain, wherein the protein interacts with a membrane surface, e.g., through electropstatic or ionic interactions)

[00062] In some embodiments, a fusosome that delivers a membrane protein payload agent to the membrane of a target cell, as described herein, is further capable of delivering (e.g., delivers) one or more agents, e.g., proteins, nucleic acids (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, etc.), organelles, or and/or metabolites to the cytosol of the target cell. Thus, in some embodiments, a method provided herein comprises delivering an agent to the cytosol of a target cell; in some such embodiments, the cytosol-delivered agent is a protein (or a nucleic acid encoding, or complementary to one encoding, the protein, e.g., an e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, etc. encoding the protein).

[00063] In some embodiments, the membrane protein payload agent is or comprises a sequence of SEQ ID NOs: 8144-16131 of U.S. Patent Publication No. 2016/0289674. In some embodiments, the membrane protein payload agent is or comprises a fragment, variant, or homolog of a sequence of SEQ ID NOs: 8144-16131 of U.S. Patent Publication No. 2016/0289674. In some embodiments, the membrane protein payload agent is or comprises a nucleic acid encoding a protein comprising a sequence of SEQ ID NOs: 8144-16131 of U.S. Patent Publication No. 2016/0289674. In some embodiments, the membrane protein payload agent is or comprises a nucleic acid encoding a protein comprising a fragment, variant, or homolog of a sequence of SEQ ID NOs: 8144-16131 of U.S. Patent Publication No. 2016/0289674.

[00064] In some embodiments, the membrane protein payload agent is or comprises a protein selected from Tables 5-15. In some embodiments, the membrane protein payload agent is or comprises a fragment, variant, or homolog of a protein selected from Tables 5-15. In some embodiments, the membrane protein payload agent is or comprises a nucleic acid encoding a protein which is or comprises a protein selected from Tables 5-15. In some embodiments, the

membrane protein payload agent is or comprises a nucleic acid encoding a protein comprising a fragment, variant, or homolog of a protein selected from Tables 5-15.

[00065] In some embodiments, the membrane protein payload agent is or comprises a chimeric antigen receptor (CAR) comprising an antigen binding domain. In some embodiments, the CAR is or comprises a first generation CAR comprising an antigen binding domain, a transmembrane domain, and signaling domain (e.g., one, two or three signaling domains). In some embodiments, the CAR is or comprises a second generation CAR comprising an antigen binding domain, a transmembrane domain, and two signaling domains. In some embodiments, the CAR comprises a third generation CAR comprising an antigen binding domain, a transmembrane domain, and at least three signaling domains. In some embodiments, a fourth generation CAR comprising an antigen binding domain, a transmembrane domain, three or four signaling domains, and a domain which upon successful signaling of the CAR induces expression of a cytokine gene. In some embodiments, the antigen binding domain is or comprises an scFv or Fab.

[00066] In some embodiments, the antigen binding domain targets an antigen characteristic of a neoplastic cell. In some embodiments, the antigen characteristic of a neoplastic cell is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor, Epidermal Growth Factor Receptors (EGFR) (including ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4), Fibroblast Growth Factor Receptors (FGFR) (including FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF18, and FGF21) Vascular Endothelial Growth Factor Receptors (VEGFR) (including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF), RET Receptor and the Eph Receptor Family (including EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA9, EphA10, EphB1, EphB2. EphB3, EphB4, and EphB6), CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CFTR, CIC-1, CIC-2, CIC-4, CIC-5, CIC-7, CIC-Ka, CIC-Kb, Bestrophins, TMEM16A, GABA receptor, glycin receptor, ABC transporters, NAV1.1, NAV1.2, NAV1.3, NAV1.4, NAV1.5, NAV1.6, NAV1.7, NAV1.8, NAV1.9, sphingosin-1-phosphate receptor (S1P1R), NMDA channel, transmembrane protein, multispan transmembrane protein, T-cell

receptor motifs; T-cell alpha chains; T-cell β chains; T-cell γ chains; T-cell δ chains; CCR7; CD3; CD4; CD5; CD7; CD8; CD11b; CD11c; CD16; CD19; CD20; CD21 ; CD22; CD25; CD28; CD34; CD35; CD40; CD45RA; CD45RO; CD52; CD56; CD62L; CD68; CD80; CD95; CD117; CD127; CD133; CD137 (4-1 BB); CD163; F4/80; IL-4Ra; Sca-1 ; CTLA-4; GITR; GARP; LAP; granzyme B; LFA-1 ; transferrin receptor; NKp46, perforin, CD4+; Th1; Th2; Th17; Th40; Th22; Th9; Tfh, Canonical Treg. FoxP3+; Tr1; Th3; Treg17; $T_{RE}G$; CDCP1, NT5E, EpCAM, CEA, gpA33, Mucins, TAG-72, Carbonic anhydrase IX, PSMA, Folate binding protein, Gangliosides (e.g., CD2, CD3, GM2), Lewis-γ^2, VEGF, VEGFR 1/2/3, αVβ3, α5β1, ErbB1/EGFR, ErbB1/HER2, ErB3, c-MET, IGF1R, EphA3, TRAIL-R1, TRAIL-R2, RANKL, FAP, Tenascin, PDL-1, BAFF, HDAC, ABL, FLT3, KIT, MET, RET, IL-1β, ALK, RANKL, mTOR, CTLA-4, IL-6, IL-6R, JAK3, BRAF, PTCH, Smoothened, PIGF, ANPEP, TIMP1, PLAUR, PTPRJ, LTBR, or ANTXR1, Folate receptor alpha (FRa), ERBB2 (Her2/neu), EphA2, IL-13Ra2, epidermal growth factor receptor (EGFR), Mesothelin, TSHR, CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1, CD33, EGFRvIII , GD2, GD3, BCMA, MUC16 (CA125), L1CAM, LeY, MSLN, IL13Rα1, L1-CAM, Tn Ag, prostate specific membrane antigen (PSMA), ROR1, FLT3, FAP, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, interleukin-11 receptor a (IL-11Ra), PSCA, PRSS21, VEGFR2, LewisY, CD24, platelet-derived growth factor receptor-beta (PDGFR-beta), SSEA-4, CD20, MUC1, NCAM, Prostase, PAP, ELF2M, Ephrin B2, IGF-1 receptor, CAIX, LMP2, gplOO, bcr-abl, tyrosinase, Fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLACl, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-A1, legumain, HPV E6, E7, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, prostein, survivin, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin B1, MYCN, RhoC, TRP-2, CYPIB I, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, a neoantigen, CD133, CD15, CD184, CD24, CD56, CD26, CD29, CD44, HLA-A, HLA-B, HLA-C, (HLA-A,B,C) CD49f,

CD151 CD340, CD200, tkrA, trkB, or trkC, or an antigenic fragment or antigenic portion thereof.

[00067] In some embodiments, the antigen binding domain targets an antigen characteristic of a T-cell. In some embodiments, the antigen characteristic of a T-cell is selected from a cell surface receptor, a membrane transport protein (e.g., an active or passive transport protein such as, for example, an ion channel protein, a pore-forming protein, etc.), a transmembrane receptor, a membrane enzyme, and/or a cell adhesion protein characteristic of a T-cell. In some embodiments, an antigen characteristic of a T-cell may be a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor, AKT1; AKT2; AKT3; ATF2; BCL10; CALM1; CD3D (CD3δ); CD3E (CD3ε); CD3G (CD3γ); CD4; CD8; CD28; CD45; CD80 (B7-1); CD86 (B7-2); CD247 (CD3ζ); CTLA4 (CD152); ELK1; ERK1 (MAPK3); ERK2; FOS; FYN; GRAP2 (GADS); GRB2; HLA-DRA; HLA-DRB1; HLA-DRB3; HLA-DRB4; HLA-DRB5; HRAS; IKBKA (CHUK); IKBKB; IKBKE; IKBKG (NEMO); IL2; ITPR1; ITK; JUN; KRAS2; LAT; LCK; MAP2K1 (MEK1); MAP2K2 (MEK2); MAP2K3 (MKK3); MAP2K4 (MKK4); MAP2K6 (MKK6); MAP2K7 (MKK7); MAP3K1 (MEKK1); MAP3K3; MAP3K4; MAP3K5; MAP3K8; MAP3K14 (NIK); MAPK8 (JNK1); MAPK9 (JNK2); MAPK10 (JNK3); MAPK11 (p38β); MAPK12 (p38γ); MAPK13 (p38δ); MAPK14 (p38α); NCK; NFAT1; NFAT2; NFKB1; NFKB2; NFKBIA; NRAS; PAK1; PAK2; PAK3; PAK4; PIK3C2B; PIK3C3 (VPS34); PIK3CA; PIK3CB; PIK3CD; PIK3R1; PKCA; PKCB; PKCM; PKCQ; PLCY1; PRF1 (Perforin); PTEN; RAC1; RAF1; RELA; SDF1; SHP2; SLP76; SOS; SRC; TBK1; TCRA; TEC; TRAF6; VAV1; VAV2; or ZAP70.

[00068] In some embodiments, the antigen binding domain targets an antigen characteristic of an autoimmune or inflammatory disorder. In some embodiments, the autoimmune or inflammatory disorder is selected from chronic graft-vs-host disease (GVHD), lupus, arthritis, immune complex glomerulonephritis, goodpasture, uveitis, hepatitis, systemic sclerosis or scleroderma, type I diabetes, multiple sclerosis, cold agglutinin disease, Pemphigus vulgaris, Grave's disease, autoimmune hemolytic anemia, Hemophilia A, Primary Sjogren's Syndrome, thrombotic thrombocytopenia purrpura, neuromyelits optica, Evan's syndrome, IgM mediated neuropathy, cyroglobulinemia, dermatomyositis, idiopathic thrombocytopenia, ankylosing

spondylitis, bullous pemphigoid, acquired angioedema, chronic urticarial, antiphospholipid demyelinating polyneuropathy, and autoimmune thrombocytopenia or neutropenia or pure red cell aplasias, while exemplary non-limiting examples of alloimmune diseases include allosensitization (see, for example, Blazar et al., 2015, Am. J. Transplant, 15(4):931-41) or xenosensitization from hematopoietic or solid organ transplantation, blood transfusions, pregnancy with fetal allosensitization, neonatal alloimmune thrombocytopenia, hemolytic disease of the newborn, sensitization to foreign antigens such as can occur with replacement of inherited or acquired deficiency disorders treated with enzyme or protein replacement therapy, blood products, and gene therapy. In some embodiments, the antigen characteristic of an an autoimmune or inflammatory disorder is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, or histidine kinase associated receptor. In some embodiments, a CAR antigen binding domain binds to a ligand expressed on B cells, plasma cells, plasmablasts, CD10, CD19, CD20, CD22, CD24, CD27, CD38, CD45R, CD138, CD319, BCMA, CD28, TNF, interferon receptors, GM-CSF, ZAP-70, LFA-1, CD3 gamma, CD5 or CD2.

[00069] In some embodiments, the antigen binding domain targets an antigen characteristic of an infectious disease. In some embodiments, wherein the infectious disease is selected from HIV, hepatitis B virus, hepatitis C virus, Human herpes virus, Human herpes virus 8 (HHV-8, Kaposi sarcoma-associated herpes virus (KSHV)), Human T-lymphotrophic virus-1 (HTLV-1), Merkel cell polyomavirus (MCV), Simian virus 40 (SV40), Eptstein-Barr virus, CMV, human papillomavirus. In some embodiments, the antigen characteristic of an infectious disease is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor, HIV Env, gpl20, or CD4-induced epitope on HIV-1 Env.

[00070] In some embodiments, the CAR transmembrane domain comprises at least a transmembrane region of the alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137,

CD154, or functional variant thereof. In some embodiments, the transmembrane domain comprises at least a transmembrane region(s) of CD8α, CD8β, 4-1BB/CD137, CD28, CD34, CD4, FcεRIγ, CD16, OX40/CD134, CD3ζ, CD3ε, CD3γ, CD3δ, TCRα, TCRβ, TCRζ, CD32, CD64, CD64, CD45, CD5, CD9, CD22, CD37, CD80, CD86, CD40, CD40L/CD154, VEGFR2, FAS, and FGFR2B, or functional variant thereof.

[00071] In some embodiments, the CAR comprises at least one signaling domain selected from one or more of B7-1/CD80; B7-2/CD86; B7-H1/PD-L1; B7-H2; B7-H3; B7-H4; B7-H6; B7-H7; BTLA/CD272; CD28; CTLA-4; Gi24/VISTA/B7-H5; ICOS/CD278; PD-1; PD-L2/B7-DC; PDCD6); 4-1BB/TNFSF9/CD137; 4-1BB Ligand/TNFSF9; BAFF/BLyS/TNFSF13B; BAFF R/TNFRSF13C; CD27/TNFRSF7; CD27 Ligand/TNFSF7; CD30/TNFRSF8; CD30 Ligand/TNFSF8; CD40/TNFRSF5; CD40/TNFSF5; CD40 Ligand/TNFSF5; DR3/TNFRSF25; GITR/TNFRSF18; GITR Ligand/TNFSF18; HVEM/TNFRSF14; LIGHT/TNFSF14; Lymphotoxin-alpha/TNF-beta; OX40/TNFRSF4; OX40 Ligand/TNFSF4; RELT/TNFRSF19L; TACI/TNFRSF13B; TL1A/TNFSF15; TNF-alpha; TNF RII/TNFRSF1B); 2B4/CD244/SLAMF4; BLAME/SLAMF8; CD2; CD2F-10/SLAMF9; CD48/SLAMF2; CD58/LFA-3; CD84/SLAMF5; CD229/SLAMF3; CRACC/SLAMF7; NTB-A/SLAMF6; SLAM/CD150); CD2; CD7; CD53; CD82/Kai-1; CD90/Thy1; CD96; CD160; CD200; CD300a/LMIR1; HLA Class I; HLA-DR; Ikaros; Integrin alpha 4/CD49d; Integrin alpha 4 beta 1; Integrin alpha 4 beta 7/LPAM-1; LAG-3; TCL1A; TCL1B; CRTAM; DAP12; Dectin-1/CLEC7A; DPPIV/CD26; EphB6; TIM-1/KIM-1/HAVCR; TIM-4; TSLP; TSLP R; lymphocyte function associated antigen-1 (LFA-1); NKG2C, a CD3 zeta domain, an immunoreceptor tyrosine-based activation motif (ITAM), CD27, CD28, 4-1BB, CD134/OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, or functional fragment thereof.

[00072] In some embodiments, the CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain or functional variant thereof; and (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof. In some embodiments, the CAR comprises a CD3 zeta domain or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof. In some

embodiments, the CAR comprises (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain, or a 4-1BB domain, or functional variant thereof, and/or (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof. In some embodiments, the CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain or functional variant thereof; (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof; and (iv) a cytokine or costimulatory ligand transgene.

[00073] In some embodiments, the CAR further comprises one or more spacers, e.g., wherein the spacer is a first spacer between the antigen binding domain and the transmembrane domain. In some embodiments, the first spacer includes at least a portion of an immunoglobulin constant region or variant or modified version thereof. In some embodiments, the spacer is a second spacer between the transmembrane domain and a signaling domain. In some embodiments, the second spacer is an oligopeptide, e.g., wherein the oligopeptide comprises glycine-serine doublets.

[00074] In some embodiments, the fusosome fuses to the target cell at the surface of the target cell. In some embodiments, the fusosome promotes fusion to a target cell in a lysosome-independent manner. In some embodiments, the fusosome and/or fusosome contents enters the target cell by endocytosis or via a non-endocytic pathway. In some embodiments, the fusosome enters the target cell by endocytosis, e.g., wherein the level of membrane protein payload agent delivered via an endocytic pathway for a given fusosome is 0.01-0.6, 0.01-0.1, 0.1-0.3, or 0.3-0.6, or at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than a chloroquine treated reference cell, e.g., using an assay of Example 91. In some embodiments, the fusosome enters the target cell by a non-endocytic pathway, e.g., wherein the level of membrane protein payload agent delivered via a non-endocytic pathway for a given fusosome is 0.1-0.95, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-0.95, or at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than a chloroquine treated reference cell, e.g., using an assay of Example 90.

[00075] In some embodiments, the target cell comprises an aggregated or misfolded membrane protein. In some embodiments, the fusosome and/or compositions or preparations

thereof, are capable of reducing levels (e.g., reduces levels) of the aggregated or misfolded protein in the target cell, or a method herein comprises reducing levels of the aggregated or misfolded protein in the target cell.

[00076] As described herein, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a membrane protein to the cell membrane of a target cell. Similarly, in some embodiments, a method herein comprises delivering a membrane protein to the cell membrane of a target cell. In some embodiments, delivering the protein comprises delivering a nucleic acid (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, etc.) encoding the protein to the target cell such that the target cell produces the protein and localizes it to the membrane. In some embodiments, the fusosome comprises, or the method further comprises delivering, the protein, and fusion of the fusosome with the target cell transfers the protein to the cell membrane of the target cell. In some embodiments, the agent comprises a cell surface ligand or an antibody that binds a cell surface receptor. In some embodiments, the fusosome further comprises, or the method further comprises delivering, a second agent that comprises or encodes a second cell surface ligand or antibody that binds a cell surface receptor, and optionally further comprising or encoding one or more additional cell surface ligands or antibodies that bind a cell surface receptor (e.g., 1, 2, 3, 4, 5, 10, 20, 50, or more). In some embodiments, the first agent and the second agent form a complex, wherein optionally the complex further comprises one or more additional cell surface ligands. In some embodiments, the agent comprises or encodes a cell surface receptor, e.g., a cell surface that is exogenous or overexpressed relative to the source cell. In some embodiments, provided fusosomes further comprise, or the method further comprises delivering, a second agent that comprises or encodes a second cell surface receptor, and optionally further comprises or encodes one or more additional cell surface receptors (e.g., 1, 2, 3, 4, 5, 10, 20, 50, or more cell surface receptors).

[00077] In some embodiments, the second agent, e.g., therapeutic agent, is selected from a protein, protein complex (e.g., comprising at least 2, 3, 4, 5, 10, 20, or 50 proteins, e.g., at least at least 2, 3, 4, 5, 10, 20, or 50 different proteins) polypeptide, nucleic acid (e.g., DNA, chromosome, or RNA, e.g., mRNA, siRNA, or miRNA) or small molecule.

[00078] In some embodiments, the first agent and the second agent form a complex, wherein

optionally the complex further comprises one or more additional cell surface receptors. In some embodiments, the agent comprises or encodes an antigen or an antigen presenting protein.

[00079] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a secreted agent, e.g., a secreted protein to a target site (e.g., an extracellular region), e.g., by delivering a nucleic acid (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, etc.) encoding the protein to the target cell under conditions that allow the target cell to produce and secrete the protein. Similarly, in some embodiments, a method herein comprises delivering a secreted agent as described herein. In embodiments, the secreted protein is endogenous or exogenous relative to the source cell; in some embodiments, the secreted protein is endogenous or exogenous to the target cell. In embodiments, the secreted protein comprises a protein therapeutic, e.g., an antibody molecule, a cytokine, or an enzyme. In embodiments, the secreted protein comprises an autocrine signalling molecule or a paracrine signalling molecule. In embodiments, the secreted agent comprises a secretory granule.

[00080] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a membrane protein or a secreted protein that is or comprises an antigen. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a membrane protein or a secreted protein that is or comprises an antigen antigen presenting protein, optionally together (e.g., as a complex) with an antigen.

[00081] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of donating (e.g., donate) one or more cell surface receptors to a target cell (e.g., an immune cell). Similarly, in some embodiments, a method herein comprises donating one or more cell surface receptors.

[00082] In some embodiments a target cell is or comprises a tumor cell. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a membrane or secreted protein that is or comprises an immunostimulatory ligand, an antigen presenting protein, a tumor suppressor protein, a pro-apoptotic protein, or a receptor or binding partner for any of the foregoing. In some embodiments, a fusosome comprises an agent (e.g., a membrane protein payload agent and/or at least one second

agent) that is immunomodulatory, e.g., immunostimulatory.

[00083] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of causing (e.g., cause) the target cell to present an antigen. Similarly, in some embodiments, a method herein comprises presenting an antigen on a target cell.

[00084] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a nucleic acid to a target cell, e.g., to transiently modify gene expression in the target cell or to modify, for example by integration into, the genome of the target cell, for example to cause expression of a membrane protein (or secreted protein) as described herein.

[00085] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a protein (e.g., a membrane protein such as a transporter protein or a secreted protein such as an immunosuppressive protein) to a target cell so that a protein deficiency of the target cell is rescued, at least transiently.

[00086] In embodiments, the membrane protein provided by or as a membrane protein payload agent as described herein is or comprises an immunoglobulin moiety or entity (e.g., an antibody, an Fab, an scFV, an scFab, a sdAb, a duobody, a minibody, a nanobody, a diabody, a zybody, a camelid antibody, a BiTE, a quadroma, a bsDb, etc). In some embodiments, a membrane protein may include one or more covalently-associated non-peptide moieties such as, for example, one or more carbohydrate moieties, lipid moieties, polyethylene glycol moieties, small molecules, etc, and combinations thereof.

[00087] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of causing (e.g., cause) a target cell to secrete a protein, e.g., a therapeutic protein. Similarly, in some embodiments, a method herein comprises causing a target cell to secrete a protein.

[00088] In embodiments, the membrane protein provided by or as a membrane protein payload agent as described herein is or comprises one or more cell surface ligands (e.g., 1, 2, 3, 4, 5, 10, 20, 50, or more cell surface ligands). Similarly, in some embodiments, a method herein comprises presenting one or more cell surface ligands to a target cell. In some embodiments, a

fusosome having a cell surface ligand is from a source cell chosen from a neutrophil (e.g., and the target cell is a tumor-infiltrating lymphocyte), dendritic cell (e.g., and the target cell is a naïve T cell), or neutrophil (e.g., and the target is a tumor cell or virus-infected cell). In some embodiments, such a fusosome comprises a membrane complex, e.g., a complex comprising at least 2, 3, 4, or 5 proteins, e.g., a homodimer, heterodimer, homotrimer, heterotrimer, homotetramer, or heterotetramer. In some embodiments, such a fusosome comprises an antibody, e.g., a toxic antibody, e.g., the fusosome and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) the antibody to the target site, e.g., by homing to a target site. In some embodiments, the source cell is an NK cell or neutrophil.

[00089] In some embodiments, the membrane protein is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor, Epidermal Growth Factor Receptors (EGFR) (including ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4), Fibroblast Growth Factor Receptors (FGFR) (including FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF18, and FGF21) Vascular Endothelial Growth Factor Receptors (VEGFR) (including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF), RET Receptor and the Eph Receptor Family (including EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA9, EphA10, EphB1, EphB2. EphB3, EphB4, and EphB6), CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CFTR, CIC-1, CIC-2, CIC-4, CIC-5, CIC-7, CIC-Ka, CIC-Kb, Bestrophins, TMEM16A, GABA receptor, glycin receptor, ABC transporters, NAV1.1, NAV1.2, NAV1.3, NAV1.4, NAV1.5, NAV1.6, NAV1.7, NAV1.8, NAV1.9, sphingosin-1-phosphate receptor (S1P1R), NMDA channel, transmembrane protein, multispan transmembrane protein, T-cell receptor motifs; T-cell alpha chains; T-cell β chains; T-cell γ chains; T-cell δ chains; CCR7; CD3; CD4; CD5; CD7; CD8; CD11b; CD11c; CD16; CD19; CD20; CD21 ; CD22; CD25; CD28; CD34; CD35; CD40; CD45RA; CD45RO; CD52; CD56; CD62L; CD68; CD80; CD95; CD117; CD127; CD133; CD137 (4-1 BB); CD163; F4/80; IL-4Ra; Sca-1 ; CTLA-4; GITR; GARP; LAP; granzyme B; LFA-1 ; transferrin receptor; NKp46, perforin, CD4+; Th1; Th2; Th17; Th40; Th22; Th9; Tfh, Canonical Treg. FoxP3+; Tr1; Th3; Treg17; $T_{RE}G$; CDCP1, NT5E, EpCAM, CEA, gpA33,

Mucins, TAG-72, Carbonic anhydrase IX, PSMA, Folate binding protein, Gangliosides (e.g., CD2, CD3, GM2), Lewis-γ^2, VEGF, VEGFR 1/2/3, $\alpha V\beta 3$, $\alpha 5\beta 1$, ErbB1/EGFR, ErbB1/HER2, ErB3, c-MET, IGF1R, EphA3, TRAIL-R1, TRAIL-R2, RANKL, FAP, Tenascin, PDL-1, BAFF, HDAC, ABL, FLT3, KIT, MET, RET, IL-1β, ALK, RANKL, mTOR, CTLA-4, IL-6, IL-6R, JAK3, BRAF, PTCH, Smoothened, PIGF, ANPEP, TIMP1, PLAUR, PTPRJ, LTBR, or ANTXR1, Folate receptor alpha (FRa), ERBB2 (Her2/neu), EphA2, IL-13Ra2, epidermal growth factor receptor (EGFR), Mesothelin, TSHR, CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1, CD33, EGFRvIII , GD2, GD3, BCMA, MUC16 (CA125), L1CAM, LeY, MSLN, IL13Rα1, L1-CAM, Tn Ag, prostate specific membrane antigen (PSMA), ROR1, FLT3, FAP, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, interleukin-11 receptor a (IL-11Ra), PSCA, PRSS21, VEGFR2, LewisY, CD24, platelet-derived growth factor receptor-beta (PDGFR-beta), SSEA-4, CD20, MUC1, NCAM, Prostase, PAP, ELF2M, Ephrin B2, IGF-1 receptor, CAIX, LMP2, gplOO, bcr-abl, tyrosinase, Fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-A1, legumain, HPV E6, E7, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, prostein, survivin, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin B1, MYCN, RhoC, TRP-2, CYPIB I, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, a neoantigen, CD133, CD15, CD184, CD24, CD56, CD26, CD29, CD44, HLA-A, HLA-B, HLA-C, (HLA-A,B,C) CD49f, CD151 CD340, CD200, tkrA, trkB, or trkC.

[00090] In some embodiments, the fusosome associates with and/or binds a target cell or a surface feature of a target cell.

[00091] In some embodiments, a method herein comprises causing secretion of a protein from a target cell or ligand presentation on the surface of a target cell. In some embodiments, the

fusosome and/or compositions or preparations thereof, are capable of causing cell death of the target cell. In some embodiments, the fusosome is from a NK source cell.

[00092] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, sense and/or respond to one or more local environment features such as, for example, metabolite, interleukin, antigen, etc or combinations thereof.

[00093] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of chemotaxis, extravasation, and/or one or more metabolic activities. In embodiments, the metabolic activity is selected from kyneurinine, gluconeogenesis, prostaglandin fatty acid oxidation, adenosine metabolism, urea cycle, and thermogenic respiration. In some embodiments, the source cell is a neutrophil and the fusosome and/or compositions or preparations thereof, are capable of homing to a site of injury. In some embodiments, the source cell is a macrophage and the fusosome and/or compositions or preparations thereof, are capable of phagocytosis. In some embodiments, the source cell is a brown adipose tissue cell and the fusosome and/or compositions or preparations thereof, are capable of lipolysis.

[00094] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, comprise (e.g., are capable of delivering to the target cell) a plurality of agents (e.g., at least 2, 3, 4, 5, 10, 20, or 50 agents), wherein at least one agent is or comprises a membrane protein payload; in some such embodiments, one or more of the agents is or comprises an inhibitory nucleic acid (e.g., siRNA or miRNA) and/or an mRNA.

[00095] In some embodiments, provided fusosomes, and/or compositions or preparations thereof that comprise (e.g., are capable of delivering to the target cell) a membrane protein payload agent are capable of reprogramming or transdifferentiating a target cell, e.g., the fusosome (and/or composition thereof) comprises one or more agents that induce reprogramming or transdifferentiation of a target cell.

[00096] In some embodiments, the fusosome fuses at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses at a higher rate with a target cell than with a non-target cell by at least at least 10%, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses at a higher rate with a target cell

than other fusosomes, e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses at a higher rate with a target cell than other fusosomes by at least 50%, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses with target cells at a rate such that an agent in the fusosome is delivered to at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of target cells after 24, 48, or 72 hours, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses with target cells at a rate such that an agent in the fusosome is delivered to at least 10% of target cells after 24 hours, e.g., in an assay of Example 54.

[00097] In some embodiments, the fusogen is present, per fusosome, at a copy number of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 29. In some embodiments, the fusogen is present at a copy number of at least 1,000 copies, e.g., as measured by an assay of Example 29. In some embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% of the fusogen comprised by the fusosome is disposed in the cell membrane. In embodiments, the fusosome also comprises fusogen internally, e.g., in the cytoplasm or an organelle.

[00098] In some embodiments, the fusosome comprises a therapeutic agent (e.g., a therapeutic membrane protein payload agent) at a copy number per fusosome of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 43. In some embodiments, the fusosome comprises a protein therapeutic agent at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies, e.g., as measured by an assay of Example 43. In some embodiments, the fusosome comprises a nucleic acid therapeutic agent at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In some embodiments, the fusosome comprises a DNA

therapeutic agent at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In some embodiments, the fusosome comprises an RNA therapeutic agent at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In some embodiments, the fusosome comprises a therapeutic agent that is exogenous relative to the source cell at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In some embodiments, the fusosome comprises a protein therapeutic agent that is exogenous relative to the source cell at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In some embodiments, the fusosome comprises a nucleic acid (e.g., DNA or RNA) therapeutic agent that is exogenous relative to the source cell at a copy number of at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In some embodiments, the ratio of the copy number of the fusogen to the copy number of the therapeutic agent is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000. In some embodiments, the ratio of the copy number of the fusogen to the copy number of the membrane protein payload agent is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000.

[00099] In some embodiments, the fusosome delivers to a target cell at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies of a

therapeutic agent (e.g., a therapeutic membrane protein payload agent). In some embodiments, the fusosome delivers to a target cell at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies of a protein therapeutic agent. In some embodiments, the fusosome delivers to a target cell at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies of a nucleic acid therapeutic agent. In some embodiments, the fusosome delivers to a target cell at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies of an RNA therapeutic agent. In some embodiments, the fusosome delivers to a target cell at least 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies of a DNA therapeutic agent.

[000100] In some embodiments, the fusosome delivers to a target cell at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% of a membrane protein payload agent (e.g., a therapeutic agent, e.g., a therapeutic agent that is endogenous or exogenous relative to the source cell) comprised by the fusosome. In some embodiments, the fusosomes that fuse with the target cell(s) deliver to the target cell an average of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% of the membrane protein payload agent (e.g., a therapeutic membrane protein payload agent, e.g., an endogenous therapeutic membrane protein payload agent or a therapeutic membrane protein payload agent that is exogenous relative to the source cell) comprised by the fusosomes that fuse with the target cell(s). In some embodiments, the fusosome composition delivers to a target tissue at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% of the membrane protein payload agent (e.g., a membrane protein payload agent agent, e.g., a therapeutic membrane protein payload agent that is exogenous relative ot he source cell) comprised by the fusosome composition.

[000101] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, comprise 0.00000001 mg fusogen to 1 mg fusogen per mg of total protein in fusosome, e.g., 0.00000001 - 0.0000001, 0.0000001 - 0.000001, 0.000001 - 0.00001, 0.00001 - 0.0001,

0.0001 - 0.001, 0.001 - 0.01, 0.01 - 0.1, or 0.1 – 1 mg fusogen per mg of total protein in fusosome. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, comprise 0.00000001 mg fusogen to 5 mg fusogen per mg of lipid in fusosome, e.g., 0.00000001 - 0.0000001, 0.0000001 - 0.000001, 0.000001 - 0.00001, 0.00001 - 0.0001, 0.0001 - 0.001, 0.001 - 0.01, 0.01 - 0.1, 0.1 – 1, or 1-5 mg fusogen per mg of lipid in fusosome.

[000102] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a lipid composition substantially similar to that of the source cell or wherein one or more of CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG is within 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%, e.g., within 75%, of the corresponding lipid level in the source cell.

[000103] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a ratio of cardiolipin: ceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin: ceramide in the source cell; or by a ratio of cardiolipin: diacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin: diacylglycerol in the source cell; or by a ratio of cardiolipin: hexosylceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin: hexosylceramide in the source cell; or by a ratio of cardiolipin:lysophosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin: lysophosphatidate in the source cell; or by a ratio of cardiolipin: lyso-phosphatidylcholine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin: lyso-phosphatidylcholine in the source cell; or by a ratio of cardiolipin: lyso-phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin: lyso-phosphatidylethanolamine in the source cell; or by a ratio of cardiolipin: lyso-phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : lyso-phosphatidylglycerol in the source cell; or by a ratio of cardiolipin: lyso-phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : lyso-phosphatidylinositol in the source cell; or by a ratio of cardiolipin: lyso-phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : lyso-phosphatidylserine in the source cell; or by a ratio of cardiolipin: phosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidate in the source cell; or by a ratio of cardiolipin: phosphatidylcholine that is within

10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidylcholine in the source cell; or by a ratio of cardiolipin: phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidylethanolamine in the source cell; or by a ratio of cardiolipin: phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidylglycerol in the source cell; or by a ratio of cardiolipin: phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidylinositol in the source cell; or by a ratio of cardiolipin: phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidylserine in the source cell; or by a ratio of cardiolipin: cholesterol ester that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : cholesterol ester in the source cell; or by a ratio of cardiolipin: sphingomyelin that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : sphingomyelin in the source cell; or by a ratio of cardiolipin: triacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : triacylglycerol in the source cell; or by a ratio of phosphatidylcholine: ceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine: ceramide in the source cell; or by a ratio of phosphatidylcholine: diacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine: diacylglycerol in the source cell; or by a ratio of phosphatidylcholine: hexosylceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine: hexosylceramide in the source cell; or by a ratio of phosphatidylcholine:lysophosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine: lysophosphatidate in the source cell; or by a ratio of phosphatidylcholine: lyso-phosphatidylcholine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine: lyso-phosphatidylcholine in the source cell; or by a ratio of phosphatidylcholine: lyso-phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine: lyso-phosphatidylethanolamine in the source cell; or by a ratio of phosphatidylcholine: lyso-phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : lyso-phosphatidylglycerol in the source cell; or by a ratio of phosphatidylcholine: lyso-phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : lyso-phosphatidylinositol in the source cell; or by a ratio of phosphatidylcholine: lyso-phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : lyso-phosphatidylserine in the source cell; or by a ratio of

phosphatidylcholine: phosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cardiolipin : phosphatidate in the source cell; or by a ratio of phosphatidylcholine: phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : phosphatidylethanolamine in the source cell; or by a ratio of cardiolipin: phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : phosphatidylglycerol in the source cell; or by a ratio of phosphatidylcholine: phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : phosphatidylinositol in the source cell; or by a ratio of phosphatidylcholine: phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : phosphatidylserine in the source cell; or by a ratio of phosphatidylcholine: cholesterol ester that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : cholesterol ester in the source cell; or by a ratio of phosphatidylcholine: sphingomyelin that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : sphingomyelin in the source cell; or by a ratio of phosphatidylcholine: triacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylcholine : triacylglycerol in the source cell; or by a ratio of phosphatidylethanolamine: ceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine: ceramide in the source cell; or by a ratio of phosphatidylethanolamine: diacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine: diacylglycerol in the source cell; or by a ratio of phosphatidylethanolamine: hexosylceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine: hexosylceramide in the source cell; or by a ratio of phosphatidylethanolamine:lysophosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine: lysophosphatidate in the source cell; or by a ratio of phosphatidylethanolamine: lyso-phosphatidylcholine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine: lyso-phosphatidylcholine in the source cell; or by a ratio of phosphatidylethanolamine: lyso-phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine: lyso-phosphatidylethanolamine in the source cell; or by a ratio of phosphatidylethanolamine: lyso-phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : lyso-phosphatidylglycerol in the source cell; or by a ratio of phosphatidylethanolamine: lyso-phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of

phosphatidylethanolamine : lyso-phosphatidylinositol in the source cell; or by a ratio of phosphatidylethanolamine: lyso-phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : lyso-phosphatidylserine in the source cell; or by a ratio of phosphatidylethanolamine: phosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : phosphatidate in the source cell; or by a ratio of phosphatidylethanolamine: phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : phosphatidylglycerol in the source cell; or by a ratio of phosphatidylethanolamine: phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : phosphatidylinositol in the source cell; or by a ratio of phosphatidylethanolamine: phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : phosphatidylserine in the source cell; or by a ratio of phosphatidylethanolamine: cholesterol ester that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : cholesterol ester in the source cell; or by a ratio of phosphatidylethanolamine: sphingomyelin that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : sphingomyelin in the source cell; or by a ratio of phosphatidylethanolamine: triacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylethanolamine : triacylglycerol in the source cell; or by a ratio of phosphatidylserine: ceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine: ceramide in the source cell; or by a ratio of phosphatidylserine: diacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine: diacylglycerol in the source cell; or by a ratio of phosphatidylserine: hexosylceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine: hexosylceramide in the source cell; or by a ratio of phosphatidylserine:lysophosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine: lysophosphatidate in the source cell; or by a ratio of phosphatidylserine: lyso-phosphatidylcholine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine: lyso-phosphatidylcholine in the source cell; or by a ratio of phosphatidylserine: lyso-phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine: lyso-phosphatidylethanolamine in the source cell; or by a ratio of phosphatidylserine: lyso-phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : lyso-phosphatidylglycerol in the source cell; or by a ratio of

phosphatidylserine: lyso-phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : lyso-phosphatidylinositol in the source cell; or by a ratio of phosphatidylserine: lyso-phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : lyso-phosphatidylserine in the source cell; or by a ratio of phosphatidylserine: phosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : phosphatidate in the source cell; or by a ratio of phosphatidylserine: phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : phosphatidylglycerol in the source cell; or by a ratio of phosphatidylserine: phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : phosphatidylinositol in the source cell; or by a ratio of phosphatidylserine: cholesterol ester that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : cholesterol ester in the source cell; or by a ratio of phosphatidylserine: sphingomyelin that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : sphingomyelin in the source cell; or by a ratio of phosphatidylserine: triacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of phosphatidylserine : triacylglycerol in the source cell; or by a ratio of sphingomyelin: ceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin: ceramide in the source cell; or by a ratio of sphingomyelin: diacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin: diacylglycerol in the source cell; or by a ratio of sphingomyelin: hexosylceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin: hexosylceramide in the source cell; or by a ratio of sphingomyelin:lysophosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin: lysophosphatidate in the source cell; or by a ratio of sphingomyelin: lyso-phosphatidylcholine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin: lyso-phosphatidylcholine in the source cell; or by a ratio of sphingomyelin: lyso-phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin: lyso-phosphatidylethanolamine in the source cell; or by a ratio of sphingomyelin: lyso-phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : lyso-phosphatidylglycerol in the source cell; or by a ratio of sphingomyelin: lyso-phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : lyso-phosphatidylinositol in the source cell; or by a ratio of sphingomyelin: lyso-phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin :

lyso-phosphatidylserine in the source cell; or by a ratio of sphingomyelin: phosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : phosphatidate in the source cell; or by a ratio of sphingomyelin: phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : phosphatidylglycerol in the source cell; or by a ratio of sphingomyelin: phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : phosphatidylinositol in the source cell; or by a ratio of sphingomyelin: cholesterol ester that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : cholesterol ester in the source cell; or by a ratio of sphingomyelin: triacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of sphingomyelin : triacylglycerol in the source cell; or by a ratio of cholesterol ester: ceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester: ceramide in the source cell; or by a ratio of cholesterol ester: diacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester: diacylglycerol in the source cell; or by a ratio of cholesterol ester: hexosylceramide that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester: hexosylceramide in the source cell; or by a ratio of cholesterol ester:lysophosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester: lysophosphatidate in the source cell; or by a ratio of cholesterol ester: lyso-phosphatidylcholine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester: lyso-phosphatidylcholine in the source cell; or by a ratio of cholesterol ester: lyso-phosphatidylethanolamine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester: lyso-phosphatidylethanolamine in the source cell; or by a ratio of cholesterol ester: lyso-phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester : lyso-phosphatidylglycerol in the source cell; or by a ratio of cholesterol ester: lyso-phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester : lyso-phosphatidylinositol in the source cell; or by a ratio of cholesterol ester: lyso-phosphatidylserine that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester : lyso-phosphatidylserine in the source cell; or by a ratio of cholesterol ester: phosphatidate that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester : phosphatidate in the source cell; or by a ratio of cholesterol ester: phosphatidylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester : phosphatidylglycerol in the source cell; or by a ratio of cholesterol ester: phosphatidylinositol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of

cholesterol ester : phosphatidylinositol in the source cell; or by a ratio of cholesterol ester: triacylglycerol that is within 10%, 20%, 30%, 40%, or 50% of the ratio of cholesterol ester : triacylglycerol in the source cell.

[000104] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a proteomic composition similar to that of the source cell, e.g., using an assay of Example 42. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by ratio of lipids to proteins that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 49. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a ratio of proteins to nucleic acids (e.g., DNA or RNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 50. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a ratio of proteins to DNA that is greater than the corresponding ratio in the source cell, e.g., at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% greater, e.g., as measured using an assay of Example 50. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a ratio of lipids to nucleic acids (e.g., DNA) that is within 10%, 20%, 30%, 40%, or 50% of the corresponding ratio in the source cell, e.g., as measured using an assay of Example 51. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a ratio of lipids to nucleic acids (e.g., DNA) that is greater than the corresponding ratio in the source cell, e.g., at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% greater, e.g., as measured using an assay of Example 51.

[000105] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a half-life in a subject, e.g., in a mouse, that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the half life of a reference cell, e.g., the source cell, e.g., by an assay of Example 75. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a half-life in a subject, e.g., in a mouse, that is at least 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, or 24 hours, e.g., in a human subject or in a mouse, e.g., by an assay of Example 75. In

some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of delivering (e.g., deliver) a membrane protein payload agent (e.g., a therapeutic agent) that is characterized by a half-life in a subject that is longer than the half-life of the fusosome, e.g., by at least 10%, 20%, 50%, 2-fold, 5-fold, or 10-fold. For instance, the fusosome may deliver the therapeutic agent to the target cell, and the agent may be present after the fusosome is no longer present or detectable.

[000106] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, transport glucose (e.g., labeled glucose, e.g., 2-NBDG) across a membrane, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of glucose, e.g., as measured using an assay of Example 64. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized byesterase activity in the lumen that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of that of the esterase activity in a reference cell, e.g., the source cell or a mouse embryonic fibroblast, e.g., using an assay of Example 66. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a metabolic activity level (e.g., citrate synthase activity) that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the metabolic activity level in a reference cell, e.g., the source cell, e.g., as described in Example 68. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a metabolic activity level (e.g., citrate synthase activity) that is at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the metabolic activity level in a reference cell, e.g., the source cell, e.g., as described in Example 68. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a respiration level (e.g., oxygen consumption rate) that is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the respiration level in a reference cell, e.g., the source cell, e.g., as described in Example 69. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a respiration level (e.g., oxygen consumption rate) that is at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the respiration level in a reference cell, e.g., the source cell, e.g., as described in Example 69. In some embodiments, provided fusosomes, and/or compositions or

preparations thereof, are characterized by an Annexin-V staining level of at most 18,000, 17,000, 16,000, 15,000, 14,000, 13,000, 12,000, 11,000, or 10,000 MFI, e.g., using an assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% lower than the Annexin-V staining level of otherwise similar fusosomes, or a composition or preparation thereof, treated with menadione in the assay of Example 70, or wherein the fusosome comprises an Annexin-V staining level at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% lower than the Annexin-V staining level of a macrophage treated with menadione in the assay of Example 70.

[000107] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a miRNA content level of at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., by an assay of Example 39. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a miRNA content level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater of the miRNA content level of the source cell (e.g., up to 100% of the miRNA content level of the source cell), e.g., by an assay of Example 39. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a total RNA content level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater of the total RNA content level of the source cell (e.g., up to 100% of the total RNA content level of the source cell), e.g., as measured by an assay of Example 108. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a soluble : non-soluble protein ratio is within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of the source cell, e.g., within 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of that of the source cell, e.g., by an assay of Example 47. In some embodiments, the fusosome has a soluble : non-soluble protein ratio within 90% of that of the source cell, e.g., by an assay of Example 47. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by an LPS level less than 5%, 1%, 0.5%, 0.01%, 0.005%, 0.0001%, 0.00001% or less of the lipid content of fusosomes, e.g., as measured by an assay of Example 48. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of signal transduction, e.g.,

transmitting an extracellular signal, e.g., AKT phosphorylation in response to insulin, or glucose (e.g., labeled glucose, e.g., 2-NBDG) uptake in response to insulin, e.g., by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% more than a negative control, e.g., an otherwise similar fusosome in the absence of insulin, e.g., using an assay of Example 63. In some embodiments, the fusosome targets a tissue, e.g., liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, or eye, when administered to a subject, e.g., a mouse, e.g., wherein at least 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, or 90% of the fusosomes in a population of administered fusosomes are present in the target tissue after 24, 48, or 72 hours, e.g., by an assay of Example 87 or 100. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a juxtacrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a juxtacrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (e.g., up to100%) of the level of juxtacrine signaling induced by a reference cell, e.g., the source cell or a bone marrow stromal cell (BMSC), e.g., by an assay of Example 71. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a paracrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% greater than the level of paracrine signaling induced by a reference cell, e.g., the source cell or a macrophage, e.g., by an assay of Example 72. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a paracrine-signaling level of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (e.g., up to100%) of the level of paracrine signaling induced by a reference cell, e.g., the source cell or a macrophage, e.g., by an assay of Example 72. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized bypolymerizes actin at a level within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% compared to the level of polymerized actin in a reference cell, e.g., the source cell or a C2C12 cell,

e.g., by the assay of Example 73. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a membrane potential within about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the membrane potential of a reference cell, e.g., the source cell or a C2C12 cell, e.g., by an assay of Example 74, or wherein provided fusosomes, and/or compositions or preparations thereof, are characterized by a membrane potential of about -20 to -150mV, -20 to -50mV, -50 to -100mV, or -100 to -150mV, or wherein the fusosome has a membrane potential of less than -1mv, -5mv, -10mv, -20mv, -30mv, -40mv, -50mv, -60mv, -70mv, -80mv, -90mv, -100mv. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of extravasation from blood vessels, e.g., at a rate at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% the rate of extravasation of the source cell, e.g., using an assay of Example 57, e.g., wherein the source cell is a neutrophil, lymphocyte, B cell, macrophage, or NK cell. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of chemotaxis, e.g., of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (e.g., up to 100%) compared to a reference cell, e.g., a macrophage, e.g., using an assay of Example 58. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of phagocytosis, e.g., at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (e.g., up to 100%) compared to a reference cell, e.g., a macrophage, e.g., using an assay of Example 60. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of crossing a cell membrane, e.g., an endothelial cell membrane or the blood brain barrier. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of secreting a protein, e.g., at a rate at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than a reference cell, e.g., a mouse embryonic fibroblast, e.g., using an assay of Example 62. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are capable of secreting a protein, e.g., at a rate at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (e.g., up to 100%) compared to a reference cell, e.g., a mouse embryonic fibroblast, e.g., using an assay of Example 62.

[000108] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are not capable of transcription or have transcriptional activity of less than 1%, 2.5% 5%,

10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of that of the transcriptional activity of a reference cell, e.g., the source cell, e.g., using an assay of Example 19. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are not capable of nuclear DNA replication or has nuclear DNA replication of less than 1%, 2.5% 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the nuclear DNA replication of a reference cell, e.g., the source cell, e.g., using an assay of Example 20. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, lack chromatin or have a chromatin content of less than 1%, 2.5% 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the of the chromatin content of a reference cell, e.g., the source cell, e.g., using an assay of Example 37.

[000109] In some embodiments, a characteristic of a provided fusosome, and/or of a composition or preparatios thereof, is described by comparison to a reference cell. In embodiments, the reference cell is the source cell. In embodiments, the reference cell is a HeLa, HEK293, HFF-1, MRC-5, WI-38, IMR 90, IMR 91, PER.C6, HT-1080, or BJ cell. In some embodiments, a characteristic of a population of fusosomes, and/or of a composition or preparation thereof, is described by comparison to a population of reference cells, e.g., a population of source cells, or a population of HeLa, HEK293, HFF-1, MRC-5, WI-38, IMR 90, IMR 91, PER.C6, HT-1080, or BJ cells.

[000110] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, meet a pharmaceutical or good manufacturing practices (GMP) standard. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, were made according to good manufacturing practices (GMP). In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by a pathogen level below a predetermined reference value, e.g., are substantially free of pathogens. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, have a contaminant (e.g., nuclear component such as nuclear DNA) level below a predetermined reference value, e.g., are substantially free of one or more specified contaminants. In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by low immunogenicity, e.g., as described herein.

[000111] In some embodiments, the source cell or target cell is an endothelial cell, a

fibroblast, a blood cell (e.g., a macrophage, a neutrophil, a granulocyte, a leukocyte), a stem cell (e.g., a mesenchymal stem cell, an umbilical cord stem cell, bone marrow stem cell, a hematopoietic stem cell, an induced pluripotent stem cell e.g., an induced pluripotent stem cell derived from a subject's cells), an embryonic stem cell (e.g., a stem cell from embryonic yolk sac, placenta, umbilical cord, fetal skin, adolescent skin, blood, bone marrow, adipose tissue, erythropoietic tissue, hematopoietic tissue), a myoblast, a parenchymal cell (e.g., hepatocyte), an alveolar cell, a neuron (e.g., a retinal neuronal cell) a precursor cell (e.g., a retinal precursor cell, a myeloblast, myeloid precursor cells, a thymocyte, a meiocyte, a megakaryoblast, a promegakaryoblast, a melanoblast, a lymphoblast, a bone marrow precursor cell, a normoblast, or an angioblast), a progenitor cell (e.g., a cardiac progenitor cell, a satellite cell, a radial gial cell, a bone marrow stromal cell, a pancreatic progenitor cell, an endothelial progenitor cell, a blast cell), or an immortalized cell (e.g., HeLa, HEK293, HFF-1, MRC-5, WI-38, IMR 90, IMR 91, PER.C6, HT-1080, or BJ cell). In some embodiments, the source cell is other than a 293 cell, HEK cell, human endothelial cell, or a human epithelial cell, monocyte, macrophage, dendritic cell, or stem cell. In some embodiments, the source cell or target cell is a white blood cell or a stem cell. In some embodiments, the source cell or target cell is selected from a neutrophil, a lymphocyte (e.g., a T cell, a B cell, a natural killer cell), a macrophage, a granulocyte, a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell, or a myeloblast.

[000112] In some embodiments, the source cell is a cell grown under adherent or suspension conditions. In some embodiments, the source cell is a primary cell, a cultured cell, an immortalized cell, or a cell line (e.g., myelobast cell line, e.g., C2C12). In some embodiments, the source cell is allogeneic, e.g., obtained from a different organism of the same species as the target cell. In some embodiments, the source cell is autologous, e.g., obtained from the same organism as the target cell. In some embodiments, the source cell is heterologous, e.g., obtained from an organism of a different species from the target cell.

[000113] In some embodiments, the source cell comprises further comprises a second agent that is exogenous to the source cell, e.g., a therapeutic agent, e.g., a protein or a nucleic acid (e.g., an RNA, e.g., an mRNA or miRNA). In some embodiments, the second agent is present at least, or no more than, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000,

200,000, 500,000 or 1,000,000 copies comprised by the fusosome, or is present at an average level of at least, or no more than, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000 or 1,000,000 copies per fusosome.

[000114] In some embodiments, the fusosome has an altered, e.g., increased or decreased level of one or more endogenous molecules as compared to the source cell, e.g., protein or nucleic acid, e.g., due to treatment of the source cell, e.g., mammalian source cell with a siRNA or gene editing enzyme. In some embodiments, the fusosome comprises at least, or no more than, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000 or 1,000,000 copies of the endogenous molecule, or is present at an average level of at least, or no more than, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000 or 1,000,000 copies of the endogenous molecule per fusosome. In some embodiments, the endogenous molecule (e.g., an RNA or protein) is present in the fusosome at a concentration of at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, 1.0 x 10^7, 5.0 x 10^7, or 1.0 x 10^8 greater than its concentration in the source cell. In some embodiments, the endogenous molecule (e.g., an RNA or protein) is present in the fusosome at a concentration of at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, 1.0 x 10^7, 5.0 x 10^7, or 1.0 x 10^8 less than its concentration in the source cell.

[000115] In some embodiments, a fusosome comprises a therapeutic membrane protein payload agent, e.g., a therapeutic membrane protein payload agent, e.g., a therapeutic membrane protein payload agent that is exogenous or endogenous relative to the source cell. In some embodiments, the therapeutic membrane protein payload agent is chosen from one or more of a protein, e.g., a transmembrane protein, a cell surface protein, a secreted protein, a receptor, an antibody; a nucleic acid, e.g., DNA, a chromosome (e.g. a human artificial chromosome), RNA, or mRNA.

[000116] In some embodiments, the target cell is in an organism. In some embodiments, the target cell is a primary cell isolated from an organism. In some embodiments, the targeting domain interacts with a target cell moiety on the target cell, e.g., a cell surface feature. In some embodiments, the fusosome does not comprise said target cell moiety. In some embodiments, the

fusosome comprises a fusogen which interacts with a fusogen binding partner on the target cell, thereby allowing the fusosome to bind or fuse to the target cell. In some embodiments, the fusosome does not comprise said fusogen binding partner. In some embodiments, the targeting domain is not part of the fusogen. In some embodiments, the fusogen comprises the targeting domain. In some embodiments, the fusogen binding partner is or is a portion of a different entity from the target cell moiety. In some embodiments, the fusogen binding partner is or is a portion of the target cell moiety.

[000117] In some embodiments, a fusosome enters the target cell by endocytosis, e.g., wherein the level of agent (e.g., membrane protein payload agent and/or second agent) delivered via an endocytic pathway is 0.01-0.6, 0.01-0.1, 0.1-0.3, or 0.3-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than a chloroquine treated reference cell contacted with similar fusosomes, e.g., using an assay of Example 91. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% of fusosomes in a fusosome composition or preparation that enter a target cell enter via a non-endocytic pathway, e.g., the fusosomes enter the target cell via fusion with the cell surface. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% of fusosomes in a fusosome composition or preparation that enter a target cell enter the cytoplasm (e.g., do not enter an endosome or lysosome). In some embodiments, less than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% of fusosomes in a fusosome composition or preparation that enter a target cell enter an endosome or lysosome. In some embodiments, the fusosome enters the target cell by a non-endocytic pathway, e.g., wherein the level of agent (e.g., membrane protein payload agent and/or second agent) delivered is at least 90%, 95%, 98%, or 99% that of a chloroquine treated reference cell, e.g., using an assay of Example 91. In some embodiments, a fusosome delivers an agent (e.g., membrane protein payload agent and/or second agent) to a target cell via a dynamin mediated pathway. In some embodiments, the level of agent (e.g., membrane protein payload agent and/or second agent) delivered via a dynamin mediated pathway is in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than Dynasore treated target cells contacted with similar fusosomes, e.g., as measured in an assay of Example 92. In some embodiments, a fusosome delivers an agent (e.g., membrane protein payload agent and/or second

agent) to a target cell via macropinocytosis. In some embodiments, the level of agent (e.g., membrane protein payload agent and/or second agent) delivered via macropinocytosis is in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than EIPA treated target cells contacted with similar fusosomes, e.g., as measured in an assay of Example 92. In some embodiments, a fusosome delivers an agent (e.g., membrane protein payload agent and/or second agent) to a target cell via an actin-mediated pathway. In some embodiments, a level of agent (e.g., membrane protein payload agent and/or second agent) delivered via an actin-mediated pathway will be in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than Latrunculin B treated target cells contacted with similar fusosomes, e.g., as measured in an assay of Example 92.

[000118] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, have a density of <1, 1-1.1, 1.05-1.15, 1.1-1.2, 1.15-1.25, 1.2-1.3, 1.25-1.35, or >1.35 g/mL, e.g., by an assay of Example 33.

[000119] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, comprise less than 0.01%, 0.05%, 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, or 10% source cells by protein mass or less than 0.01%, 0.05%, 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, or 10% of cells have a functional nucleus. In some embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% of fusosomes in the fusosome composition or preparation comprise an organelle, e.g., a mitochondrion.

[000120] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, comprise at least 0.01%-0.05%, 0.05%-0.1%, 0.1%-0.5%, 0.5%- 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% fusosomes wherein: i) the fusogen is present at a copy number of at least 1,000 copies per fusosome, e.g., as measured by an assay of Example 29, ii) the ratio of the copy number of the fusogen to the copy number of the membrane protein payload agent per fusosome is between 1,000,000:1 and 100,000:1, 100,000:1 and 10,000:1, 10,000:1 and 1,000:1, 1,000:1 and 100:1, 100:1 and 50:1, 50:1 and 20:1, 20:1 and 10:1, 10:1 and 5:1, 5:1 and 2:1, 2:1 and 1:1, 1:1 and 1:2, 1:2 and 1:5, 1:5 and 1:10, 1:10 and 1:20, 1:20 and 1:50, 1:50 and 1:100, 1:100 and 1:1,000, 1:1,000 and 1:10,000, 1:10,000 and 1:100,000, or 1:100,000 and 1:1,000,000, or iii) the membrane

protein payload agent is present at a copy number of at least 1,000 copies per fusosome, e.g., as measured by an assay of Example 43.

[000121] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, comprise a therapeutic agent that is exogenous relative to the source cell. In some embodiments, the therapeutic agent is exogenous relative to the target cell. In some embodiments, the exogenous therapeutic agent is chosen from one or more of a protein, e.g., a transmembrane protein, a cell surface protein, a secreted protein, a receptor, an antibody; a nucleic acid, e.g., DNA, a chromosome (e.g. a human artificial chromosome), RNA, mRNA, siRNA, miRNA, or a small molecule.

[000122] In embodiments, a provided fusosome enters the cell by endocytosis or a non-endocytic pathway.

[000123] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, do not comprise a nucleus. In some embodiments, the fusosome is substantially free of nuclear DNA.

[000124] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, are refrigerated or frozen. In embodiments, provided fusosomes do not comprise a functional nucleus, and/or provided fusosome compositions or preparations comprise one or more fusosomes without a functional nucleus. In some embodiments, provided fusosome compositions or preparations comprise less than 0.01%, 0.05%, 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, or 10% source cells by protein mass or less than 0.01%, 0.05%, 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, or 10% of cells have a functional nucleus. In embodiments, provided fusosomes, and/or compositions or preparations thereof, have been maintained at said temperature for at least 1, 2, 3, 6, or 12 hours; 1, 2, 3, 4, 5, or 6 days; 1, 2, 3, or 4 weeks; 1, 2, 3, or 6 months; or 1, 2, 3, 4, or 5 years. In embodiments, provided fusosomes, and/or compositions or preparations thereof, are characterized by an activity of at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the activity of the population before maintenance at said temperature, e.g., by one or more of:

i) fusing at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, e.g., in an assay of Example 54;

ii) fusing at a higher rate with a target cell than with other fusosomes, e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., in an assay of Example 54;

iii) fusing with target cells at a rate such that an agent in the fusosome is delivered to at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of target cells after 24, 48, or 72 hours, e.g., in an assay of Example 54; or

iv) level of fusogen at a copy number of at least, or no more than, 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000 or 1,000,000 copies, e.g., as measured by an assay of Example 29.

[000125] In embodiments, a provided fusosome composition or preparation is stable at a temperature of less than 4 C for at least 1, 2, 3, 6, or 12 hours; 1, 2, 3, 4, 5, or 6 days; 1, 2, 3, or 4 weeks; 1, 2, 3, or 6 months; or 1, 2, 3, 4, or 5 years. In embodiments, the fusosome composition or preparation is stable at a temperature of less than -20°C for at least 1, 2, 3, 6, or 12 hours; 1, 2, 3, 4, 5, or 6 days; 1, 2, 3, or 4 weeks; 1, 2, 3, or 6 months; or 1, 2, 3, 4, or 5 years. In embodiments, the fusosome composition or preparation is stable at a temperature of less than -80°C for at least 1, 2, 3, 6, or 12 hours; 1, 2, 3, 4, 5, or 6 days; 1, 2, 3, or 4 weeks; 1, 2, 3, or 6 months; or 1, 2, 3, 4, or 5 years.

[000126] In embodiments, one or more of the following is true of provided fusosomes, and/or compositions or preparations thereof:

i) the source cell is other than a 293 cell;

ii) the source cell is not transformed or immortalized;

iii) the source cell is transformed or immortalized using a method other than adenovirus-mediated immortalization, e.g., immortalized by spontaneous mutation or telomerase expression;

iv) the fusogen is other than VSVG, a SNARE protein, or a secretory granule protein;

v) the therapeutic agent is other than Cre or GFP, e.g., EGFP;

vi) the therapeutic agent is a nucleic acid (e.g., RNA, e.g., mRNA, miRNA, or siRNA) or a protein exogenous to the source cell (e.g., an antibody, e.g., an antibody), e.g., in the lumen; or

vii) the fusosome does not comprise mitochondria.

[000127] Alternatively or additionally, in embodiments, one or more of the following is true of provided fusosomes and/or compositions or preparations thereof:

i) the source cell is other than a 293 or HEK cell;

ii) the source cell is not transformed or immortalized;

iii) the source cell is transformed or immortalized using a method other than adenovirus-mediated immortalization, e.g., immortalized by spontaneous mutation or telomerase expression;

iv) the fusogen is not a viral fusogen;

v) the fusosome has a diameter of other than between 40 and 150 nm, e.g., greater than 150 nm, 200 nm, 300 n, 400 nm, or 500 nm; or

vi) the fusosome has a diameter of at least about 10 nm, 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 150 nm, or 200 nm, e.g., as measured by an assay of Example 32.

[000128] Alternatively or additionally, in embodiments, one or more of the following is true of provided fusosomes and/or compositions or preparations thereof:

i) the membrane protein is expressed by the source cell;

ii) the fusogen is other than TAT, TAT-HA2, HA-2, gp41, Alzheimer's beta-amyloid peptide, a Sendai virus protein, or amphipathic net-negative peptide (WAE 11);

iii) the fusogen is a mammalian fusogen;

iv) the fusosome comprises in its lumen a polypeptide selected from an enzyme, antibody, or anti-viral polypeptide;

v) the fusosome does not comprise a therapeutic transmembrane protein, e.g., a therapeutic transmembrane protein that is exogenous relative to the source cell; or

vi) the fusosome does not comprise CD63 or GLUT4.

[000129] Alternatively or additionally, in embodiments, one or more of the following is true of provided fusosomes and/or compositions or preparations thereof:

i) the fusogen is other than a viral protein;

ii) the fusogen is other than a fusogenic glycoprotein;

iii) the fusogen is a mammalian protein other than fertilin-beta;

iv) the fusogen is other than VSVG, a SNARE protein, or a secretory granule protein; or

v) the fusogen is other than TAT, TAT-HA2, HA-2, gp41, Alzheimer's beta-amyloid peptide, a Sendai virus protein, or amphipathic net-negative peptide (WAE 11).

[000130] Alternatively or additionally, in embodiments, one or more of the following is true of provided fusosomes and/or compositions or preparations thereof:

i) does not comprise a virus, is not infectious, or does not propagate in a host cell;

ii) is not a VLP (virus like particle);

iii) does not comprise a viral structural protein, e.g., a viral capsid protein, e.g., a viral nucleocapsid protein, or wherein the amount of viral capsid protein is less than 10%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.2%, or 0.1% of total protein, e.g., by an assay of Example 53;

iv) does not comprise a viral matrix protein;

v) does not comprise a viral non-structural protein;

vi) comprises less than 10, 50, 100, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, 1,000,000,000 copies per vesicle of a viral structural protein; or

vii) the fusosome is not a virosome.

[000131] Alternatively or additionally, in embodiments, the ratio of the copy number of the fusogen to the copy number of viral structural protein on the fusosome is at least 1,000,000:1, 100,000:1, 10,000:1, 1,000:1, 100:1, 50:1 1, 20:1, 10:1, 5:1, or 1:1. In embodiments, the ratio of the copy number of the fusogen to the copy number of viral matrix protein on the fusosome is at least 1,000,000:1, 100,000:1, 10,000:1, 1,000:1, 100:1, 50:1, 20:1, 10:1, 5:1, or 1:1.

[000132] Alternatively or additionally, in embodiments, one or more of the following is true of provided fusosomes and/or compositions or preparations thereof:

i) the fusosome does not comprise a water-immiscible droplet;

ii) the fusosome comprises an aqueous lumen and a hydrophilic exterior;

iii) the fusogen is a protein fusogen.

[000133] Alternatively or additionally, in embodiments, one or more of the following is true of provided fusosomes and/or compositions or preparations thereof:

i) the fusogen is a mammalian fusogen or a viral fusogen;

ii) the fusosome was not made by loading the fusosome with a therapeutic or diagnostic substance;

iii) the source cell was not loaded with a therapeutic or diagnostic substance;

iv) the fusosome does not comprise doxorubicin, dexamethasone, cyclodextrin; polyethylene glycol, a micro RNA e.g., miR125, VEGF receptor, ICAM-1, E-selectin, iron oxide, a fluorescent protein e.g., GFP or RFP, a nanoparticle, or an RNase, or does not comprise an exogenous form of any of the foregoing that is exogenous to the source cell; or

v) the fusosome further comprises a therapeutic agent that is exogenous to the source cell, having one or more post-translational modifications, e.g., glycosylation.

[000134] Alternatively or additionally, in embodiments, the fusosome is unilamellar or multilamellar.

[000135] Alternatively or additionally, in embodiments, provided fusosomes and/or compositions or preparations thereof are characterized by a diameter within about 0.01%, 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, of that of the source cell, e.g., as measured by an assay of Example 30. In embodiments, the diameter that is less than about 0.01%, 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, of that of the source cell, e.g., as measured by an assay of Example 30. In embodiments, the diameter within about 0.01%-0.05%, 0.05%-0.1%, 0.1%-0.5%, 0.5%- 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% the diameter of the source cell, e.g., as measured by an assay of Example 30. In embodiments, the fusosome has a diameter that is less than about 0.01%-0.05%, 0.05%-0.1%, 0.1%-0.5%, 0.5%- 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the diameter of the source cell, e.g., as measured by an assay of Example 30. In embodiments, the diameter is at least about 10 nm, 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 150 nm, 200 nm, or 250 nm e.g., as measured by an assay of Example 32. In embodiments, the diameter

is about 10 nm, 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 150 nm, 200 nm, or 250 nm (e.g., ±20%) e.g., as measured by an assay of Example 32. In embodiments, the diameter is at least about 500 nm, 750 nm, 1,000 nm, 1,500 nm, 2,000 nm, 2,500 nm, 3,000 nm, 5,000 nm, 10,000 nm, or 20,000 nm, e.g., as measured by an assay of Example 32. In embodiments, the diameter is about 500 nm, 750 nm, 1,000 nm, 1,500 nm, 2,000 nm, 2,500 nm, 3,000 nm, 5,000 nm, 10,000 nm, or 20,000 nm (e.g., ±20%), e.g., as measured by an assay of Example 32. In embodiments, the diameter is greater than 5 μm, 6 μm, 7 μm, 8 μm, 10 μm, 20 μm, 50 μm, 100 μm, 150 μm, or 200 μm.

[000136] In some embodiments, provided fusosomes and/or compositions or preparations thereof have a volume that is less than about 0.01%-0.05%, 0.05%-0.1%, 0.1%-0.5%, 0.5%- 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the volume of the source cell.

[000137] In some embodiments, the provided fusosomes and/or compositions or preparations thereof have a density of other than between 1.08 g/mL and 1.12 g/mL. In some embodiments, the density is 1.25 g/mL +/- 0.05, e.g., as measured by an assay of Example 33. In some embodiments, the density is <1, 1-1.1, 1.05-1.15, 1.1-1.2, 1.15-1.25, 1.2-1.3, 1.25-1.35, or >1.35 g/mL, e.g., by an assay of Example 33.

[000138] In embodiments, one or more of the following is true of provided fusosomes, and/or compositions or preparations thereof:

i) the fusosome is not an exosome;
ii) the fusosome is a microvesicle;
iii) the fusosome comprises a non-mammalian fusogen;
iv) the fusosome has been engineered to comprise or incorporate a fusogen;
v) the fusosome comprises a fusogen that is exogenous relative to the source cell or an overexpressed fusogen;
vi) the fusosome has a diameter of at least 80 nm, 100 nm, 200 nm, 500 nm, 1000 nm, 1200 nm, 1400 nm, or 1500 nm, or a population or plurality of fusosomes has an average diameter of at least 80 nm, 100 nm, 200 nm, 500 nm, 1000 nm, 1200 nm, 1400 nm, or 1500 nm;

vii) the fusosome comprises one or more organelles, e.g., a mitochondrion, Golgi apparatus, lysosome, endoplasmic reticulum, vacuole, endosome, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, cnidocyst, peroxisome, proteasome, vesicle, and stress granule;

viii) the fusosome comprises a cytoskeleton or a component thereof, e.g., actin, Arp2/3, formin, coronin, dystrophin, keratin, myosin, or tubulin;

ix) a preparation comprising a plurality of the fusosomes does not have a flotation density of 1.08–1.22 g/mL, or has a density of at least 1.18–1.25 g/mL, or 1.05–1.12 g/mL, e.g., in a sucrose gradient centrifugation assay, e.g., as described in Théry et al., "Isolation and characterization of exosomes from cell culture supernatants and biological fluids." Curr Protoc Cell Biol. 2006 Apr; Chapter 3:Unit 3.22;

x) the lipid bilayer is enriched for ceramides or sphingomyelins or a combination thereof compared to the source cell, or the lipid bilayer is not enriched (e.g., is depleted) for glycolipids, free fatty acids, or phosphatidylserine, or a combination thereof, compared to the source cell;

xi) the fusosome comprises Phosphatidyl serine (PS) or CD40 ligand or both of PS and CD40 ligand, e.g., when measured in an assay of Example 52;

xii) the fusosome is enriched for PS compared to the source cell, e.g., in a population of fusosomes at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% are positive for PS by an assay of Kanada M, et al. (2015) Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc Natl Acad Sci USA 112:E1433–E1442;

xiii) the fusosome is substantially free of acetylcholinesterase (AChE), or contains less than 0.001, 0.002, 0.005, 0.01,0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, or 1000 AChE activity units/μg of protein , e.g., by an assay of Example 67;

xiv) the fusosome is substantially free of a Tetraspanin family protein (e.g., CD63, CD9, or CD81), an ESCRT-related protein (e.g., TSG101, CHMP4A-B, or VPS4B), Alix, TSG101, MHCI, MHCII, GP96, actinin-4, mitofilin, syntenin-1, TSG101, ADAM10, EHD4, syntenin-1, TSG101, EHD1, flotillin-1, heat-shock 70-kDa proteins (HSC70/HSP73, HSP70/HSP72), or any combination thereof, or contains less than 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 5%, or 10% of any individual exosomal marker protein and/or less than

0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% of total exosomal marker proteins of any of said proteins, or is de-enriched for any one or more of these proteins compared to the source cell, or is not enriched for any one or more of these proteins, e.g., by an assay of Example 44;

xv) the fusosome comprises a level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that is below 500, 250, 100, 50, 20, 10, 5, or 1 ng GAPDH/μg total protein or below the level of GAPDH in the source cell, e.g., less than 1%, 2.5%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, less than the level of GAPDH per total protein in ng/μg in the source cell, e.g., using an assay of Example 45;

xvi) the fusosome is enriched for one or more endoplasmic reticulum proteins (e.g., calnexin), one or more proteasome proteins, or one or more mitochondrial proteins, or any combination thereof, e.g., wherein the amount of calnexin is less than 500, 250, 100, 50, 20, 10, 5, or 1 ng Calnexin / μg total protein, or wherein the fusosome comprises less Calnexin per total protein in ng/μg compared to the source cell by 1%, 2.5%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., using an assay of Example 46;

xvii) the fusosome comprises an agent (e.g., protein, mRNA, or siRNA) that is exogenous relative to the source cell, e.g., as measured using an assay of Example 39 or 40; or

xviii) the fusosome can be immobilized on a mica surface by atomic force microscopy for at least 30 min, e.g., by an assay of Kanada M, et al. (2015) Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc Natl Acad Sci USA 112:E1433–E1442.

[000139] In embodiments, one or more of:

i) the fusosome is an exosome;

ii) the fusosome is not a microvesicle;

iii) the fusosome has a diameter of less than 80 nm, 100 nm, 200 nm, 500 nm, 1000 nm, 1200 nm, 1400 nm, or 1500 nm, or a population of fusosomes has an average diameter of at least 80 nm, 100 nm, 200 nm, 500 nm, 1000 nm, 1200 nm, 1400 nm, or 1500 nm;

iv) the fusosome does not comprise an organelle;

v) the fusosome does not comprise a cytoskeleton or a component thereof, e.g., actin, Arp2/3,

formin, coronin, dystrophin, keratin, myosin, or tubulin;

vi) a preparation comprising a plurality of the fusosomes has a flotation density of 1.08–1.22 g/mL, e.g., in a sucrose gradient centrifugation assay, e.g., as described in Théry et al., "Isolation and characterization of exosomes from cell culture supernatants and biological fluids." Curr Protoc Cell Biol. 2006 Apr; Chapter 3:Unit 3.22;

vii) the lipid bilayer is not enriched (e.g., is depleted) for ceramides or sphingomyelins or a combination thereof compared to the source cell, or the lipid bilayer is enriched for glycolipids, free fatty acids, or phosphatidylserine, or a combination thereof, compared to the source cell;

viii) the fusosome does not comprise, or is depleted for relative to the source cell, Phosphatidyl serine (PS) or CD40 ligand or both of PS and CD40 ligand, e.g., when measured in an assay of Example 52;

ix) the fusosome is not enriched (e.g., is depleted) for PS compared to the source cell, e.g., in a population of fusosomes less than 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% are positive for PS by an assay of Kanada M, et al. (2015) Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc Natl Acad Sci USA 112:E1433–E1442;

x) the fusosome comprises acetylcholinesterase (AChE), e.g. at least 0.001, 0.002, 0.005, 0.01,0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, or 1000 AChE activity units/μg of protein , e.g., by an assay of Example 67;

xi) the fusosome comprises a Tetraspanin family protein (e.g., CD63, CD9, or CD81), an ESCRT-related protein (e.g., TSG101, CHMP4A-B, or VPS4B), Alix, TSG101, MHCI, MHCII, GP96, actinin-4, mitofilin, syntenin-1, TSG101, ADAM10, EHD4, syntenin-1, TSG101, EHD1, flotillin-1, heat-shock 70-kDa proteins (HSC70/HSP73, HSP70/HSP72), or any combination thereof, e.g., contains more than 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 5%, or 10% of any individual exosomal marker protein and/or less than 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% of total exosomal marker proteins of any of said proteins, or is enriched for any one or more of these proteins compared to the source cell, e.g., by an assay of Example 44;

xii) the fusosome comprises a level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

that is above 500, 250, 100, 50, 20, 10, 5, or 1 ng GAPDH/μg total protein or below the level of GAPDH in the source cell, e.g., at least 1%, 2.5%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, greater than the level of GAPDH per total protein in ng/μg in the source cell, e.g., using an assay of Example 45;

xiii) the fusosome is not enriched for (e.g., is depleted for) one or more endoplasmic reticulum proteins (e.g., calnexin), one or more proteasome proteins, or one or more mitochondrial proteins, or any combination thereof, e.g., wherein the amount of calnexin is less than 500, 250, 100, 50, 20, 10, 5, or 1 ng Calnexin / μg total protein, or wherein the fusosome comprises less Calnexin per total protein in ng/μg compared to the source cell by 1%, 2.5%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., using an assay of Example 46; or

xiv) the fusosome can not be immobilized on a mica surface by atomic force microscopy for at least 30 min, e.g., by an assay of Kanada M, et al. (2015) Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc Natl Acad Sci USA 112:E1433–E1442.

[000140] In embodiments, one or more of:

i) the fusosome does not comprise a VLP;

ii) the fusosome does not comprise a virus;

iii) the fusosome does not comprise a replication-competent virus;

iv) the fusosome does not comprise a viral protein, e.g., a viral structural protein, e.g., a capsid protein or a viral matrix protein;

v) the fusosome does not comprise a capsid protein from an enveloped virus;

vi) the fusosome does not comprise a nucleocapsid protein; or

vii) the fusogen is not a viral fusogen.

[000141] In embodiments, the fusosome comprises cytosol.

[000142] In embodiments, the fusosome comprises or is comprised by a cytobiologic.

[000143] In embodiments, the fusosome comprises or is comprised by an enucleated cell.

[000144] In embodiments, the fusosome is or comprises a chondrisome.

[000145] In embodiments, one or more of:

i) the fusosome or the source cell does not form a teratoma when implanted into subject, e.g., by an assay of Example 102;

ii) the fusosome and/or compositions or preparations thereof, are capable of chemotaxis, e.g., at a speed at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% compared to a reference cell, e.g., a macrophage, e.g., using an assay of Example 58;

iii) the fusosome and/or compositions or preparations thereof, are capable of homing, e.g., at the site of an injury, wherein the cytobiologic is from a human cell, e.g., using an assay of Example 59, e.g., wherein the source cell is a neutrophil; or

iv) the fusosome and/or compositions or preparations thereof, are capable of phagocytosis, e.g., wherein phagocytosis by the fusosome is detectable within .5, 1, 2, 3, 4, 5, or 6 hours in using an assay of Example 60, e.g., wherein the source cell is a macrophage.

[000146] In embodiments, the fusosome or fusosome composition retains one, two, three, four, five six or more of any of the characteristics for 5 days or less, e.g., 4 days or less, 3 days or less, 2 days or less, 1 day or less, e.g., about 12-72 hours, after administration into a subject, e.g., a human subject.

[000147] In embodiments, the fusosome has one or more of the following characteristics:

a) comprises one or more endogenous proteins from a source cell, e.g., membrane proteins or cytosolic proteins;

b) comprises at least 10, 20, 50, 100, 200, 500, 1000, 2000, or 5000 different proteins;

c) comprises at least 1, 2, 5, 10, 20, 50, or 100 different glycoproteins;

d) at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% by mass of the proteins in the fusosome are naturally-occurring proteins;

e) comprises at least 10, 20, 50, 100, 200, 500, 1000, 2000, or 5000 different RNAs; or

f) comprises at least 2, 3, 4, 5, 10, or 20 different lipids, e.g., selected from CL, Cer, DAG, HexCer, LPA, LPC, LPE, LPG, LPI, LPS, PA, PC, PE, PG, PI, PS, CE, SM and TAG.

[000148] In embodiments, the fusosome has been manipulated to have, or the fusosome is not a naturally occurring cell and has, or wherein the nucleus does not naturally have one, two, three, four, five or more of the following properties:

a) the partial nuclear inactivation results in a reduction of at least 50%, 60%, 70%, 80%, 90% or more in nuclear function, e.g., a reduction in transcription or DNA replication, or both, e.g., wherein transcription is measured by an assay of Example 19 and DNA replication is measured by an assay of Example 20;

b) the fusosome is not capable of transcription or has transcriptional activity of less than 1%, 2.5% 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of that of the transcriptional activity of a reference cell, e.g., the source cell, e.g., using an assay of Example 19;

c) the fusosome is not capable of nuclear DNA replication or has nuclear DNA replication of less than 1%, 2.5% 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the nuclear DNA replication of a reference cell, e.g., the source cell, e.g., using an assay of Example 20;

d) the fusosome lacks chromatin or has a chromatin content of less than 1%, 2.5% 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the of the chromatin content of a reference cell, e.g., the source cell, e.g., using an assay of Example 37;

e) the fusosome lacks a nuclear membrane or has less than 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% the amount of nuclear membrane of a reference cell, e.g., the source cell or a Jurkat cell, e.g., by an assay of Example 36;

f) the fusosome lacks functional nuclear pore complexes or has reduced nuclear import or export activity, e.g., by at least 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% by an assay of Example 36, or the fusosome lacks on or more of a nuclear pore protein, e.g., NUP98 or Importin 7.

g) the fusosome does not comprise histones or has histone levels less than 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the histone level of the source cell (e.g., of H1, H2a, H2b, H3, or H4), e.g., by an assay of Example 37;

h) the fusosome comprises less than 20, 10, 5, 4, 3, 2, or 1 chromosome;

i) nuclear function is eliminated;

j) the fusosome is an enucleated mammalian cell;

k) the nucleus is removed or inactivated, e.g., extruded by mechanical force, by radiation or by chemical ablation; or

l) the fusosome is from a mammalian cell having DNA that is completely or partially removed, e.g., during interphase or mitosis.

[000149] In embodiments, the fusosome comprises mtDNA or vector DNA. In embodiments, the fusosome does not comprise DNA, or is substantially free of DNA. In some embodiments, the fusosome does not comprise a functional nucleus. In some embodiments, the fusosome does not comprise a nucleus. In some embodiments, the fusosome is substantially free of nuclear DNA.

[000150] In embodiments, the fusosome is substantially free of one or more of the following organelles: a mitochondrion, Golgi apparatus, lysosome, endoplasmic reticulum, vacuole, endosome, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, cnidocyst, peroxisome, proteasome, vesicle, and stress granules. In embodiments, the fusosome has a lower number of an organelle as compared to the source cell, where the organelle is selected from: a mitochondrion, Golgi apparatus, lysosome, endoplasmic reticulum, vacuole, endosome, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, cnidocyst, peroxisome, proteasome, vesicle, and stress granule.

[000151] In embodiments, the source cell is a primary cell, immortalized cell or a cell line (e.g., myelobast cell line, e.g., C2C12). In embodiments, the fusosome is from a source cell having a modified genome, e.g., having reduced immunogenicity (e.g., by genome editing, e.g., to remove an MHC protein, e.g., MHC complex). In embodiments, the source cell is from a cell culture treated with an immunosuppressive agent. In embodiments, the source cell is substantially non-immunogenic, e.g., using an assay described herein. In embodiments, the source cell comprises an exogenous agent, e.g., a therapeutic agent. In embodiments, the source cell is a recombinant cell.

[000152] In some embodiments, the source cell is from a cell culture treated with an anti-

inflammatory signal. In some embodiments, a method of making described herein further comprises contacting the source cell with an anti-inflammatory signal, e.g., before or after inactivating the nucleus, e.g., enucleating the cell.

[000153] In embodiments, the fusosome further comprises an agent that is exogenous relative to the source cell, e.g., a therapeutic membrane protein payload agent, e.g., a protein or a nucleic acid (e.g., a DNA, a chromosome (e.g. a human artificial chromosome), an RNA, e.g., an mRNA or miRNA). In embodiments, the exogenous agent is present at at least, or no more than, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In embodiments, the fusosome has an altered, e.g., increased or decreased level of one or more endogenous molecule, e.g., protein or nucleic acid (e.g., in some embodiments, endogenous relative to the source cell, and in some embodiments, endogenous relative to the target cell), e.g., due to treatment of the source cell, e.g., mammalian source cell with a siRNA or gene editing enzyme. In embodiments, the endogenous molecule is present at at least, or no more than, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000, 100,000,000, 500,000,000, or 1,000,000,000 copies. In embodiments, the endogenous molecule (e.g., an RNA or protein) is present at a concentration of at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, 1.0 x 10^7, 5.0 x 10^7, or 1.0 x 10^8, greater than its concentration in the source cell. In embodiments, the endogenous molecule (e.g., an RNA or protein) is present at a concentration of at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, 1.0 x 10^7, 5.0 x 10^7, or 1.0 x 10^8 less than its concentration in the source cell.

[000154] In embodiments, the fusogen is a viral fusogen, e.g., HA, HIV-1 ENV, gp120, or VSV-G. In embodiments, the fusogen is a mammalian fusogen, e.g., a SNARE, a Syncytin, myomaker, myomixer, myomerger, or FGFRL1. In embodiments, the fusogen is active at a pH of 4-5, 5-6, 6-7, 7-8, 8-9, or 9-10. In embodiments, the fusogen is active at a pH of 6-8. In embodiments, the fusogen is not active at a pH of 4-5, 5-6, 6-7, 7-8, 8-9, or 9-10. In embodiments, the fusosome fuses to a target cell at the surface of the target cell. In embodiments, the fusogen promotes fusion in a lysosome-independent manner. In embodiments, the fusogen is a protein

fusogen. In embodiments, the fusogen is a lipid fusogen, e.g., oleic acid, glycerol mono-oleate, a glyceride, diacylglycerol, or a modified unsaturated fatty acid. In embodiments, the fusogen is a chemical fusogen, e.g., PEG. In embodiments, the fusogen is a small molecule fusogen, e.g., halothane, an NSAID such as meloxicam, piroxicam, tenoxicam, and chlorpromazine. In embodiments, the fusogen is recombinant. In embodiments, the fusogen is biochemically incorporated, e.g., the fusogen is provided as a purified protein and contacted with a lipid bilayer under conditions that allow for association of the fusogen with the lipid bilayer. In embodiments, the fusogen is biosynthetically incorporated, e.g. expressed in a source cell under conditions that allow the fusogen to associate with the lipid bilayer.

[000155] In embodiments, the fusosome binds a target cell. In embodiments, the target cell is other than a HeLa cell, or the target cell is not transformed or immortalized. For instance, in some embodiments a cell that is not transformed displays contact inhibition and/or its growth is dependent on the same survival factors or growth factors as a normal cell of the same type. In some embodiments, the target cell is transformed or immortalized.

[000156] In some embodiments involving fusosome compositions or preparations, the plurality of fusosomes are the same. In some embodiments, the plurality of fusosomes are different. In some embodiments the plurality of fusosomes are from one, two or more types of source cells. In some embodiments, the plurality of fusosomes are the same if at least 0.01%-0.05%, 0.05%-0.1%, 0.1%-0.5%, 0.5%- 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the fusosomes in the fusosome composition share at least one property selected from: comprise the same fusogen; produced using the same type of source cell; or comprise the same membrane protein payload agent. In some embodiments at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of fusosomes in the plurality have a diameter within 10%, 20%, 30%, 40%, or 50% of the mean diameter of the fusosomes in the fusosome composition or preparation. In some embodiments, at least 50% of fusosomes in the plurality have a diameter within 10%, 20%, 30%, 40%, or 50% of the mean diameter of the fusosomes in the fusosome composition. In some embodiments, the plurality of fusosomes has a mean diameter of at least about 50 nm, about 80 nm, about 100 nm, about 200 nm, about 500 nm, about 1000 nm, about 1200 nm, about 1400 nm, or about 1500 nm.

In some embodiments, the plurality of fusosomes comprises fusosomes having a diameter within the range of about 10 nm to about 100 μm. In some embodiments, the plurality comprises fusosomes having a size within the range of about 20 nm to about 200 nm, about 50 nm to about 200 nm, about 50 nm to about 100 nm, about 50 nm to about 150 nm, or about 100 nm to about 150 nm. In some embodiments at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of fusosomes in the plurality have a volume within 10%, 20%, 30%, 40%, or 50% of the mean volume of the fusosomes in the fusosome composition or preparation. In some embodiments, at least 50% of fusosomes in the plurality have a volume within 10%, 20%, 30%, 40%, or 50% of the mean volume of the fusosomes in the fusosome composition. In some embodiments, the plurality comprises fusosomes having a volume within the range of about 500 nm^3 to about 0.0006 mm^3, or about 4,000 nm^3 to about 0.005 μm^3, about 65,000 nm^3 to about 0.005 μm^3, about 65,000 nm^3 to about 0.0006 μm^3, about 65,000 nm^3 to about 0.002 μm^3, or about 0.0006 μm^3 to about 0.002 μm^3. In some embodiments, the fusosome composition or preparation has less than about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, variability in diameter distribution within 10%, 50%, or 90% of the source cell population variability in diameter distribution, e.g., based on Example 31. In some embodiments, at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of fusosomes in the plurality have a copy number of the fusogen within 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the mean fusogen copy number in the fusosomes in the fusosome composition or preparation. In some embodiments, at least 50% of fusosomes in the plurality have a copy number of the fusogen within 10%, 20%, 30%, 40%, or 50% of the mean fusogen copy number in the fusosomes in the fusosome composition. In some embodiments, at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of fusosomes in the plurality have a copy number of the therapeutic agent within 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the mean therapeutic agent copy number in the fusosomes in the fusosome composition or preparation. In some embodiments, at least 50% of fusosomes in the plurality have a copy number of the membrane protein payload within 10%, 20%, 30%, 40%, or 50% of the mean protein membrane payload copy number in the fusosomes in the fusosome composition. In some embodiments, the fusosome composition or preparation comprises at least 10^5, 10^6, 10^7, 10^8, 10^9, or 10^{10} or fusosomes. In some embodiments, the fusosome composition or preparation is in a volume of at least 1 μL, 2 μL, 5 μL, 10 μL, 20 μL, 50 μL, 100 μL, 200 μL, 500 μL, 1 mL, 2 mL, 5 mL, or 10 mL.

[000157] In some embodiments, the plurality of fusosomes comprises at least 0.01%-0.05%, 0.05%-0.1%, 0.1%-0.5%, 0.5%- 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% fusosomes which have one or more of the following characteristics:

(i) do not comprise a nucleus or a functional nucleus;

(ii) are substantially free of nuclear DNA; or

(iii) do not comprise mitochondria or functional mitochondria.

[000158] In embodiments, a pharmaceutical composition described herein has one or more of the following characteristics:

a) the pharmaceutical composition meets a pharmaceutical or good manufacturing practices (GMP) standard;
b) the pharmaceutical composition was made according to good manufacturing practices (GMP);
c) the pharmaceutical composition has a pathogen level below a predetermined reference value, e.g., is substantially free of pathogens;
d) the pharmaceutical composition has a contaminant level (e.g., nuclear DNA) below a predetermined reference value, e.g., is substantially free of contaminants; or
e) the pharmaceutical composition has low immunogenicity, e.g., as described herein.

[000159] In embodiments, the biological function is selected from:

a) modulating, e.g., inhibiting or stimulating, an enzyme;
b) modulating, e.g., increasing or decreasing levels of, a molecule (e.g., a protein, nucleic acid, or metabolite, drug, or toxin) in the subject, e.g., by inhibiting or stimulating synthesis or by inhibiting or stimulating degradation of the factor;
c) modulating, e.g., increasing or decreasing, viability of a target cell or tissue; or
d) modulating a protein state, e.g., increasing or decreasing phosphorylation of the protein, or modulating the protein conformation;
e) promoting healing of an injury;
f) modulating, e.g., increasing or decreasing, an interaction between two cells;
g) modulating, e.g., promoting or inhibiting, cell differentiation;

h) altering distribution of a factor (e.g., a protein, nucleic acid, metabolite, drug, or toxin) in the subject;

i) modulating, e.g. increasing or decreasing, an immune response; or

j) modulating, e.g. increasing or decreasing, recruitment of cells to a target tissue.

[000160] In some embodiments of the therapeutic methods herein, the plurality of fusosomes has a local effect. In some embodiments, the plurality of fusosomes has a distal effect. In some embodiments, the plurality of fusosomes has a systemic effect.

[000161] In some embodiments, the subject has a cancer, an inflammatory disorder, autoimmune disease, a chronic disease, inflammation, damaged organ function, an infectious disease, metabolic disease, degenerative disorder, genetic disease (e.g., a genetic deficiency or a dominant genetic disorder), or an injury. In some embodiments, the subject has an infectious disease and the fusosome comprises an antigen for the infectious disease. In some embodiments, the subject has a genetic deficiency and the fusosome comprises a protein for which the subject is deficient, or a nucleic acid (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, etc.) encoding the protein, or a DNA encoding the protein, or a chromosome encoding the protein, or a nucleus comprising a nucleic acid encoding the protein. In some embodiments, the subject has a dominant genetic disorder, and the fusosome comprises a nucleic acid inhibitor (e.g., siRNA or miRNA) of the dominant mutant allele. In some embodiments, the subject has a dominant genetic disorder, and the fusosome comprises a nucleic acid inhibitor (e.g., siRNA or miRNA) of the dominant mutant allele, and the fusosome also comprises an mRNA encoding a non-mutated allele of the mutated gene that is not targeted by the nucleic acid inhibitor. In some embodiments, the subject is in need of vaccination. In some embodiments, the subject is in need of regeneration, e.g., of an injured site.

[000162] In some embodiments, the fusosome comprises a nucleic acid which further comprises one or more sequences encoding one or more signal sequences, e.g., wherein a target cell translocates a protein comprising a signal sequence to the cell membrane of the target cell.

[000163] In some embodiments, the fusosome composition or preparation is administered to the subject at least 1, 2, 3, 4, or 5 times.

[000164] In some embodiments, the fusosome composition or preparation is administered to the subject systemically (e.g., orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally) or locally. In some embodiments, the fusosome composition or preparation is administered to the subject such that the fusosome composition or preparation reaches a target tissue selected from liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, or eye. In some embodiments (e.g., wherein the subject has an autoimmune disease), the fusosome composition or preparation is co-administered with an immunosuppressive agent, e.g., a glucocorticoid, cytostatic, antibody, or immunophilin modulator. In some embodiments (e.g., wherein the subject has a cancer or an infectious disease), the fusosome composition or preparation is co-administered with an immunostimulatory agent, e.g., an adjuvant, interleukin, cytokine, or chemokine. In some embodiments, administration of the fusosome composition or preparation results in upregulation or downregulation of a gene in a target cell in the subject, e.g., wherein the fusosome comprises a transcriptional activator or repressor, a translational activator or repressor, or an epigenetic activator or repressor.

[000165] In some embodiments of the methods of making herein, providing a source cell expressing a fusogen comprises expressing an exogenous fusogen in the source cell or upregulating expression of an endogenous fusogen in the source cell. In some embodiments, the method comprises inactivating the nucleus of the source cell.

[000166] In some embodiments, at least one fusosome of the plurality of fusosomes is derived from a source cell.

[000167] In some embodiments a fusosome is at a temperature of less than 4, 0, -4, -10, -12, -16, -20, -80, or -160 °C.

[000168] In embodiments, a fusosome preparation comprises at least about 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, or 10^{15} fusosomes. In embodiments, the fusosome preparation comprises a volume of at least 10 mL, 20 mL, 50 mL, 100 mL, 200 mL, 500 mL, 1 L, 2 L, 5 L, 10 L, 20 L, or 50 L. In embodiments, the method comprises enucleating the source cell, e.g., a mammalian cell, e.g., by chemical enucleation, use of mechanical force e.g., use of a filter or centrifuge, at least partial disruption of the cytoskeleton, or a combination thereof. In

embodiments, the method comprises expressing a fusogen or other membrane protein in the source cell. In embodiments, the method comprises one or more of: vesiculation, hypotonic treatment, extrusion, or centrifugation. In embodiments, the method comprises genetically expressing an exogenous agent in the source cell or loading the exogenous agent into the source cell or fusosome. In embodiments, the method comprises contacting the source cell with DNA encoding a polypeptide agent, e.g., before inactivating the nucleus, e.g., enucleating the cell. In embodiments, the method comprises contacting the source cell with RNA encoding a polypeptide agent, e.g., before or after inactivating the nucleus, e.g., enucleating the cell. In embodiments, the method comprises introducing a therapeutic agent (e.g., a nucleic acid or protein, e.g., a membrane protein payload agent) into a fusosome, e.g., by electroporation.

[000169] In embodiments, the fusosome is from a mammalian cell having a modified genome, e.g., to reduce immunogenicity (e.g., by genome editing, e.g., to remove an MHC protein). In embodiments, the method further comprises contacting the source cell of step a) with an immunosuppressive agent, e.g., before or after inactivating the nucleus, e.g., enucleating the cell.

[000170] In some embodiments, if a detectable level, e.g., a value above a reference value, is determined, a sample containing the plurality of fusosomes or fusosome composition or preparation is discarded.

[000171] In some embodiments, the first fusogen is not a lipopeptide.

[000172] In some embodiments, provided fusosomes, and/or compositions or preparations thereof, have partial or complete nuclear inactivation (e.g. nuclear removal).

[000173] In some embodiments, the source cell is a cell grown under adherent or suspension conditions. In some embodiments, the source cell is a primary cell, a cultured cell, an immortalized cell, or a cell line (e.g., myelobast cell line, e.g., C2C12). In some embodiments, the source cell is allogeneic, e.g., obtained from a different organism of the same species as the target cell. In some embodiments, the source cell is is autologous, e.g., obtained from the same organism as the target cell. In some embodiments, the source cell is heterologous, e.g., obtained from an organism of a different species from the target cell.

[000174] In some embodiments, the fusosome is not captured by the scavenger system in circulation or by Kupffer cells in the sinus of the liver. In some embodiments, the fusosome is not captured by the reticulo-endothelial system (RES) in a subject, e.g., by an assay of Example 76. In some embodiments, when a plurality of fusosomes are administered to a subject, less than 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, of the plurality are not captured by the RES after 24 hours, e.g., by an assay of Example 76. In some embodiments, when a plurality of fusosomes are administered to a subject less than 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, of the plurality are not captured by the RES after 24 hours, e.g., by an assay of Example 76.

[000175] In some embodiments, the fusosome comprises a viral structural protein and/or a viral matrix protein.

[000176] In some embodiments, the fusosome is substantially free of, or has a lower number of one or more of the following organelles: a mitochondrion, Golgi apparatus, lysosome, endoplasmic reticulum, vacuole, endosome, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, cnidocyst, peroxisome, proteasome, vesicle, and stress granule, e.g., as compared to the source cell.

[000177] In some embodiments, the fusosome does not comprise Cre or GFP, e.g., EGFP.

[000178] In some embodiments, the fusosome composition or pharmaceutical composition has been maintained at a predetermined temperature for at least 1, 2, 3, 6, or 12 hours; 1, 2, 3, 4, 5, or 6 days; 1, 2, 3, or 4 weeks; 1, 2, 3, or 6 months; or 1, 2, 3, 4, or 5 years. In some embodiments, the predetermined temperature is selected from about 4, 0, -4, -10, -12, -16, -20, -80, or -160 °C.

[000179] In some embodiments, the fusosome composition or pharmaceutical composition, has an activity of at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the activity of the plurality before maintenance at said temperature, e.g., by one or more of:

i) the fusosome fuses at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 10%, e.g., in an assay of Example 54;

ii) the fusosome fuses at a higher rate with a target cell than with other fusosomes, e.g., by at least 50%, e.g., in an assay of Example 54;

iii) the fusosome fuses with target cells at a rate such that an agent in the fusosome is delivered to at least 10% of target cells after 24 hours, e.g., in an assay of Example 54; or

iv) the fusogen is present at a copy number of at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the fusogen copy number of the plurality before maintenance at said temperature, e.g., as measured by an assay of Example 29.

[000180] In some embodiments, the fusosome composition or pharmaceutical composition is considered stable if it has an activity of at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the activity of the plurality before storage at said temperature for said time period, e.g., by one or more of:

i) the fusosome fuses at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 10%, e.g., in an assay of Example 54;

ii) the fusosome fuses at a higher rate with a target cell than with other fusosomes, e.g., by at least 50%, e.g., in an assay of Example 54;

iii) the fusosome fuses with target cells at a rate such that an agent in the fusosome is delivered to at least 10% of target cells after 24 hours, e.g., in an assay of Example 54; or

iv) the fusogen is present at a copy number of at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the fusogen copy number of the plurality before maintenance at said temperature, e.g., as measured by an assay of Example 29.

[000181] In some embodiments, the disease or disorder is selected from cancer, autoimmune disorder, or infectious disease. In some embodiments, the subject has a cancer. In some embodiments, fusosome comprises a neoantigen. In some embodiments, the fusosome composition is administered to the subject at least 1, 2, 3, 4, or 5 times. In some embodiments, the fusosome composition is administered to the subject systemically (e.g., orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally) or locally. In some embodiments, wherein the fusosome composition is administered to the subject such that the fusosome composition reaches a target tissue selected from liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, or eye. In some embodiments,

the fusosome composition is co-administered with an immunosuppressive agent, e.g., a glucocorticoid, cytostatic, antibody, or immunophilin modulator. In some embodiments, the fusosome composition is co-administered with an immunostimulatory agent, e.g., an adjuvant, interleukin, cytokine, or chemokine.

[000182] In some embodiments, the plurality of fusosomes has a local, distal, or systemic effect.

[000183] In some embodiments, any of the methods disclosed herein, further comprises a step of monitoring one or more of cancer progression, tumor recession, tumor volume, decrease in neoplastic cell number, quantity of fused cells, quantity of fused cells comprising a membrane protein payload agent, quantity of fused cells expressing a nucleic acid protein payload, and quantity of membrane protein disposed in membrane of a fused cell.

[000184] In some embodiments, any of the methods disclosed herein, further comprises a step of monitoring adverse events in the organism. In some embodiments, the adverse event includes one or more of cytokine release syndrome, fever, tachycardia, chills, anorexia, nausea, vomiting, myalgia, headaches, capillary leak syndrome, hypotension, pulmonary edema, coagulopathy, renal dysfunction, kidney injury, macrophage-activation syndrome, hemophagocytic lymphohistiocytosis, organ failure, cerebral edema, bystander inflammation from T cell activation, neurologic symptoms, encephalopathy, confusion, hallucination, delirium, obtundation, aphasia, seizures, B-cell aplasia, tumor lysis syndrome, and graft versus host disease.

[000185] In some embodiments, the organism is a human. In some embodiments, the human has a disease, disorder, or condition. In some embodiments, presence of the membrane protein payload agent in the cell membrane lipid bilayer of the target cell improves one or more symptoms of the disease, disorder, or condition.

[000186] Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

[000187] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications, patent applications, patents, and other references mentioned herein are

incorporated by reference in their entirety. For example, all GenBank, Unigene, and Entrez sequences referred to herein, e.g., in any Table herein, are incorporated by reference. Unless otherwise specified, the sequence accession numbers specified herein, including in any Table herein, refer to the database entries current as of February 17, 2018. When one gene or protein references a plurality of sequence accession numbers, all of the sequence variants are encompassed. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

[000188] The following detailed description of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings described herein certain embodiments, which are presently exemplified. It should be understood, however, that the invention is not limited to the precise arrangement and instrumentalities of the embodiments shown in the drawings.

[000189] FIG. 1 quantifies staining of fusosomes with a dye for endoplasmic reticulum.

[000190] FIG. 2 quantifies staining of fusosomes with a dye for mitochondria.

[000191] FIG. 3 quantifies staining of fusosomes with a dye for lysosomes.

[000192] FIG. 4 quantifies staining of fusosomes with a dye for F-actin.

[000193] FIG. 5 is a graph showing recovery of GFP fluorescence after photobleaching of cells contacted with fusogens expressing Cre and GFP.

[000194] FIG. 6 is a graph showing the percentage of target cells expressing RFP after contacting with fusosomes or negative controls.

[000195] FIG. 7 is an image of a positive organelle delivery via fusion between donor and recipient HeLa cells. The intracellular areas indicated in white indicate overlap between donor and recipient mitochondria. The intracellular regions in grey indicate where donor and recipient organelles do not overlap.

[000196] FIG. 8 is an image of a positive organelle delivery via fusion between donor and recipient HeLa cells. The intracellular areas indicated in white indicate overlap between donor

and recipient mitochondria. The intracellular regions in grey indicate where donor and recipient organelles do not overlap.

[000197] FIG. 9 shows microscopy images of the indicated tissues from mice injected with fusosomes. White indicates represent RFP-fluorescent cells, indicating delivery of a protein cargo to the cells in vivo.

[000198] FIG. 10 is a series of images showing successful delivery of fusosomes to murine tissues in vivo by the indicated routes of administration, resulting in expression of luciferase by targeted cells.

[000199] FIG. 11 shows microscopy images of tdTomato fluorescence in murine muscle tissue, indicating delivery of a protein cargo to muscle cells by cytobiologics.

DETAILED DESCRIPTION

[000200] The invention describes fusosomes that include a membrane protein payload agent, and related methods.

Definitions

[000201] ***Agent:*** In general, the term "agent", as used herein, may be used to refer to a compound or entity including, for example, a peptide, a polypeptide, a nucleic acid (e.g., DNA, a chromosome (e.g. a human artificial chromosome), RNA, mRNA, siRNA, miRNA), a saccharide or a polysaccharide, a lipid, a small molecule, or a combination or complex thereof. The term may refer to an entity that is or comprises an organelle, or a fraction, extract, or component thereof.

[000202] ***Antibody:*** As used herein, the term "antibody" refers to a polypeptide that includes canonical immunoglobulin sequence elements sufficient to confer specific binding to a particular target antigen. For purposes of the present invention, in certain embodiments, any polypeptide or complex of polypeptides that includes sufficient immunoglobulin domain sequences to confer specific binding to an antigen can be referred to and/or used as an "antibody", whether such polypeptide is naturally produced (e.g., generated by an organism reacting to an antigen), or produced by recombinant engineering, chemical synthesis, or other artificial system or methodology. In some embodiments, an antibody is polyclonal; in some embodiments, an antibody is monoclonal. In some embodiments, an antibody has constant region sequences that

are characteristic of mouse, rabbit, primate, or human antibodies. In some embodiments, antibody sequence elements are humanized, primatized, chimeric, etc. In embodiments, an antibody utilized in accordance with the present invention is in a format selected from, but not limited to, intact IgA, IgG, IgE or IgM antibodies; bi- or multi- specific antibodies (e.g., Zybodies®, etc); antibody fragments such as Fab fragments, Fab' fragments, F(ab')2 fragments, Fd' fragments, Fd fragments, and isolated CDRs or sets thereof; single chain Fvs; polypeptide-Fc fusions; single domain antibodies (e.g., shark single domain antibodies such as IgNAR or fragments thereof); cameloid antibodies; masked antibodies (e.g., Probodies®); Small Modular ImmunoPharmaceuticals ("SMIPs™"); single chain or Tandem diabodies (TandAb®); VHHs; Anticalins®; Nanobodies®; minibodies; BiTE®s; ankyrin repeat proteins or DARPINs®; Avimers®; DARTs; TCR-like antibodies;, Adnectins®; Affilins®; Trans-bodies®; Affibodies®; TrimerX®; MicroProteins; Fynomers®, Centyrins®; and KALBITOR®s. In some embodiments, an antibody may lack a covalent modification (e.g., attachment of a glycan) that it would have if produced naturally. In some embodiments, an antibody may contain a covalent modification (e.g., attachment of a glycan, a payload [e.g., a detectable moiety, a therapeutic moiety, a catalytic moiety, etc.], or other pendant group [e.g., poly-ethylene glycol, etc.]. In some embodiments, an antibody of any of the above-described formats comprises one or more complement determining regions, e.g., CDR1, CD2, and/or CDR3.

[000203] ***Antigen binding domain:*** The term "antigen binding domain" as used herein refers to that portion of antibody or a chimeric antigen receptor which binds an antigen. In some embodiments, an antigen binding domain binds to a cell surface antigen of a cell. In some embodiments an antigen binding domain binds an antigen characteristic of a cancer, e.g., a tumor associated antigen in a neoplastic cell. In some embodiments, an antigen binding domain binds an antigen characteristic of an infectious disease, e.g. a virus associated antigen in a virus infected cell. In some embodiments, an antigen binding domain binds an antigen characteristic of a cell targeted by a subject's immune system in an autoimmune disease, e.g., a self-antigen. In some embodiments, an antigen binding domain is or comprises an antibody or antigen-binding portion thereof. In some embodiments, an antigen binding domain is or comprises an scFv or Fab.

[000204] ***Associated with:*** In some embodiments, two or more entities are physically

"associated" with one another if they interact, directly or indirectly, so that they are and/or remain in physical proximity with one another. In some embodiments, two or more entities that are physically associated with one another are covalently linked to one another; in some embodiments, two or more entities that are physically associated with one another are not covalently linked to one another but are non-covalently associated, for example by means of hydrogen bonds, van der Waals interaction, hydrophobic interactions, magnetism, and combinations thereof.

[000205] ***Cancer***: The terms "cancer", "malignancy", "neoplasm", "tumor", and "carcinoma", are used herein to refer to cells that exhibit relatively abnormal, uncontrolled, and/or autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. In some embodiments, a tumor may be or comprise cells that are precancerous (e.g., benign), malignant, pre-metastatic, metastatic, and/or non-metastatic. The present disclosure specifically identifies certain cancers to which its teachings may be particularly relevant. In some embodiments, a relevant cancer may be characterized by a solid tumor. In some embodiments, a tumor may be a disperse tumor or a liquid tumor. In some embodiments, a relevant cancer may be characterized by a hematologic tumor. In general, examples of different types of cancers known in the art include, for example, leukemias, lymphomas (Hodgkin's and non-Hodgkin's), myelomas and myeloproliferative disorders; sarcomas, melanomas, adenomas, carcinomas of solid tissue, squamous cell carcinomas of the mouth, throat, larynx, and lung, liver cancer, genitourinary cancers such as prostate, cervical, bladder, uterine, and endometrial cancer and renal cell carcinomas, bone cancer, pancreatic cancer, skin cancer, cutaneous or intraocular melanoma, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, head and neck cancers, breast cancer, gastro-intestinal cancers and nervous system cancers, benign lesions such as papillomas, and the like.

[000206] **Cargo**: As used herein, "cargo" or "payload" comprises an agent which may be delivered by a fusosome to a target cell. In some embodiments a cargo comprises one or more of a therapeutic agent, e.g., a therapeutic agent that is endogenous or exogenous to the source cell. In some embodiments, the therapeutic agent is chosen from one or more of a protein, e.g., an enzyme, a transmembrane protein, a receptor, an antibody; a nucleic acid, e.g., DNA, a chromosome (e.g. a human artificial chromosome), RNA, mRNA, siRNA, miRNA, or a small

molecule. In some embodiments, a cargo is or comprises a membrane protein payload agent. In some embodiments, a cargo is or comprises an organelle.

[000207] ***CDR:*** As used herein, "CDR" refers to a complementarity determining region, e.g., which can be situated within an antibody variable region. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the variable regions. A "*set of CDRs*" or "*CDR set*" refers to a group of three or six CDRs that occur in either a single variable region capable of binding the antigen or the CDRs of cognate heavy and light chain variable regions capable of binding the antigen. Certain systems have been established in the art for defining CDR boundaries (e.g., Kabat, Chothia, etc.); those skilled in the art appreciate the differences between and among these systems and are capable of understanding CDR boundaries to the extent required to understand and to practice the claimed invention.

[000208] ***Cell Membrane***: As used herein, a "cell membrane" refers to a membrane derived from a cell, e.g., a source cell or a target cell.

[000209] ***Cytobiologic***: As used herein, "cytobiologic" refers to a portion of a cell that comprises a lumen and a cell membrane, or a cell having partial or complete nuclear inactivation. In some embodiments, the cytobiologic comprises one or more of a cytoskeleton component, an organelle, and a ribosome. In embodiments, the cytobiologic is an enucleated cell, a microvesicle, or a cell ghost.

[000210] ***Cytosol:*** As used herein, "cytosol" refers to the aqueous component of the cytoplasm of a cell. The cytosol may comprise proteins, RNA, metabolites, and ions.

[000211] ***Endogenous*:** As used herein, the term "endogenous" refers to an agent, e.g., a protein or lipid that is naturally found in a relevant system (e.g., cell, tissue, organism, source cell, or target cell, etc). For example, in some embodiments, a fusosome or a membrane-enclosed preparation may be said to contain one or more "endogenous" lipids and/or proteins when the relevant lipids and/or proteins are naturally found in a source cell from which the fusosome or membrane-enclosed preparation is obtained or derived (e.g., the source cell of the fusosome or membrane-enclosed preparation). In some embodiments, an endogenous agent is overexpressed in a source cell.

[000212] ***Exogenous***: As used herein, the term "exogenous" refers to an agent (e.g., a protein or lipid) that is not naturally found in a relevant system (e.g., a cell, a tissue, an organism, a source cell or a target cell, etc.). In embodiments, the agent is engineered and/or introduced into the relevant system, For example, in some embodiments, a fusosome or a membrane-enclosed preparation may be said to contain one or more "exogenous" lipids and/or proteins when the relevant lipids and/or proteins are not naturally found in a source cell from which the fusosome or membrane-enclosed preparation is obtained or derived (e.g., the source cell of the fusosome or membrane-enclosed. In some embodiments, an exogenous agent is a variant of an endogenous agent, such as, for example, a protein variant that differs in one or more structural aspects such as amino acid sequence, post-translational modification, etc from a reference endogenous protein, etc).

[000213] ***Functional variant:*** The term "functional variant" refers to a polypeptide that has a substantially identical amino acid sequence to a reference amino acid sequence, or is encoded by a substantially identical nucleotide sequence, and is capable of having one or more activities of the reference amino acid sequence.

[000214] ***Fused Cell***: As used herein, a "fused cell" refers to a cell produced by the contacting of one or more fusosomes with a target cell. In some embodiments of the fused cell, at least a portion of the lipid bilayer of one or more fusosomes is associated with a membrane of the target cell.

[000215] ***Fusogen:*** As used herein, "fusogen" refers to an agent or molecule that creates an interaction between two membrane enclosed lumens. In embodiments, the fusogen facilitates fusion of the membranes. In other embodiments, the fusogen creates a connection, e.g., a pore, between two lumens (e.g., the lumen of the fusosome and a cytoplasm of a target cell). In some embodiments, the fusogen comprises a complex of two or more proteins, e.g., wherein neither protein has fusogenic activity alone.

[000216] ***Fusogen binding partner***: As used herein, "fusogen binding partner" refers to an agent or molecule that interacts with a fusogen to facilitate fusion between two membranes. In some embodiments, a fusogen binding partner may be or comprise a surface feature of a cell.

[000217] ***Fusosome Composition:*** As used herein, "fusosome composition" refers to a

composition comprising one or more fusosomes.

[000218] ***Membrane protein payload agent***: As used herein, "membrane protein payload agent" refers to a cargo that is or comprises a membrane protein and/or a nucleic acid encoding a membrane protein, which cargo may be included in a fusosome or membrane-enclosed preparation as described herein (e.g., for delivery to a target cell). A membrane protein is a protein which associates with (e.g., is localized in and/or on) or is capable of associating with a cell membrane. In some embodiments a membrane protein is a transmembrane protein. In some embodiments, a membrane protein comprises a domain that at least partially (e.g., completely) spans a membrane, e.g., cell membrane. In some embodiments, a membrane protein is associated with an interior (e.g., cytosolic) portion of a membrane lipid bilayer. In some embodiments a membrane protein is associated with an exterior portion of a membrane lipid bilayer (e.g., with a cell surface or with a surface of a fusosome or a membrane-enclosed preparation as described herein). In some embodiments, a membrane protein is associated with an exterior portion of a membrane lipid bilayer is a cell surface protein. In some embodiments a membrane protein passes through a membrane lipid bilayer and is secreted. In some embodiments a membrane protein is a naturally occurring protein. In some embodiments a membrane protein is an engineered and/or synthetic protein (e.g., a chimeric antigen receptor). In some embodiments a membrane protein is a therapeutic agent.

[000219] ***Pharmaceutical composition:*** As used herein, the term "pharmaceutical composition" refers to an active agent, formulated together with one or more pharmaceutically acceptable carriers. In some embodiments, active agent is present in unit dose amount appropriate for administration in a therapeutic regimen to a relevant subject. In some embodiments, pharmaceutical compositions may be specially formulated for parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation.

[000220] ***Pharmaceutically acceptable carrier:*** As used herein, the term "pharmaceutically acceptable carrier" means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, or excipient.

[000221] ***Purified:*** As used herein, the term "purified" means altered or removed from the

natural state. For example, a cell or cell fragment naturally present in a living animal is not "purified," but the same cell or cell fragment partially or completely separated from the coexisting materials of its natural state is "purified." A purified fusosome composition can exist in substantially pure form, or can exist in a non-native environment such as, for example, a culture medium such as a culture medium comprising cells.

[000222] ***Source cell:*** As used herein, a "source cell" refers to a cell from which a fusosome is derived, e.g., obtained. In some embodiments, derived includes obtaining a membrane enclosed preparation from a source cell and adding a fusogen.

[000223] ***Substantially identical:*** In the context of a nucleotide sequence, the term "substantially identical" is used herein to refer to a first nucleic acid sequence that contains a sufficient or minimum number of nucleotides that are identical to aligned nucleotides in a second nucleic acid sequence such that the first and second nucleotide sequences encode a polypeptide having common functional activity, or encode a common structural polypeptide domain or a common functional polypeptide activity, for example, nucleotide sequences having at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to a reference sequence, *e.g.*, a sequence provided herein. The compositions and methods herein encompass polypeptides and nucleic acids having the sequences specified, or sequences substantially identical or similar thereto, *e.g.*, sequences at least 85%, 90%, or 95% identical or higher to the sequence specified. In the context of an amino acid sequence, the term "substantially identical" is used herein to refer to a first amino acid sequence that contains a sufficient or minimum number of amino acid residues that are i) identical to, or ii) conservative substitutions of aligned amino acid residues in a second amino acid sequence such that the first and second amino acid sequences can have a common structural domain and/or common functional activity, for example, amino acid sequences that contain a common structural domain having at least about 85%, 90%. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to a reference sequence, *e.g.*, a sequence provided herein.

[000224] ***Target cell moiety:*** As used herein, the term "target cell moiety" is used to refer to a feature of a cell (e.g., a target cell) which may be used to specifically (relative to at least one other cell in the relevant system) target a fusosome to the cell. In some embodiments, a target cell moiety is a surface feature of a target cell. In some embodiments, a target cell moiety is or is a

portion of a protein associated with the cell membrane of a target cell. In some embodiments, a target cell moiety is, or is a portion of, a peptide or protein associated with the membrane of a target cell. In some embodiments, a target cell moiety is or is a portion of a lipid associated with the membrane of a target cell. In some embodiments, a target cell moiety is or is a portion of a saccharide associated with the membrane of a target cell.

[000225] ***Targeting domain***: As used herein, the term "targeting domain" is a feature of a fusosome which associates or interacts with a target cell moiety. In some embodiments, a targeting domain specifically (under conditions of exposure) associates or interacts with a target cell moiety. In some embodiments, a targeting domain specifically binds to a target cell moiety present on a target cell. In some embodiments, a targeting domain is or comprises a domain of a fusogen e.g., is covalently linked to a fusogen, e.g., is part of a fusogen polypeptide. In some embodiments, a targeting domain is is a separate entity from any fusogen, e.g., is not covalently linked to a fusogen, e.g., is not part of a fusogen polypeptide.

[000226] ***Stable:*** The term "stable," when applied to compositions herein, means that the compositions maintain one or more aspects of their physical structure and/or activity over a period of time under a designated set of conditions. In some embodiments, the designated conditions are under cold storage (e.g., at or below about 4 ℃, -20 ℃, or -80 ℃).

[000227] ***Target cell***: As used herein "target cell" refers to a cell which a fusosome fuses to.

[000228] ***TCR domain***: As used herein, a "TCR domain" refers to a portion of a T-cell receptor polypeptide, or a functional fragment or variant thereof, which can cause activate the TCR complex for at least some aspect of the T-cell signaling pathway. In some embodiments, activation of the TCR complex leads to one or more of T cell proliferation, activation, differentiation, cytokine secretion, or cytolytic activity.

[000229] ***Variant:*** The term "variant" refers to a polypeptide that has a substantially identical amino acid sequence to a reference amino acid sequence, or is encoded by a substantially identical nucleotide sequence. In some embodiments, the variant is a functional variant.

Fusosomes

[000230] The fusosome compositions and methods described herein comprise (a) a lipid bilayer, (b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer; (c) a fusogen that is exogenous or overexpressed relative to the source cell , e.g., wherein the fusogen is disposed in the lipid bilayer, and (d) a membrane protein payload agent. In embodiments, the fusosome is derived from a non-plant cell, e.g., a mammalian cell, or derivative thereof (e.g., a mitochondrion, a chondrisome, an organelle, a vesicle, or an enucleated cell), and comprises a fusogen, e.g., protein, lipid or chemical fusogen.

Encapsulation

[000231] In some embodiments of the compositions and methods described herein include fusosomes, e.g., naturally derived bilayers of amphipathic lipids with a fusogen. Fusosomes may comprise several different types of lipids, e.g., amphipathic lipids, such as phospholipids. Fusosomes may comprise a lipid bilayer as the outermost surface. Such compositions can surprisingly be used in the methods of the invention. In some instances, membranes may take the form of an autologous, allogeneic, xenogeneic or engineered cell such as is described in Ahmad et al. 2014 Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. EMBO Journal. 33(9):994-1010. In some embodiments, the compositions include engineered membranes such as described in, e.g. in Orive. et al. 2015. Cell encapsulation: technical and clinical advances. Trends in Pharmacology Sciences; 36 (8):537-46; and in Mishra. 2016. Handbook of Encapsulation and Controlled Release. CRC Press. In some embodiments, the compositions include naturally occurring membranes (McBride et al. 2012. A Vesicular Transport Pathway Shuttles Cargo from mitochondria to lysosomes. Current Biology 22:135-141).

[000232] In some embodiments, a composition described herein includes a naturally derived membrane, e.g., membrane vesicles prepared from cells or tissues. In some embodiments, a fusosome is a vesicle derived from MSCs or astrocytes.

[000233] In some embodiments, a fusosome is an exosome.

[000234] Exemplary exosomes and other membrane-enclosed bodies are described, e.g., in US2016137716, which is herein incorporated by reference in its entirety. In some embodiments,

the fusosome comprises a vesicle that is, for instance, obtainable from a cell, for instance a microvesicle, an exosome, an apoptotic body (from apoptotic cells), a microparticle (which may be derived from e.g. platelets), an ectosome (derivable from, e.g., neutrophiles and monocytes in serum), a prostatosome (obtainable from prostate cancer cells), a cardiosome (derivable from cardiac cells), and the like.

[000235] Exemplary exosomes and other membrane-enclosed bodies are also described in WO/2017/161010, WO/2016/077639, US20160168572, US20150290343, and US20070298118, each of which is incorporated by reference herein in its entirety. In some embodiments, the fusosome comprises an extracellular vesicle, nanovesicle, or exosome. In some embodiments a fusosome comprises an extracellular vesicle, e.g., a cell-derived vesicle comprising a membrane that encloses an internal space and has a smaller diameter than the cell from which it is derived. In embodiments the extracellular vesicle has a diameter from 20nm to 1000 nm. In embodiments the fusosome comprises an apoptotic body, a fragment of a cell, a vesicle derived from a cell by direct or indirect manipulation, a vesiculated organelle, and a vesicle produced by a living cell (e.g., by direct plasma membrane budding or fusion of the late endosome with the plasma membrane). In embodiments the extracellular vesicle is derived from a living or dead organism, explanted tissues or organs, or cultured cells. In embodiments, the fusosome comprises a nanovesicle, e.g., a cell-derived small (e.g., between 20-250 nm in diameter, or 30-150 nm in diameter) vesicle comprising a membrane that encloses an internal space, and which is generated from said cell by direct or indirect manipulation. The production of nanovesicles can, in some instances, result in the destruction of the source cell. The nanovesicle may comprise a lipid or fatty acid and polypeptide. In embodiments, the fusosome comprises an exosome. In embodiments, the exosome is a cell-derived small (e.g., between 20-300 nm in diameter, or 40-200nm in diameter) vesicle comprising a membrane that encloses an internal space, and which is generated from said cell by direct plasma membrane budding or by fusion of the late endosome with the plasma membrane. In embodiments, production of exosomes does not result in the destruction of the source cell. In embodiments, the exosome comprises lipid or fatty acid and polypeptide.

[000236] Exemplary exosomes and other membrane-enclosed bodies are also described in US 20160354313, which is herein incorporated by reference in its entirety. In embodiments, the

fusosome comprises a Biocompatible Delivery Module, an exosome (e.g., about 30 nm to about 200 nm in diameter), a microvesicle (e.g., about 100 nm to about 2000 nm in diameter) an apoptotic body (e.g., about 300 nm to about 2000 nm in diameter), a membrane particle, a membrane vesicle, an exosome-like vesicle, an ectosome-like vesicle, an ectosome, or an exovesicle.

[000237] In some embodiments, a fusosome is a microvesicle. In some embodiments, a fusosome is a cell ghost. In some embodiments, a vesicle is a plasma membrane vesicle, e.g. a giant plasma membrane vesicle.

[000238] Fusosomes can be made from several different types of lipids, e.g., amphipathic lipids, such as phospholipids. The fusosome may comprise a lipid bilayer as the outermost surface. This bilayer may be comprised of one or more lipids of the same or different type. Examples include without limitation phospholipids such as phosphocholines and phosphoinositols. Specific examples include without limitation DMPC, DOPC, and DSPC.

Fusogens

[000239] In some embodiments, the fusosome described herein (e.g., comprising a vesicle or a portion of a cell) includes one or more fusogens, e.g., to facilitate the fusion of the fusosome to a membrane, e.g., a cell membrane. Also these compositions may include surface modifications made during or after synthesis to include one or more fusogens. The surface modification may comprise a modification to the membrane, e.g., insertion of a lipid or protein into the membrane.

[000240] In some embodiments, the fusosomes comprise one or more fusogens on their exterior surface (e.g., integrated into the cell membrane) to target a specific cell or tissue type (e.g., cardiomyocytes). Fusosomes may comprise a targeting domain. Fusogens include without limitation protein based, lipid based, and chemical based fusogens. The fusogen may bind a partner, e.g., a feature on a target cells' surface. In some embodiments the partner on a target cells' surface is a target cell moiety. In some embodiments, the fusosome comprising the fusogen will integrate the membrane into a lipid bilayer of a target cell.

[000241] In some embodiments, one or more of the fusogens described herein may be included in the fusosome.

Protein Fusogens

[000242] In some embodiments, the fusogen is a protein fusogen, e.g., a mammalian protein or a homologue of a mammalian protein (e.g., having 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater identity), a non-mammalian protein such as a viral protein or a homologue of a viral protein (e.g., having 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater identity), a native protein or a derivative of a native protein, a synthetic protein, a fragment thereof, a variant thereof, a protein fusion comprising one or more of the fusogens or fragments, and any combination thereof.

[000243] In some embodiments, the fusogen results in mixing between lipids in the fusosome and lipids in the target cell. In some embodiments, the fusogen results in formation of one or more pores between the lumen of the fusosome and the cytosol of the target cell, e.g., the fusosome is, or comprises, a connexin as described herein.

(i) Mammalian Proteins

[000244] In some embodiments, the fusogen may include a mammalian protein, see Table 1. Examples of mammalian fusogens may include, but are not limited to, a SNARE family protein such as vSNAREs and tSNAREs, a syncytin protein such as Syncytin-1 (DOI: 10.1128/JVI.76.13.6442–6452.2002), and Syncytin-2, myomaker (biorxiv.org/content/early/2017/04/02/123158, doi.org/10.1101/123158, doi: 10.1096/fj.201600945R, doi:10.1038/nature12343), myomixer (www.nature.com/nature/journal/v499/n7458/full/nature12343.html, doi:10.1038/nature12343), myomerger (science.sciencemag.org/content/early/2017/04/05/science.aam9361, DOI: 10.1126/science.aam9361), FGFRL1 (fibroblast growth factor receptor-like 1), Minion (doi.org/10.1101/122697), an isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (e.g., as disclosed in US 6,099,857A), a gap junction protein such as connexin 43, connexin 40, connexin 45, connexin 32 or connexin 37 (e.g., as disclosed in US 2007/0224176, Hap2, any protein capable of inducing syncytium formation between heterologous cells (see Table 2), any protein with fusogen properties (see Table 3), a homologue thereof, a fragment thereof, a variant thereof, and a protein fusion comprising one or more proteins or fragments thereof. In some embodiments, the fusogen is encoded by a human endogenous retroviral element (hERV) found

in the human genome. Additional exemplary fusogens are disclosed in US 6,099,857A and US 2007/0224176, the entire contents of which are hereby incorporated by reference.

Table 1: Non-limiting examples of human and non-human fusogens.

Human and Non-Human Fusogen Classes		
Fusogen Class	**Uniprot Protein Family ID**	**# of sequences**
EFF-AFF	PF14884	191
SNARE	PF05739	5977
DC-STAMP	PF07782	633
ENV	PF00429	312

Table 2: Genes that encode proteins with fusogen properties.

Human genes with the gene ontology annotation of: Syncytium formation by plasma membrane fusion proteins	
ID	**Symbol**
A0A024R0I0	DYRK1B
A0A024R1N1	MYH9
A0A024R2D8	CAV3
A0A096LNV2	FER1L5
A0A096LPA8	FER1L5
A0A096LPB1	FER1L5
A0AVI2	FER1L5
A6NI61	TMEM8C (myomaker)
B3KSL7	-
B7ZLI3	FER1L5
H0YD14	MYOF
O43184	ADAM12
O60242	ADGRB3
O60500	NPHS1
O95180	CACNA1H
O95259	KCNH1
P04628	WNT1

Human genes with the gene ontology annotation of: Syncytium formation by plasma membrane fusion proteins	
ID	**Symbol**
P15172	MYOD1
P17655	CAPN2
P29475	NOS1
P35579	MYH9
P56539	CAV3
Q2NNQ7	FER1L5
Q4KMG0	CDON
Q53GL0	PLEKHO1
Q5TCZ1	SH3PXD2A
Q6YHK3	CD109
Q86V25	VASH2
Q99697	PITX2
Q9C0D5	TANC1
Q9H295	DCSTAMP
Q9NZM1	MYOF
Q9Y463	DYRK1B

Table 3: Human Fusogen Candidates

Fusogen Class	Gene ID
SNARE	O15400
	Q16623
	K7EQB1
	Q86Y82
	E9PN33
	Q96NA8
	H3BT82
	Q9UNK0
	P32856
	Q13190

Fusogen Class	Gene ID
	O14662
	P61266
	O43752
	O60499
	Q13277
	B7ZBM8
	A0AVG3
	Q12846
DC-STAMP	Q9H295
	Q5T1A1
	Q5T197
	E9PJX3
	Q9BR26
ENV	Q9UQF0
	Q9N2K0
	P60507
	P60608
	B6SEH9
	P60508
	B6SEH8
	P61550
	P60509
	Q9N2J8
Muscle Fusion (Myomaker)	H0Y5B2
	H7C1S0
	Q9HCN3
	A6NDV4
	K4DI83
Muscle Fusion (Myomixer)	NP_001302423.1
	ACT64390.1
	XP_018884517.1
	XP_017826615.1

Fusogen Class	Gene ID
	XP_020012665.1
	XP_017402927.1
	XP_019498363.1
	ELW65617.1
	ERE90100.1
	XP_017813001.1
	XP_017733785.1
	XP_017531750.1
	XP_020142594.1
	XP_019649987.1
	XP_019805280.1
	NP_001170939.1
	NP_001170941.1
	XP_019590171.1
	XP_019062106.1
	EPQ04443.1
	EPY76709.1
	XP_017652630.1
	XP_017459263.1
	OBS58441.1
	XP_017459262.1
	XP_017894180.1
	XP_020746447.1
	ELK00259.1
	XP_019312826.1
	XP_017200354.1
	BAH40091.1
HA	P03452
	Q9Q0U6
	P03460
GAP JUNCTION	P36382
	P17302

Fusogen Class	Gene ID
	P36383
	P08034
	P35212
Other	FGFRL1
	GAPDH

[000245] In some embodiments, the fusosome comprises a curvature-generating protein, e.g., Epsin1, dynamin, or a protein comprising a BAR domain. See, e.g., Kozlovet al, CurrOp StrucBio 2015, Zimmerberget al. Nat Rev 2006, Richard et al, Biochem J 2011.

(ii) Non-mammalian Proteins

Viral Proteins

[000246] In some embodiments, the fusogen may include a non-mammalian protein, e.g., a viral protein. In some embodiments, a viral fusogen is a Class I viral membrane fusion protein, a Class II viral membrane fusion protein, a Class III viral membrane fusion protein, a viral membrane glycoprotein, or other viral fusion proteins, or a homologue thereof, a fragment thereof, a variant thereof, or a protein fusion comprising one or more proteins or fragments thereof.

[000247] In some embodiments, Class I viral membrane fusion proteins include, but are not limited to, Baculovirus F protein, e.g., F proteins of the nucleopolyhedrovirus (NPV) genera, e.g., Spodoptera exigua MNPV (SeMNPV) F protein and Lymantria dispar MNPV (LdMNPV), and paramyxo virus F proteins.

[000248] In some embodiments, Class II viral membrane proteins include, but are not limited to, tick bone encephalitis E (TBEV E), Semliki Forest Virus E1/E2.

[000249] In some embodiments, Class III viral membrane fusion proteins include, but are not limited to, rhabdovirus G (e.g., fusogenic protein G of the Vesicular Stomatatis Virus (VSV-G)), herpesvirus glycoprotein B (e.g., Herpes Simplex virus 1 (HSV-1) gB)), Epstein Barr Virus glycoprotein B (EBV gB), thogotovirus G, baculovirus gp64 (e.g., Autographa California multiple NPV (AcMNPV) gp64), and Borna disease virus (BDV) glycoprotein (BDV G).

[000250] Examples of other viral fusogens, e.g., membrane glycoproteins and viral fusion

proteins, include, but are not limited to: viral syncytia proteins such as influenza hemagglutinin (HA) or mutants, or fusion proteins thereof; human immunodeficiency virus type 1 envelope protein (HIV-1 ENV), gp120 from HIV binding LFA-1 to form lymphocyte syncytium, HIV gp41, HIV gp160, or HIV Trans-Activator of Transcription (TAT); viral glycoprotein VSV-G, viral glycoprotein from vesicular stomatitis virus of the Rhabdoviridae family; glycoproteins gB and gH-gL of the varicella-zoster virus (VZV); murine leukaemia virus (MLV)-10A1; Gibbon Ape Leukemia Virus glycoprotein (GaLV); type G glycoproteins in Rabies, Mokola, vesicular stomatitis virus and Togaviruses; murine hepatitis virus JHM surface projection protein; porcine respiratory coronavirus spike- and membrane glycoproteins; avian infectious bronchitis spike glycoprotein and its precursor; bovine enteric coronavirus spike protein; the F and H, HN or G genes of Measles virus; canine distemper virus, Newcastle disease virus, human parainfluenza virus 3, simian virus 41, Sendai virus and human respiratory syncytial virus; gH of human herpesvirus 1 and simian varicella virus, with the chaperone protein gL; human, bovine and cercopithicine herpesvirus gB; envelope glycoproteins of Friend murine leukaemia virus and Mason Pfizer monkey virus; mumps virus hemagglutinin neuraminidase, and glyoproteins F1 and F2; membrane glycoproteins from Venezuelan equine encephalomyelitis; paramyxovirus F protein; SIV gp160 protein; Ebola virus G protein; or Sendai virus fusion protein, or a homologue thereof, a fragment thereof, a variant thereof, and a protein fusion comprising one or more proteins or fragments thereof.

[000251] Non-mammalian fusogens include viral fusogens, homologues thereof, fragments thereof, and fusion proteins comprising one or more proteins or fragments thereof. Viral fusogens include class I fusogens, class II fusogens, class III fusogens, and class IV fusogens. In embodiments, class I fusogens such as human immunodeficiency virus (HIV) gp41, have a characteristic postfusion conformation with a signature trimer of α-helical hairpins with a central coiled-coil structure. Class I viral fusion proteins include proteins having a central postfusion six-helix bundle. Class I viral fusion proteins include influenza HA, parainfluenza F, HIV Env, Ebola GP, hemagglutinins from orthomyxoviruses, F proteins from paramyxoviruses (e.g. Measles, (Katoh et al. BMC Biotechnology 2010, 10:37)), ENV proteins from retroviruses, and fusogens of filoviruses and coronaviruses. In embodiments, class II viral fusogens such as dengue E glycoprotein, have a structural signature of β- sheets forming an elongated ectodomain that refolds

to result in a trimer of hairpins. In embodiments, the class II viral fusogen lacks the central coiled coil. Class II viral fusogen can be found in alphaviruses (e.g., E1 protein) and flaviviruses (e.g., E glycoproteins). Class II viral fusogens include fusogens from Semliki Forest virus, Sinbis, rubella virus, and dengue virus. In embodiments, class III viral fusogens such as the vesicular stomatitis virus G glycoprotein, combine structural signatures found in classes I and II. In embodiments, a class III viral fusogen comprises α helices (e.g., forming a six-helix bundle to fold back the protein as with class I viral fusogens), and β sheets with an amphiphilic fusion peptide at its end, reminiscent of class II viral fusogens. Class III viral fusogens can be found in rhabdoviruses and herpesviruses. In embodiments, class IV viral fusogens are fusion-associated small transmembrane (FAST) proteins (doi:10.1038/sj.emboj.7600767, Nesbitt, Rae L., "Targeted Intracellular Therapeutic Delivery Using Liposomes Formulated with Multifunctional FAST proteins" (2012). Electronic Thesis and Dissertation Repository. Paper 388), which are encoded by nonenveloped reoviruses. In embodiments, the class IV viral fusogens are sufficiently small that they do not form hairpins (doi: 10.1146/annurev-cellbio-101512-122422, doi:10.1016/j.devcel.2007.12.008).

[000252] In some embodiments the fusogen is a paramyxovirus fusogen. In some embodiments the fusogen is a Nipah virus protein F, a measles virus F protein, a tupaia paramyxovirus F protein, a paramyxovirus F protein, a Hendra virus F protein, a Henipavirus F protein, a Morbilivirus F protein, a respirovirus F protein, a Sendai virus F protein, a rubulavirus F protein, or an avulavirus F protein.

[000253] Additional exemplary fusogens are disclosed in US 9,695,446, US 2004/0028687, US 6,416,997, US 7,329,807, US 2017/0112773, US 2009/0202622, WO 2006/027202, and US 2004/0009604, the entire contents of all of which are hereby incorporated by reference.

(iii) Other Proteins

[000254] In some embodiments, the fusogen may include a pH dependent (e.g., as in cases of ischemic injury) protein, a homologue thereof, a fragment thereof, and a protein fusion comprising one or more proteins or fragments thereof. Fusogens may mediate membrane fusion at the cell surface or in an endosome or in another cell-membrane bound space.

[000255] In some embodiments, the fusogen includes a EFF-1, AFF-1, gap junction protein,

e.g., a connexin (such as Cn43, GAP43, CX43) (DOI: 10.1021/jacs.6b05191), other tumor connection proteins, a homologue thereof, a fragment thereof, a variant thereof, and a protein fusion comprising one or more proteins or fragments thereof.

Modifications to Protein Fusogens

[000256] In some embodiments protein fusogens can be altered to reduce immunoreactivity. For instance, protein fusogens may be decorated with molecules that reduce immune interactions, such as PEG (DOI: 10.1128/JVI.78.2.912–921.2004). Thus, in some embodiments, the fusogen comprises PEG, e.g., is a PEGylated polypeptide. Amino acid residues in the fusogen that are targeted by the immune system may be altered to be unrecognized by the immune system (doi: 10.1016/j.virol.2014.01.027, doi:10.1371/journal.pone.0046667). In some embodiments the protein sequence of the fusogen is altered to resemble amino acid sequences found in humans (humanized). In some embodiments the protein sequence of the fusogen is changed to a protein sequence that binds MHC complexes less strongly. In some embodiments, the protein fusogens are derived from viruses or organisms that do not infect humans (and which humans have not been vaccinated against), increasing the likelihood that a patient's immune system is naïve to the protein fusogens (e.g., there is a negligible humoral or cell-mediated adaptive immune response towards the fusogen) (doi:10.1006/mthe.2002.0550, doi:10.1371/journal.ppat.1005641, doi:10.1038/gt.2011.209, DOI 10.1182/blood-2014-02-558163). In some embodiments, glycosylation of the fusogen may be changed to alter immune interactions or reduce immunoreactivity. Without wishing to be bound by theory, in some embodiments, a protein fusogen derived from a virus or organism that do not infect humans does not have a natural fusion targets in patients, and thus has high specificity.

Lipid Fusogens

[000257] In some embodiments, the fusosome may be treated with fusogenic lipids, such as saturated fatty acids. In some embodiments, the saturated fatty acids have between 10-14 carbons. In some embodiments, the saturated fatty acids have longer-chain carboxylic acids. In some embodiments, the saturated fatty acids are mono-esters.

[000258] In some embodiments, the fusosome may be treated with unsaturated fatty acids. In some embodiments, the unsaturated fatty acids have between C16 and C18 unsaturated fatty

acids. In some embodiments, the unsaturated fatty acids include oleic acid, glycerol mono-oleate, glycerides, diacylglycerol, modified unsaturated fatty acids, and any combination thereof.

[000259] Without wishing to be bound by theory, in some embodiments negative curvature lipids promote membrane fusion. In some embodiments, the fusosome comprises one or more negative curvature lipids, e.g., negative curvature lipids that are exogenous relative to the source cell, in the membrane. In embodiments, the negative curvature lipid or a precursor thereof is added to media comprising source cells or fusosomes. In embodiments, the source cell is engineered to express or overexpress one or more lipid synthesis genes. The negative curvature lipid can be, e.g., diacylglycerol (DAG), cholesterol, phosphatidic acid (PA), phosphatidylethanolamine (PE), or fatty acid (FA).

[000260] Without wishing to be bound by theory, in some embodiments positive curvature lipids inhibit membrane fusion. In some embodiments, the fusosome comprises reduced levels of one or more positive curvature lipids, e.g., exogenous positive curvature lipids, in the membrane. In embodiments, the levels are reduced by inhibiting synthesis of the lipid, e.g., by knockout or knockdown of a lipid synthesis gene, in the source cell. The positive curvature lipid can be, e.g., lysophosphatidylcholine (LPC), phosphatidylinositol (PtdIns), lysophosphatidic acid (LPA), lysophosphatidylethanolamine (LPE), or monoacylglycerol (MAG).

Chemical Fusogens

[000261] In some embodiments, the fusosome may be treated with fusogenic chemicals. In some embodiments, the fusogenic chemical is polyethylene glycol (PEG) or derivatives thereof.

[000262] In some embodiments, the chemical fusogen induces a local dehydration between the two membranes that leads to unfavorable molecular packing of the bilayer. In some embodiments, the chemical fusogen induces dehydration of an area near the lipid bilayer, causing displacement of aqueous molecules between cells and allowing interaction between the two membranes together.

[000263] In some embodiments, the chemical fusogen is a positive cation. Some nonlimiting examples of positive cations include Ca2+, Mg2+, Mn2+, Zn2+, La3+, Sr3+, and H+.

[000264] In some embodiments, the chemical fusogen binds to the target membrane by

modifying surface polarity, which alters the hydration-dependent intermembrane repulsion.

[000265] In some embodiments, the chemical fusogen is a soluble lipid soluble. Some nonlimiting examples include oleoylglycerol, dioleoylglycerol, trioleoylglycerol, and variants and derivatives thereof.

[000266] In some embodiments, the chemical fusogen is a water-soluble chemical. Some nonlimiting examples include polyethylene glycol, dimethyl sulphoxide, and variants and derivatives thereof.

[000267] In some embodiments, the chemical fusogen is a small organic molecule. A nonlimiting example includes n-hexyl bromide.

[000268] In some embodiments, the chemical fusogen does not alter the constitution, cell viability, or the ion transport properties of the fusogen or target membrane.

[000269] In some embodiments, the chemical fusogen is a hormone or a vitamin. Some nonlimiting examples include abscisic acid, retinol (vitamin A1), a tocopherol (vitamin E), and variants and derivatives thereof.

[000270] In some embodiments, the fusosome comprises actin and an agent that stabilizes polymerized actin. Without wishing to be bound by theory, stabilized actin in a fusosome can promote fusion with a target cell. In embodiments, the agent that stabilizes polymerized actin is chosen from actin, myosin, biotin-streptavidin, ATP, neuronal Wiskott–Aldrich syndrome protein (N-WASP), or formin. See, e.g., Langmuir. 2011 Aug 16;27(16):10061-71 and Wen et al., Nat Commun. 2016 Aug 31;7. In embodiments, the fusosome comprises actin that is exogenous or overexpressed relative to the source cell, e.g., wild-type actin or actin comprising a mutation that promotes polymerization. In embodiments, the fusosome comprises ATP or phosphocreatine, e.g., exogenous ATP or phosphocreatine.

Small Molecule Fusogens

[000271] In some embodiments, the fusosome may be treated with fusogenic small molecules. Some nonlimiting examples include halothane, nonsteroidal anti-inflammatory drugs (NSAIDs) such as meloxicam, piroxicam, tenoxicam, and chlorpromazine.

[000272] In some embodiments, the small molecule fusogen may be present in micelle-like aggregates or free of aggregates.

Fusogen Modifications

[000273] In some embodiments, the fusogen is linked to a cleavable protein. In some cases, a cleavable protein may be cleaved by exposure to a protease. An engineered fusion protein may bind any domain of a transmembrane protein. The engineered fusion protein may be linked by a cleavage peptide to a protein domain located within the intermembrane space. The cleavage peptide may be cleaved by one or a combination of intermembrane proteases (e.g. HTRA2/OMI which requires a non-polar aliphatic amino acid - valine, isoleucine or methionine are preferred - at position P1, and hydrophilic residues - arginine is preferred - at the P2 and P3 positions).

[000274] In some embodiments the fusogen is linked to an affinity tag. In some embodiments the affinity tag aids in fusosome separation and isolation. In some embodiments the affinity tag is cleavable. In some embodiments the affinity tag is non-covalently linked to the fusogen. In some embodiments the affinity tag is present on the fusosome and separate from the fusogen.

[000275] In some embodiments, fusogen proteins are engineered by any methods known in the art or any method described herein to comprise a proteolytic degradation sequence, e.g., a mitochondrial or cytosolic degradation sequence. Fusogen proteins may be engineered to include, but is not limited to a proteolytic degradation sequence, e.g., a Caspase 2 protein sequence (e.g., Val-Asp-Val-Ala-Asp-l- (SEQ ID NO: 1)) or other proteolytic sequences (see, for example, Gasteiger et al., The Proteomics Protocols Handbook; 2005: 571-607), a modified proteolytic degradation sequence that has at least 75%, 80%, 85%, 90%, 95% or greater identity to the wildtype proteolytic degradation sequence, a cytosolic proteolytic degradation sequence, e.g., ubiquitin, or a modified cytosolic proteolytic degradation sequence that has at least 75%, 80%, 85%, 90%, 95% or greater identity to the wildtype proteolytic degradation sequence. In some embodiments, a composition comprises mitochondria in a source cell or chondrisome comprising a protein modified with a proteolytic degradation sequence, e.g., at least 75%, 80%, 85%, 90%, 95% or greater identity to the wildtype proteolytic degradation sequence, a cytosolic proteolytic degradation sequence, e.g., ubiquitin, or a modified cytosolic proteolytic degradation sequence that has at least 75%, 80%, 85%, 90%, 95% or greater identity to the wildtype proteolytic

degradation sequence.

[000276] In some embodiments, the fusogen may be modified with a protease domain that recognizes specific proteins, e.g., over-expression of a protease, e.g., an engineered fusion protein with protease activity. For example, a protease or protease domain from a protease, such as MMP, mitochondrial processing peptidase, mitochondrial intermediate peptidase, inner membrane peptidase.

[000277] See, Alfonzo, J.D. & Soll, D. Mitochondrial tRNA import – the challenge to understand has just begun. Biological Chemistry 390: 717-722. 2009; Langer, T. et al. Characterization of Peptides Released from Mitochondria. THE JOURNAL OF BIOLOGICAL CHEMISTRY. Vol. 280, No. 4. 2691–2699, 2005; Vliegh, P. et al. Synthetic therapeutic peptides: science and market. Drug Discovery Today. 15(1/2). 2010; Quiros P.M.m et al., New roles for mitochondrial proteases in health, ageing and disease. Nature Reviews Molecular Cell Biology. V16, 2015; Weber-Lotfi, F. et al. DNA import competence and mitochondrial genetics. Biopolymers and Cell. Vol. 30. N 1. 71–73, 2014.

Non-Endocytic Entry into Target Cells

[000278] In some embodiments, a fusosome or fusosome composition described herein delivers a cargo to a target cell via a non-endocytic pathway. Without wishing to be bound by theory, a non-endocytic delivery route can improve the amount or percentage of cargo delivered to the cell, e.g., to the desired compartment of the cell.

[000279] Accordingly, in some embodiments, a plurality of fusosomes described herein, when contacted with a target cell population in the presence of an inhibitor of endocytosis, and when contacted with a reference target cell population not treated with the inhibitor of endocytosis, delivers the cargo to at least 30%, 40%, 50%, 60%, 70%, or 80% of the number of cells in the target cell population compared to the reference target cell population.

[000280] In some embodiments, less than 10% of cargo enters the cell by endocytosis.

[000281] In some embodiments, the inhibitor of endocytosis is an inhibitor of lysosomal acidification, e.g., bafilomycin A1.

[000282] In some embodiments, cargo delivered is determined using an endocytosis

inhibition assay, e.g., an assay of Example 135 of International Application WO2018/208728, which is herein incorporated by reference in its entirety.

[000283] In some embodiments, cargo enters the cell through a dynamin-independent pathway or a lysosomal acidification-independent pathway, a macropinocytosis-independent pathway, or an actin-independent pathway.

[000284] In some embodiments (e.g., embodiments for assaying non-endocytic delivery of cargo) cargo delivery is assayed using one or more of (e.g., all of) the following steps: (a) placing 30,000 HEK-293T target cells into a first well of a 96-well plate comprising 100 nM bafilomycin A1, and placing a similar number of similar cells into a second well of a 96-well plate lacking bafilomycin A1, (b) culturing the target cells for four hours in DMEM media at 37°C and 5% CO_2, (c) contacting the target cells with 10 ug of fusosomes that comprise cargo, (d) incubating the target cells and fusosomes for 24 hrs at 37°C and 5% CO2, and (e) determining the percentage of cells in the first well and in the second well that comprise the cargo. Step (e) may comprise detecting the cargo using microscopy, e.g., using immunofluorescence. Step (e) may comprise detecting the cargo indirectly, e.g., detecting a downstream effect of the cargo, e.g., presence of a reporter protein. In some embodiments, one or more of steps (a)-(e) above is performed as described in Example 135 of International Application WO2018/208728.

[000285] In some embodiments, an inhibitor of endocytosis (e.g., chloroquine or bafilomycin A1) inhibits inhibits endosomal acidification. In some embodiments, cargo delivery is independent of lysosomal acidification. In some embodiments, an inhibitor of endocytosis (e.g., Dynasore) inhibits dynamin. In some embodiments, cargo delivery is independent of dynamin activity.

[000286] In some embodiments, the fusosome enters the target cell by endocytosis, e.g., wherein the level of therapeutic agent delivered via an endocytic pathway is 0.01-0.6, 0.01-0.1, 0.1-0.3, or 0.3-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than a chloroquine treated reference cell contacted with similar fusosomes, e.g., using an assay of Example 91 herein. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% of fusosomes in a fusosome composition that enter a target cell enter via a non-endocytic pathway, e.g., the fusosomes enter the target cell via fusion with the cell surface. In some embodiments, the level of a therapeutic agent delivered via a non-

endocytic pathway for a given fusosome is 0.1-0.95, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-0.95, or at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than a chloroquine treated reference cell, e.g., using an assay of Example 90 herein. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% of fusosomes in a fusosome composition that enter a target cell enter the cytoplasm (e.g., do not enter an endosome or lysosome). In some embodimetns, after the membrane protein payload agent enters the cytoplasm, the membrane protein payload agent or polypeptide encoded therein localizes to the cell membrane or is secreted. In some embodiments, less than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% of fusosomes in a fusosome composition that enter a target cell enter an endosome or lysosome. In some embodiments, the fusosome enters the target cell by a non-endocytic pathway, e.g., wherein the level of therapeutic agent delivered is at least 90%, 95%, 98%, or 99% that of a chloroquine treated reference cell, e.g., using an assay of Example 91 herein. In an embodiment, a fusosome delivers an agent to a target cell via a dynamin mediated pathway. In an embodiment, the level of agent delivered via a dynamin mediated pathway is in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than Dynasore treated target cells contacted with similar fusosomes, e.g., as measured in an assay of Example 92 herein. In an embodiment, a fusosome delivers an agent to a target cell via macropinocytosis. In an embodiment, the level of agent delivered via macropinocytosis is in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than EIPA treated target cells contacted with similar fusosomes, e.g., as measured in an assay of Example 92 herein. In an embodiment, a fusosome delivers an agent to a target cell via an actin-mediated pathway. In an embodiment, the level of agent delivered via an actin-mediated pathway will be in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than Latrunculin B treated target cells contacted with similar fusosomes, e.g., as measured in an assay of Example 92 herein.

[000287] In some embodiments, the cargo delivered to the target cell is determined using an endocytosis inhibition assay, e.g., an assay of Example 55, 90, or 92 herein.

[000288] In some embodiments, cargo enters the target cell through a dynamin-independent

pathway or a lysosomal acidification-independent pathway, a macropinocytosis-independent pathway (e.g., wherein the inhibitor of endocytosis is an inhibitor of macropinocytosis, e.g., 5-(N-ethyl-N-isopropyl)amiloride (EIPA), e.g., at a concentration of 25 μM), or an actin-independent pathway (e.g., wherein the inhibitor of endocytosis is an inhibitor of actin polymerization is, e.g., Latrunculin B, e.g., at a concentration of 6 μM).

[000289] In some embodiments, the fusosome, when contacted with a target cell population, delivers cargo to a target cell location other than an endosome or lysosome, e.g., to the cytosol or the cell membrane. In embodiments, less 50%, 40%, 30%, 20%, or 10% of the cargo is delivered to an endosome or lysosome.

Specific Delivery to Target Cells

[000290] In some embodiments, a fusosome composition described herein delivers cargo preferentially to a target cell compared to a non-target cell. Accordingly, in certain embodiments, a fusosome described herein has one or both of the following properties: (i) when the plurality of fusosomes are contacted with a cell population comprising target cells and non-target cells, the cargo is present in at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold more target cells than non-target cells, or (ii) the fusosomes of the plurality fuse at a higher rate with a target cell than with a non-target cell by at least at least 50%.

[000291] In some embodiments, presence of cargo is measured by microscopy, e.g., using an assay of Example 124 of Internation Application WO2018/208728, which is herein incorporated by reference in its entirety. In some embodiments, fusion is measured by microscopy, e.g., using an assay of Example 54 herein. In some embodiments, the targeting moiety is specific for a cell surface marker on the target cell. In some embodiments, the cell surface marker is a cell surface marker of a skin cell, cardiomyocyte, hepatocyte, intestinal cell (e.g., cell of the small intestine), pancreatic cell, brain cell, prostate cell, lung cell, colon cell, or bone marrow cell.

[000292] In some embodiments (e.g., embodiments for specific delivery of cargo to a target cell versus a non-target cell), cargo delivery is assayed using one or more of (e.g., all of) the following steps: (a) placing 30,000 HEK-293T target cells that over-express CD8a and CD8b into a first well of a 96-well plate and placing 30,000 HEK-293T non-target cells that do not over-express CD8a and CD8b into a second well of a 96-well plate, (b) culturing the cells for four hours

in DMEM media at 37°C and 5% CO_2, (c) contacting the target cells with 10 ug of fusosomes that comprise cargo, (d) incubating the target cells and fusosomes for 24 hrs at 37°C and 5% CO_2, and (e) determining the percentage of cells in the first well and in the second well that comprise the cargo. Step (e) may comprise detecting the cargo using microscopy, e.g., using immunofluorescence. Step (e) may comprise detecting the cargo indirectly, e.g., detecting a downstream effect of the cargo, e.g., presence of a reporter protein. In some embodiments, one or more of steps (a)-(e) above is performed as described in Example 124 of Internation Application WO2018/208728.

[000293] In some embodiments, the fusosome fuses at a higher rate with a target cell than with a non-target cell, e.g., by at least at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses at a higher rate with a target cell than with other fusosomes, e.g., by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, e.g., in an assay of Example 54. In some embodiments, the fusosome fuses with target cells at a rate such that an agent in the fusosome is delivered to at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of target cells after 24, 48, or 72 hours, e.g., in an assay of Example 54. In embodiments, the amount of targeted fusion is about 30%-70%, 35%-65%, 40%-60%, 45%-55%, or 45%-50%, e.g., about 48.8% e.g., in an assay of Example 54. In embodiments, the amount of targeted fusion is about 20%-40%, 25%-35%, or 30%-35%, e.g., about 32.2% e.g., in an assay of Example 55.

[000294] In some embodiments, the fusosome composition delivers at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the cargo to the target cell population compared to the reference target cell population or to a non-target cell population. In some embodiments, the fusosome composition delivers at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% more of the cargo to the target cell population compared to the reference target cell population or to a non-target cell population.

Fusosome Generation

Fusosomes Generated from Cells

[000295] Compositions of fusosomes may be generated from cells in culture, for example cultured mammalian cells, e.g., cultured human cells. The cells may be progenitor cells or non-progenitor (e.g., differentiated) cells. The cells may be primary cells or cell lines (e.g., a mammalian, e.g., human, cell line described herein). In embodiments, the cultured cells are progenitor cells, e.g., bone marrow stromal cells, marrow derived adult progenitor cells (MAPCs), endothelial progenitor cells (EPC), blast cells, intermediate progenitor cells formed in the subventricular zone, neural stem cells, muscle stem cells, satellite cells, liver stem cells, hematopoietic stem cells, bone marrow stromal cells, epidermal stem cells, embryonic stem cells, mesenchymal stem cells, umbilical cord stem cells, precursor cells, muscle precursor cells, myoblast, cardiomyoblast, neural precursor cells, glial precursor cells, neuronal precursor cells, hepatoblasts.

[000296] In some embodiments, the source cell is an endothelial cell, a fibroblast, a blood cell (e.g., a macrophage, a neutrophil, a granulocyte, a leukocyte), a stem cell (e.g., a mesenchymal stem cell, an umbilical cord stem cell, bone marrow stem cell, a hematopoietic stem cell, an induced pluripotent stem cell e.g., an induced pluripotent stem cell derived from a subject's cells), an embryonic stem cell (e.g., a stem cell from embryonic yolk sac, placenta, umbilical cord, fetal skin, adolescent skin, blood, bone marrow, adipose tissue, erythropoietic tissue, hematopoietic tissue), a myoblast, a parenchymal cell (e.g., hepatocyte), an alveolar cell, a neuron (e.g., a retinal neuronal cell) a precursor cell (e.g., a retinal precursor cell, a myeloblast, myeloid precursor cells, a thymocyte, a meiocyte, a megakaryoblast, a promegakaryoblast, a melanoblast, a lymphoblast, a bone marrow precursor cell, a normoblast, or an angioblast), a progenitor cell (e.g., a cardiac progenitor cell, a satellite cell, a radial gial cell, a bone marrow stromal cell, a pancreatic progenitor cell, an endothelial progenitor cell, a blast cell), or an immortalized cell (e.g., HeLa, HEK293, HFF-1, MRC-5, WI-38, IMR 90, IMR 91, PER.C6, HT-1080, or BJ cell).

[000297] The cultured cells may be from epithelial, connective, muscular, or nervous tissue or cells, and combinations thereof. Fusosome can be generated from cultured cells from any

eukaryotic (e.g., mammalian) organ system, for example, from the cardiovascular system (heart, vasculature); digestive system (esophagus, stomach, liver, gallbladder, pancreas, intestines, colon, rectum and anus); endocrine system (hypothalamus, pituitary gland, pineal body or pineal gland, thyroid, parathyroids, adrenal glands); excretory system (kidneys, ureters, bladder); lymphatic system (lymph, lymph nodes, lymph vessels, tonsils, adenoids, thymus, spleen); integumentary system (skin, hair, nails); muscular system (e.g., skeletal muscle); nervous system (brain, spinal cord, nerves); reproductive system (ovaries, uterus, mammary glands, testes, vas deferens, seminal vesicles, prostate); respiratory system (pharynx, larynx, trachea, bronchi, lungs, diaphragm); skeletal system (bone, cartilage), and combinations thereof. In embodiments, the cells are from a highly mitotic tissue (e.g., a highly mitotic healthy tissue, such as epithelium, embryonic tissue, bone marrow, intestinal crypts). In embodiments, the tissue sample is a highly metabolic tissue (e.g., skeletal tissue, neural tissue, cardiomyocytes).

[000298] In some embodiments a cell is a suspension cell. In some embodiments a cell is an adherent cell.

[000299] In some embodiments, the cells are from a young donor, e.g., a donor 25 years, 20 years, 18 years, 16 years, 12 years, 10 years, 8 years of age, 5 years of age, 1 year of age, or less. In some embodiments, the cells are from fetal tissue.

[000300] In some embodiments, the cells are derived from a subject and administered to the same subject or a subject with a similar genetic signature (e.g., MHC-matched).

[000301] In certain embodiments, the cells have telomeres of average size greater than 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10000 nucleotides in length (e.g., between 4,000-10,000 nucleotides in length, between 6,000-10,000 nucleotides in length).

[000302] Fusosomes may be generated from cells generally cultured according to methods known in the art. In some embodiments, the cells may be cultured in 2 or more "phases", e.g., a growth phase, wherein the cells are cultured under conditions to multiply and increase biomass of the culture, and a "production" phase, wherein the cells are cultured under conditions to alter cell phenotype (e.g., to maximize mitochondrial phenotype, to increase number or diameter of mitochondria, to increase oxidative phosphorylation status). There may also be an "expression" phase, wherein the cells are cultured under conditions to maximize expression of protein fusogens

or agents exogenous relative to the source cell, on the cell membrane and to restrict unwanted fusion in other phases.

[000303] In some embodiments, fusosomes are generated from cells synchronized, e.g., during a growth phase or the production phase. For example, cells may be synchronized at G1 phase by elimination of serum from the culture medium (e.g., for about 12- 24 hours) or by the use in the culture media of DNA synthesis inhibitors such as thymidine, aminopterin, hydroxyurea and cytosine arabinoside. Additional methods for mammalian cell cycle synchronization are known and disclosed, e.g., in Rosner et al. 2013. Nature Protocols 8:602–626 (specifically Table 1 in Rosner).

[000304] In some embodiments, the cells can be evaluated and optionally enriched for a desirable phenotype or genotype for use as a source for fusosome composition as described herein. For example, cells can be evaluated and optionally enriched, e.g., before culturing, during culturing (e.g., during a growth phase or a production phase) or after culturing but before fusosome production, for example, for one or more of: membrane potential (e.g., a membrane potential of -5 to -200 mV; cardiolipin content (e.g., between 1-20% of total lipid); cholesterol, phosphatidylethanolamine (PE), diglyceride (DAG), phosphatidic acid (PA), or fatty acid (FA) content; genetic quality > 80%, >85%, > 90%; fusogen expression or content; cargo expression or content.

[000305] In some embodiments, fusosomes are generated from a cell clone identified, chosen, or selected based on a desirable phenotype or genotype for use as a source for fusosome composition described herein. For example, a cell clone is identified, chosen, or selected based on low mitochondrial mutation load, long telomere length, differentiation state, or a particular genetic signature (e.g., a genetic signature to match a recipient).

[000306] A fusosome composition described herein may be comprised of fusosomes from one cellular or tissue source, or from a combination of sources. For example, a fusosome composition may comprise fusosomes from xenogeneic sources (e.g., animals, tissue culture of the aforementioned species' cells), allogeneic, autologous, from specific tissues resulting in different protein concentrations and distributions (liver, skeletal, neural, adipose, etc.), from cells of different metabolic states (e.g., glycolytic, respiring). A composition may also comprise

fusosomes in different metabolic states, e.g. coupled or uncoupled, as described elsewhere herein.

[000307] In some embodiments, fusosomes are generated from source cells expressing a fusogen, e.g., a fusogen described herein. In some embodiments, the fusogen is disposed in a membrane of the source cell, e.g., a lipid bilayer membrane, e.g., a cell surface membrane, or a subcellular membrane (e.g., lysosomal membrane). In some embodiments, fusosomes are generated from source cells with a fusogen disposed in a cell surface membrane.

[000308] In some embodiments, fusosomes are generated by inducing budding of an exosome, microvesicle, membrane vesicle, extracellular membrane vesicle, plasma membrane vesicle, giant plasma membrane vesicle, apoptotic body, mitoparticle, pyrenocyte, lysosome, or other membrane enclosed vesicle.

[000309] In some embodiments, fusosomes are generated by inducing cell enucleation. Enucleation may be performed using assays such as genetic, chemical (e.g., using Actinomycin D, see Bayona-Bafaluyet al., "A chemical enucleation method for the transfer of mitochondrial DNA to ρ° cells" Nucleic Acids Res. 2003 Aug 15; 31(16): e98), mechanical methods (e.g., squeezing or aspiration, see Lee et al., "A comparative study on the efficiency of two enucleation methods in pig somatic cell nuclear transfer: effects of the squeezing and the aspiration methods." Anim Biotechnol. 2008;19(2):71-9), or combinations thereof. Enucleation refers not only to a complete removal of the nucleus but also the displacement of the nucleus from its typical location such that the cell contains the nucleus but it is non-functional.

[000310] In embodiments, making a fusosome comprises producing cell ghosts, giant plasma membrane vesicle, or apoptotic bodies. In embodiments, a fusosome composition comprises one or more of cell ghosts, giant plasma membrane vesicle, and apoptotic bodies.

[000311] In some embodiments, fusosomes are generated by inducing cell fragmentation. In some embodiments, cell fragmentation can be performed using the following methods, including, but not limited to: chemical methods, mechanical methods (e.g., centrifugation (e.g., ultracentrifugation, or density centrifugation), freeze-thaw, or sonication), or combinations thereof.

[000312] In some embodiments, a fusosome can be generated from a source cell expressing

a fusogen, e.g., as described herein, by any one, all of, or a combination of the following methods:

i) inducing budding of a mitoparticle, exosome, or other membrane enclosed vesicle;

ii) inducing nuclear inactivation, e.g., enucleation, by any of the following methods or a combination thereof:

a) a genetic method;

b) a chemical method, e.g., using Actinomycin D; or

c) a mechanical method, e.g., squeezing or aspiration; or

iii) inducing cell fragmentation, e.g., by any of the following methods or a combination thereof:

a) a chemical method;

b) a mechanical method, e.g., centrifugation (e.g., ultracentrifugation or density centrifugation); freeze thaw; or sonication.

[000313] For avoidance of doubt, it is understood that in many cases the source cell actually used to make the fusosome will not be available for testing after the fusosome is made. Thus, a comparison between a source cell and a fusosome does not need to assay the source cell that was actually modified (e.g., enucleated) to make the fusosome. Rather, cells otherwise similar to the source cell, e.g., from the same culture, the same genotype same tissue type, or any combination thereof, can be assayed instead.

Modifications to Cells Prior to Fusosome Generation

[000314] In some aspects, a modification is made to a cell, such as modification of a subject, tissue or cell, prior to fusosome generation. Such modifications can be effective to, e.g., improve fusion, fusogen expression or activity, structure or function of the cargo, or structure or function of the target cell.

(i) Physical Modifications

[000315] In some embodiments, a cell is physically modified prior to generating the fusosome. For example, as described elsewhere herein, a fusogen may be linked to the surface of the cell.

[000316] In some embodiments, a cell is treated with a chemical agent prior to generating the fusosome. For example, the cell may be treated with a chemical or lipid fusogen, such that the

chemical or lipid fusogen non-covalently or covalently interacts with the surface of the cell or embeds within the surface of the cell. In some embodiments, the cell is treated with an agent to enhance fusogenic properties of the lipids in the cell membrane.

[000317] In some embodiments, the cell is physically modified prior to generating the fusosome with one or more covalent or non-covalent attachment sites for synthetic or endogenous small molecules or lipids on the cell surface that enhance targeting of the fusosome to an organ, tissues, or cell-type.

[000318] In embodiments, a fusosome comprises increased or decreased levels of an endogenous molecule. For instance, the fusosome may comprise an endogenous molecule that also naturally occurs in the naturally occurring source cell but at a higher or lower level than in the fusosome. In some embodiments, the polypeptide is expressed from an exogenous nucleic acid in the source cell or fusosome. In some embodiments, the polypeptide is isolated from a source and loaded into or conjugated to a source cell or fusosome.

[000319] In some embodiments, a cell is treated with a chemical agent, e.g., small molecule, prior to generating the fusosome to increase the expression or activity of an endogenous fusogen in the cell (e.g., in some embodiments, endogenous relative to the source cell, and in some embodiments, endogenous relative to the target cell). In some embodiments, a small molecule may increase expression or activity of a transcriptional activator of the endogenous fusogen. In some embodiments, a small molecule may decrease expression or activity of a transcriptional repressor of the endogenous fusogen. In some embodiments, a small molecule is an epigenetic modifier that increases expression of the endogenous fusogen.

[000320] In some embodiments, fusosomes are generated from cells treated with fusion arresting compounds, e.g., lysophosphatidylcholine. In some embodiments, fusosomes are generated from cells treated with dissociation reagents that do not cleave fusogens, e.g., Accutase.

[000321] In some embodiments, a source cell is physically modified with, e.g., CRISPR activators, prior to generating a fusosome to add or increase the concentration of fusogens.

[000322] In some embodiments, the cell is physically modified to increase or decrease the quantity, or enhance the structure or function of organelles, e.g., mitochondria, Golgi apparatus,

endoplasmic reticulum, intracellular vesicles (such as lysosomes, autophagosomes).

(ii) Genetic Modifications

[000323] In some embodiments, a cell is genetically modified prior to generating the fusosome to increase the expression of an endogenous fusogen in the cell (e.g., in some embodiments, endogenous relative to the source cell, and in some embodiments, endogenous relative to the target cell . In some embodiments, a genetic modification may increase expression or activity of a transcriptional activator of the endogenous fusogen. In some embodiments, a genetic modification may decrease expression or activity of a transcriptional repressor of the endogenous fusogen. In some embodiments the activator or repressor is a nuclease-inactive cas9 (dCas9) linked to a transcriptional activator or repressor that is targeted to the endogenous fusogen by a guide RNA. In some embodiments, a genetic modification epigenetically modifies an endogenous fusogen gene to increase its expression. In some embodiments the epigenetic activator a nuclease-inactive cas9 (dCas9) linked to an epigenetic modifier that is targeted to the endogenous fusogen by a guide RNA.

[000324] In some embodiments, a cell is genetically modified prior to generating the fusosome to increase the expression of an exogenous fusogen in the cell, e.g., delivery of a transgene. In some embodiments, a nucleic acid, e.g., DNA, mRNA or siRNA, is transferred to the cell prior to generating the fusosome, e.g., to increase or decrease the expression of a cell surface molecule (protein, glycan, lipid or low molecular weight molecule) used for organ, tissue, or cell targeting. In some embodiments, the nucleic acid targets a repressor of a fusogen, e.g., an shRNA, siRNA construct. In some embodiments, the nucleic acid encodes an inhibitor of a fusogen repressor.

[000325] In some embodiments, the method comprises introducing a nucleic acid , that is exogenous relative to the source cell encoding a fusogen into a source cell. The exogenous nucleic acid may be, e.g., DNA or RNA. In some embodiments the exogenous nucleic acid may be e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-mRNA, an mRNA, an miRNA, an siRNA, etc. In some embodiments, the exogenous DNA may be linear DNA, circular DNA, or an artificial chromosome. In some embodiments the DNA is maintained episomally. In some embodiments the

DNA is integrated into the genome. The exogenous RNA may be chemically modified RNA, e.g., may comprise one or more backbone modification, sugar modifications, noncanonical bases, or caps. Backbone modifications include, e.g., phosphorothioate, N3′ phosphoramidite, boranophosphate, phosphonoacetate, thio-PACE, morpholino phosphoramidites, or PNA. Sugar modifications include, e.g., 2′-O-Me, 2′F, 2′F-ANA, LNA, UNA, and 2′-O-MOE. Noncanonical bases include, e.g., 5-bromo-U, and 5-iodo-U, 2,6-diaminopurine, C-5 propynyl pyrimidine, difluorotoluene, difluorobenzene, dichlorobenzene, 2-thiouridine, pseudouridine, and dihydrouridine. Caps include, e.g., ARCA. Additional modifications are discussed, e.g., in Deleavey et al., "Designing Chemically Modified Oligonucleotides for Targeted Gene Silencing" Chemistry & Biology Volume 19, Issue 8, 24 August 2012, Pages 937-954, which is herein incorporated by reference in its entirety.

[000326] In some embodiments, a cell is treated with a chemical agent, e.g. a small molecule, prior to generating the fusosome to increase the expression or activity of a fusogen that is exogenous relative to the source cell in the cell. In some embodiments, a small molecule may increase expression or activity of a transcriptional activator of the exogenous fusogen. In some embodiments, a small molecule may decrease expression or activity of a transcriptional repressor of the exogenous fusogen. In some embodiments, a small molecule is an epigenetic modifier that increases expression of the exogenous fusogen.

[000327] In some embodiments, the nucleic acid encodes a modified fusogen. For example, a fusogen that has regulatable fusogenic activity, e.g., specific cell-type, tissue-type or local microenvironment activity. Such regulatable fusogenic activity may include, activation and/or initiation of fusogenic activity by low pH, high pH, heat, infrared light, extracellular enzyme activity (eukaryotic or prokaryotic), or exposure of a small molecule, a protein, or a lipid. In some embodiments, the small molecule, protein, or lipid is displayed on a target cell.

[000328] In some embodiments, a cell is genetically modified prior to generating the fusosome to alter (i.e., upregulate or downregulate) the expression of signaling pathways (e.g., the Wnt/Beta-catenin pathway). In some embodiments, a cell is genetically modified prior to generating the fusosome to alter (e.g., upregulate or downregulate) the expression of a gene or genes of interest. In some embodiments, a cell is genetically modified prior to generating the

fusosome to alter (e.g., upregulate or downregulate) the expression of a nucleic acid (e.g. a miRNA or mRNA) or nucleic acids of interest. In some embodiments, nucleic acids, e.g., DNA, mRNA or siRNA, are transferred to the cell prior to generating the fusosome, e.g., to increase or decrease the expression of signaling pathways, genes, or nucleic acids. In some embodiments, the nucleic acid targets a repressor of a signaling pathway, gene, or nucleic acid, or represses a signaling pathway, gene, or nucleic acid. In some embodiments, the nucleic acid encodes a transcription factor that upregulates or downregulates a signaling pathway, gene, or nucleic acid. In some embodiments the activator or repressor is a nuclease-inactive cas9 (dCas9) linked to a transcriptional activator or repressor that is targeted to the signaling pathway, gene, or nucleic acid by a guide RNA. In some embodiments, a genetic modification epigenetically modifies an endogenous signaling pathway, gene, or nucleic acid to its expression. In some embodiments the epigenetic activator a nuclease-inactive cas9 (dCas9) linked to a epigenetic modifier that is targeted to the signaling pathway, gene, or nucleic acid by a guide RNA. In some embodiments, a cell's DNA is edited prior to generating the fusosome to alter (e.g., upregulate or downregulate) the expression of signaling pathways (e.g. the Wnt/Beta-catenin pathway), gene, or nucleic acid. In some embodiments, the DNA is edited using a guide RNA and CRISPR-Cas9/Cpf1 or other gene editing technology.

[000329] A cell may be genetically modified using recombinant methods. A nucleic acid sequence coding for a desired gene can be obtained using recombinant methods, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, a gene of interest can be produced synthetically, rather than cloned.

[000330] Expression of natural or synthetic nucleic acids is typically achieved by operably linking a nucleic acid encoding the gene of interest to a promoter, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration in eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for expression of the desired nucleic acid sequence.

[000331] In some embodiments, a cell may be genetically modified with one or more

expression regions, e.g., a gene. In some embodiments, the cell may be genetically modified with an exogenous gene (e.g., capable of expressing an exogenous gene product such as an RNA or a polypeptide product) and/or an exogenous regulatory nucleic acid. In some embodiments, the cell may be genetically modified with an exogenous sequence encoding a gene product that is endogenous to a target cell and/or an exogenous regulatory nucleic acid capable of modulating expression of an endogenous gene. In some embodiments, the cell may be genetically modified with an exogenous gene and/or a regulatory nucleic acid that modulates expression of an exogenous gene. In some embodiments, the cell may be genetically modified with an exogenous gene and/or a regulatory nucleic acid that modulates expression of an endogenous gene. It will be understood by one of skill in the art that the cell described herein may be genetically modified to express a variety of exogenous genes that encode proteins or regulatory molecules, which may, e.g., act on a gene product of the endogenous or exogenous genome of a target cell. In some embodiments, such genes confer characteristics to the fusosome, e.g., modulate fusion with a target cell. In some embodiments, the cell may be genetically modified to express an endogenous gene and/or regulatory nucleic acid. In some embodiments, the endogenous gene or regulatory nucleic acid modulates the expression of other endogenous genes. In some embodiments, the cell may be genetically modified to express an endogenous gene and/or regulatory nucleic acid which is expressed differently (e.g., inducibly, tissue-specifically, constitutively, or at a higher or lower level) than a version of the endogenous gene and/or regulatory nucleic acid on other chromosomes.

[000332] The promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

[000333] One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable

of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor-1α (EF-1α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter.

[000334] Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a tissue-specific promoter, metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter. In some embodiments, expression of a fusogen is upregulated before fusosomes are generated, e.g., 3, 6, 9, 12, 24, 26, 48, 60, or 72 hours before fusosomes are generated.

[000335] The expression vector to be introduced into the source can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

[000336] Reporter genes may be used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient source and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the

recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

[000337] In some embodiments, a cell may be genetically modified to alter expression of one or more proteins. Expression of the one or more proteins may be modified for a specific time, e.g., development or differentiation state of the source. In some embodiments, fusosomes are generated from a source of cells genetically modified to alter expression of one or more proteins, e.g., fusogen proteins or non-fusogen proteins that affect fusion activity, structure or function. Expression of the one or more proteins may be restricted to a specific location(s) or widespread throughout the source.

[000338] In some embodiments, the expression of a fusogen protein is modified. In some embodiments, fusosomes are generated from cells with modified expression of a fusogen protein, e.g., an increase or a decrease in expression of a fusogen by at least 10%, 15%, 20%, 30%, 40%, 50%, 60%, 75%, 80%, 90% or more.

[000339] In some embodiments, cells may be engineered to express a cytosolic enzyme (e.g., proteases, phosphatases, kinases, demethylases, methyltransferases, acetylases) that targets a fusogen protein. In some embodiments, the cytosolic enzyme affects one or more fusogens by altering post-translational modifications. Post-translational protein modifications of proteins may affect responsiveness to nutrient availability and redox conditions, and protein–protein interactions. In some embodiments, a fusosome comprises fusogens with altered post-translational modifications, e.g., an increase or a decrease in post-translational modifications by at least 10%, 15%, 20%, 30%, 40%, 50%, 60%, 75%, 80%, 90% or more.

[000340] Methods of introducing a modification into a cell include physical, biological and chemical methods. See, for example, Geng. & Lu, Microfluidic electroporation for cellular analysis and delivery. Lab on a Chip. 13(19):3803-21. 2013; Sharei, A. et al. A vector-free

microfluidic platform for intracellular delivery. PNAS vol. 110 no. 6. 2013; Yin, H. et al., Non-viral vectors for gene-based therapy. Nature Reviews Genetics. 15: 541–555. 2014. Suitable methods for modifying a cell for use in generating the fusosomes described herein include, for example, diffusion, osmosis, osmotic pulsing, osmotic shock, hypotonic lysis, hypotonic dialysis, ionophoresis, electroporation, sonication, microinjection, calcium precipitation, membrane intercalation, lipid mediated transfection, detergent treatment, viral infection, receptor mediated endocytosis, use of protein transduction domains, particle firing, membrane fusion, freeze-thawing, mechanical disruption, and filtration.

[000341] Confirming the presence of a genetic modification includes a variety of assays. Such assays include, for example, molecular biological assays, such as Southern and Northern blotting, RT-PCR and PCR; biochemical assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein.

Fusosome Modifications

[000342] In some aspects, a modification is made to the fusosome. Such modifications can be effective to, e.g., improve targeting, function, or structure.

[000343] In some embodiments, the fusosome is treated with a fusogen, e.g., a chemical fusogen described herein, that may non-covalently or covalently link to the surface of the membrane. In some embodiments, the fusosome is treated with a fusogen, e.g., a protein or a lipid fusogen, that may non-covalently or covalently link or embed itself in the membrane.

[000344] In some embodiments, a ligand is conjugated to the surface of the fusosome via a functional chemical group (carboxylic acids, aldehydes, amines, sulfhydryls and hydroxyls) that is present on the surface of the fusosome.

[000345] Such reactive groups include without limitation maleimide groups. As an example, fusosomes may be synthesized to include maleimide conjugated phospholipids such as without limitation DSPE-MaL-PEG2000.

[000346] In some embodiments, a small molecule or lipid, synthetic or native, may be covalently or non-covalent linked to the surface of the fusosome. In some embodiments, a

membrane lipid in the fusosome may be modified to promote, induce, or enhance fusogenic properties.

[000347] In some embodiments, the fusosome is modified by loading with modified proteins (e.g., enable novel functionality, alter post-translational modifications, bind to the mitochondrial membrane and/or mitochondrial membrane proteins, form a cleavable protein with a heterologous function, form a protein destined for proteolytic degradation, assay the agent's location and levels, or deliver the agent as a carrier). In some embodiments, a fusosome is loaded with one or more modified proteins.

[000348] In some embodiments, a protein exogenous relative to the source cell is non-covalently bound to the fusosome. The protein may include a cleavable domain for release. In some embodiments, the invention includes a fusosome comprising an exogenous protein with a cleavable domain.

[000349] In some embodiments, the fusosome is modified with a protein destined for proteolytic degradation. A variety of proteases recognize specific protein amino acid sequences and target the proteins for degradation. These protein degrading enzymes can be used to specifically degrade proteins having a proteolytic degradation sequence. In some embodiments, a fusosome comprises modulated levels of one or more protein degrading enzymes, e.g., an increase or a decrease in protein degrading enzymes by at least 10%, 15%, 20%, 30%, 40%, 50%, 60%, 75%, 80%, 90% or more.

[000350] As described herein, non-fusogen additives may be added to the fusosome to modify their structure and/or properties. For example, either cholesterol or sphingomyelin may be added to the membrane to help stabilize the structure and to prevent the leakage of the inner cargo. Further, membranes can be prepared from hydrogenated egg phosphatidylcholine or egg phosphatidylcholine, cholesterol, and dicetyl phosphate. (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[000351] In some embodiments, the fusosome comprises one or more targeting groups (e.g., a targeting protein) on the exterior surface to target a specific cell or tissue type (e.g., cardiomyocytes). These targeting groups include without limitation receptors, ligands, antibodies,

and the like. These targeting groups bind their partner on the target cells' surface. In embodiments, the targeting protein is specific for a cell surface marker on a target cell described herein, e.g., a skin cell, cardiomyocyte, hepatocyte, intestinal cell (e.g., cell of the small intestine), pancreatic cell, brain cell, prostate cell, lung cell, colon cell, or bone marrow cell.

[000352] In some embodiments, the targeting protein binds a cell surface marker on a target cell. In embodiments, the cell surface marker comprises a protein, glycoprotein, receptor, cell surface ligand, class I transmembrane protein, class II transmembrane protein, or class III transmembrane protein.

[000353] In some embodiments, the targeting moiety is comprised by a polypeptide that is a separate polypeptide from the fusogen. In some embodiments, the polypeptide comprising a targeting moiety comprises a transmembrane domain and an extracellular targeting domain. In embodiments, the extracellular targeting domain comprises an scFv, DARPin, nanobody, receptor ligand, or antigen. In some embodiments, the extracellular targeting domain comprises an antibody or an antigen-binding fragment thereof (e.g., Fab, Fab', F(ab')2, Fv fragments, scFv antibody fragments, disulfide-linked Fvs (sdFv), a Fd fragment consisting of the VH and CH1 domains, linear antibodies, single domain antibodies such as sdAb (either VL or VH), or camelid VHH domains), an antigen-binding fibronectin type III (Fn3) scaffold such as a fibronectin polypeptide minibody, a ligand, a cytokine, a chemokine, or a T cell receptor (TCR).

[000354] In some embodiments, the fusosome described herein is functionalized with a diagnostic agent. Examples of diagnostic agents include, but are not limited to, commercially available imaging agents used in positron emissions tomography (PET), computer assisted tomography (CAT), single photon emission computerized tomography, x-ray, fluoroscopy, and magnetic resonance imaging (MRI); and contrast agents. Examples of suitable materials for use as contrast agents in MRI include gadolinium chelates, as well as iron, magnesium, manganese, copper, and chromium.

[000355] Another example of introducing functional groups to the fusosome is during post-preparation, by direct crosslinking fusosome and ligands with homo- or heterobifunctional crosslinkers. This procedure may use a suitable chemistry and a class of crosslinkers (CDI, EDAC, glutaraldehydes, etc. as discussed herein) or any other crosslinker that couples a ligand to the

fusosome surface via chemical modification of the fusosome surface after preparation. This also includes a process whereby amphiphilic molecules such as fatty acids, lipids or functional stabilizers may be passively adsorbed and adhered to the fusosome surface, thereby introducing functional end groups for tethering to ligands.

Cargo

[000356] In some embodiments, a fusosome described herein includes a cargo that is or comprises a membrane protein payload agent. In some embodiments, the membrane protein payload agent may be or may encode a therapeutic protein. A fusosome may additionally include other cargo, e.g., in some embodiments, a fusosome described herein includes a cargo that is or comprises a therapeutic agent. In some embodiments, a fusosome described herein includes a plurality of membrane payload agents. In some embodiments, a fusosome described herein includes a cargo that is or comprises a plurality of therapeutic agents. In some embodiments, a fusosome comprises a cargo comprising one or more membrane protein payload agents and one or more therapeutic agents. In some embodiments, a cargo may be a therapeutic agent that is exogenous or endogenous relative to the source cell.

[000357] In some embodiments a fusosome comprises a cargo associated with the fusosome lipid bilayer. In some embodiments a fusosome comprises a cargo disposed within the lumen of the fusosome. In some embodiments, a fusosome comprises a cargo associated with the fusosome lipid bilayer and a cargo disposed within the lumen of the fusosome.

[000358] In some embodiments, a cargo is not expressed naturally in a cell from which the fuososme is derived. In some embodiments, a cargo is expressed naturally in the cell from which a fusosome is derived. In some embodiments, a cargo is a mutant of a wild type nucleic acid or protein expressed naturally in a cell from which the fusosome is derived or is a wild type of a mutant expressed naturally in a cell from which a fusosome is derived.

[000359] In some embodiments, a cargo is loaded into a fusosome via expression in a cell from which the fusosome is derived (e.g. expression from DNA introduced via transfection, transduction, or electroporation). In some embodiments, a cargo is expressed from DNA integrated into the genome of the cell from which the fusosome is derived or maintained episosomally in the cell from which the fusosome is derived. In some embodiments, expression of a cargo is

constitutive in the cell from which the fusosome is derived. In some embodiments, expression of a cargo in the cell from which the fusosome is derived is induced. In some embodiments, expression of the cargo is induced in the cell from which the fusosome is derived immediately prior to generating the fusosome. In some embodiments, expression of a cargo in the cell from which the fusosome is derived is induced at the same time as expression of the fusogen in the cell from which the fusosome is derived.

[000360] In some embodiments, a cargo is loaded into a fusosome via electroporation into the fusosome itself or into a cell from which the fusosome is derived. In some embodiments, a cargo is loaded into a fusosome via transfection into the fusosome itself or into a cell from which the fusosome is derived.

[000361] In some aspects, the disclosure provides a fusosome composition (e.g., a pharmaceutical composition) comprising: (i) one or more of a chondrisome (e.g., as described in international application, PCT/US16/64251), a mitochondrion, an organelle (e.g., Mitochondria, Lysosomes, nucleus, cell membrane, cytoplasm, endoplasmic reticulum, ribosomes, vacuoles, endosomes, spliceosomes, polymerases, capsids, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, myofibril, cnidocyst, peroxisome, proteasome, vesicle, stress granule, and networks of organelles), or an enucleated cell, e.g., an enucleated cell comprising any of the foregoing, and (ii) a fusogen, e.g., a myomaker protein.

[000362] In embodiments, the fusogen is present in a lipid bilayer external to the mitochondrion or chondrisome. In embodiments, the chondrisome has one or more of the properties as described, for example, in international application, PCT/US16/64251, which is herein incorporated by reference in its entirety, including the Examples and the Summary of the Invention.

[000363] In some embodiments, the cargo may include one or more nucleic acid sequences, one or more polypeptides, a combination of nucleic acid sequences and/or polypeptides, one or more organelles, and any combination thereof. In some embodiments, the cargo may include one or more cellular components. In some embodiments, the cargo includes one or more cytosolic and/or nuclear components.

[000364] In some embodiments, the cargo includes a nucleic acid, e.g., DNA, nDNA (nuclear

DNA), mtDNA (mitochondrial DNA), protein coding DNA, gene, operon, chromosome, genome, transposon, retrotransposon, viral genome, intron, exon, modified DNA, mRNA (messenger RNA), tRNA (transfer RNA), modified RNA, microRNA, siRNA (small interfering RNA), tmRNA (transfer messenger RNA), rRNA (ribosomal RNA), mtRNA (mitochondrial RNA), snRNA (small nuclear RNA), small nucleolar RNA (snoRNA), SmY RNA (mRNA trans-splicing RNA), gRNA (guide RNA), TERC (telomerase RNA component), aRNA (antisense RNA), cis-NAT (Cis-natural antisense transcript), CRISPR RNA (crRNA), lncRNA (long noncoding RNA), piRNA (piwi-interacting RNA), shRNA (short hairpin RNA), tasiRNA (trans-acting siRNA), eRNA (enhancer RNA), satellite RNA, pcRNA (protein coding RNA), dsRNA (double stranded RNA), RNAi (interfering RNA), circRNA (circular RNA), reprogramming RNAs, aptamers, and any combination thereof. In some embodiments, the nucleic acid is a wild-type nucleic acid. In some embodiments, the protein is a mutant nucleic acid. In some embodiments the nucleic acid is a fusion or chimera of multiple nucleic acid sequences.

[000365] In some embodiments, DNA in the fusosome or DNA in the cell that the fusosome is derived from is edited to correct a genetic mutation using a gene editing technology, e.g. a guide RNA and CRISPR-Cas9/Cpf1, or using a different targeted endonuclease (e.g., Zinc-finger nucleases, transcription-activator-like nucleases (TALENs)). In some embodiments, the genetic mutation is linked to a disease in a subject. Examples of edits to DNA include small insertions/deletions, large deletions, gene corrections with template DNA, or large insertions of DNA. In some embodiments, gene editing is accomplished with non-homologous end joining (NHEJ) or homology directed repair (HDR). In some embodiments, the edit is a knockout. In some embodiments, the edit is a knock-in. In some embodiments, both alleles of DNA are edited. In some embodiments, a single allele is edited. In some embodiments, multiple edits are made. In some embodiments, the fusosome or cell is derived from a subject, or is genetically matched to the subject, or is immunologically compatible with the subject (e.g. having similar MHC).

[000366] In some embodiments, the cargo may include a nucleic acid. For example, the cargo may comprise RNA to enhance expression of an endogenous protein (e.g., in some embodiments, endogenous relative to the source cell, and in some embodiments, endogenous relative to the target cell), or a siRNA or miRNA that inhibits protein expression of an endogenous

protein. For example, the endogenous protein may modulate structure or function in the target cells. In some embodiments, the cargo may include a nucleic acid encoding an engineered protein that modulates structure or function in the target cells. In some embodiments, the cargo is a nucleic acid that targets a transcriptional activator that modulate structure or function in the target cells.

[000367] In some embodiments, the cargo comprises a self-replicating RNA, e.g., as described herein. In some embodiments, the self-replicating RNA is single stranded RNA and/or linear RNA. In some embodiments, the self-replicating RNA encodes one or more proteins, e.g., a protein described herein, e.g., a membrane protein or a secreted protein. In some embodiments, the self-replicating RNA comprises a partial or complete genome from arterivirus or alphavirus, or a variant thereof.

[000368] In some embodiments, the cargo can comprise an RNA that can be delivered into a target cell, and RNA is replicated inside the target cell. Replication of the self-replicating RNA can involve RNA replication machinery that is exogenous to the host cell, and/or RNA replication machinery that is endogenous to the host cell.

[000369] In some embodiments, the self-replicating RNA comprises a viral genome, or a self-replicating portion or analog thereof. In some embodiments, the self-replicating RNA is from a positive-sense single-stranded RNA virus. In some embodiments, the self-replicating RNA comprises a partial or complete arterivirus genome, or a variant thereof. In some embodiments, the arterivirus comprises Equine arteritis virus (EAV), Porcine respiratory and reproductive syndrome virus (PRRSV), Lactate dehydrogenase elevating virus (LDV), and Simian hemorrhagic fever virus (SHFV). In some embodiments, the self-replicating RNA comprises a partial or complete alphavirus genome, or a variant thereof. In some embodiments, the alphavirus belongs to the VEEV/EEEV group (e.g., Venezuelan equine encephalitis virus), the SF group, or the SIN group.

[000370] In some embodiments, the fusosome that comprises the self-replicating RNA further comprises: (i) one or more proteins that promote replication of the RNA, or (ii) a nucleic acid encoding one or more proteins that promote replication of the RNA, e.g., as part of the self-replicating RNA or in a separate nucleic acid molecule.

[000371] In some embodiments, the self-replicating RNA lacks at least one functional gene

encoding one or more viral structural protein relative to the corresponding wild-type genome. For instance, in some embodiments the self-replicating RNA fully lacks one or more genes for viral structural proteins or comprises a non-functional mutant gene for a viral structural protein. In some embodiments, the self-replicating RNA does not comprise any genes for viral structural proteins.

[000372] In some embodiments, the self-replicating RNA comprises a viral capsid enhancer, e.g., as described in International Application WO2018/106615, which is hereby incorporated by reference in its entirety. In some embodiments, the viral capsid enhancer is an RNA structure that increases translation of a coding sequence in cis, e.g., by allowing eIF2alpha independent translation of the coding sequence. In some embodiments, a host cell has impaired translation, e.g., due to PKR-mediated phosphorylation of eIF2alpha. In embodiments, the viral capsid enhancer comprises a Downstream Loop (DLP) from a viral capsid protein, or a variant of the DLP. In some embodiments, the viral capsid enhancer is from a virus belonging to the Togaviridae family, e.g., the Alphavirus genus of the Togaviridae family. In some embodiments, the viral capsid enhancer has a sequence of SEQ ID NO: 1 of WO2018/106615 (which sequence is herein incorporated by reference in its entirety), or a sequence having at least 70%, 80%, 85%, 90%, 95%, or 99% identity thereto. In some embodiments, the sequence has the same secondary structure shown in Fig. 1 of WO2018/106615.

[000373] In some embodiments, the self-replicating RNA comprises one or more arterivirus sequences, e.g., as described in International Application WO2017/180770, which is hereby incorporated by reference in its entirety. In some embodiments, the self-replicating RNA comprises ORF7 (or a functional fragment or variant thereof) and/or the self-replicating RNA lacks a functional ORF2a (e.g., fully lacks ORF2a, or comprises a non-functional mutant of ORF2a) of an arterivirus. In some embodiments, the self-replicating RNA lacks a functional ORF2b, ORF3, ORF4, ORF5a, ORF5, or ORF6 or any combination thereof (e.g., fully lacks the sequence(s) or comprises a non-functional mutant of the sequence(s)). In some embodiments, the self-replicating RNA lacks a portion of one or more of ORF2a, ORF2b, ORF3, ORF4, ORF5a, ORF5, or ORF6. In some embodiments, the self-replicating RNA comprises one or more subgenomic (sg) promoters, e.g., situated at a non-native site. In some embodiments, the promoter comprises sg

promoter 1, sg promoter 2, sg promoter 3, sg promoter 4, sg promoter 5, sg promoter 6, sg promoter 7, or a functional fragment or variant thereof. In some embodiments, the self-replicating RNA comprises one or more transcriptional termination signals, e.g., T7 transcriptional termination signals, e.g., a mutant T7 transcription termination signal, e.g., a mutant T7 transcription termination signal comprising one or more of (e.g., any two of, or all of) T9001G, T3185A, or G3188A.

[000374] In some embodiments, the self-replicating RNA comprises a 5' UTR, e.g., a mutant alphavirus 5' UTR, e.g., as described in International Application WO2018/075235, which is hereby incorporated by reference in its entirety. In some embodiments, the mutant alphavirus 5' UTR comprises one or more nucleotide substitutions at position 1, 2, 4, or a combination thereof. In some embodiments, the mutant alphavirus 5' UTR comprises a U-> G substitution at position 2.

[000375] In some embodiments, the cargo includes a polypeptide, e.g., enzymes, structural polypeptides, signaling polypeptides, regulatory polypeptides, transport polypeptides, sensory polypeptides, motor polypeptides, defense polypeptides, storage polypeptides, transcription factors, antibodies, cytokines, hormones, catabolic polypeptides, anabolic polypeptides, proteolytic polypeptides, metabolic polypeptides, kinases, transferases, hydrolases, lyases, isomerases, ligases, enzyme modulator polypeptides, protein binding polypeptides, lipid binding polypeptides, membrane fusion polypeptides, cell differentiation polypeptides, epigenetic polypeptides, cell death polypeptides, nuclear transport polypeptides, nucleic acid binding polypeptides, reprogramming polypeptides, DNA editing polypeptides, DNA repair polypeptides, DNA recombination polypeptides, transposase polypeptides, DNA integration polypeptides, targeted endonucleases (e.g. Zinc-finger nucleases, transcription-activator-like nucleases (TALENs), cas9 and homologs thereof), recombinases, and any combination thereof. In some embodiments the protein targets a protein in the cell for degredation. In some embodiments the protein targets a protein in the cell for degredation by localizing the protein to the proteasome. In some embodiments, the protein is a wild-type protein. In some embodiments, the protein is a mutant protein. In some embodiments the protein is a fusion or chimeric protein.

[000376] In some embodiments, the cargo includes a small molecule, e.g., ions (e.g. Ca^{2+},

Cl^-, Fe^{2+}), carbohydrates, lipids, reactive oxygen species, reactive nitrogen species, isoprenoids, signaling molecules, heme, polypeptide cofactors, electron accepting compounds, electron donating compounds, metabolites, ligands, and any combination thereof. In some embodiments the small molecule is a pharmaceutical that interacts with a target in the cell. In some embodiments the small molecule targets a protein in the cell for degredation. In some embodiments the small molecule targets a protein in the cell for degredation by localizing the protein to the proteasome. In some embodiments that small molecule is a proteolysis targeting chimera molecule (PROTAC).

[000377] In some embodiments, the cargo includes a mixture of proteins, nucleic acids, or metabolites, e.g., multiple polypeptides, multiple nucleic acids, multiple small molecules; combinations of nucleic acids, polypeptides, and small molecules; ribonucleoprotein complexes (e.g. Cas9-gRNA complex); multiple transcription factors, multiple epigenetic factors, reprogramming factors (e.g. Oct4, Sox2, cMyc, and Klf4); multiple regulatory RNAs; and any combination thereof.

[000378] In some embodiments, the cargo includes one or more organelles, e.g., chondrisomes, mitochondria, lysosomes, nucleus, cell membrane, cytoplasm, endoplasmic reticulum, ribosomes, vacuoles, endosomes, spliceosomes, polymerases, capsids, acrosome, autophagosome, centriole, glycosome, glyoxysome, hydrogenosome, melanosome, mitosome, myofibril, cnidocyst, peroxisome, proteasome, vesicle, stress granule, networks of organelles, and any combination thereof.

[000379] In some embodiments, the cargo is enriched at the fusosome or cell membrane. In some embodiments, the cargo is enriched by targeting to the membrane via a peptide signal sequence. In some embodiments, the cargo is enriched by binding with a membrane associated protein, lipid, or small molecule. In some embodiments, the cargo is enriched by dimerizing with a membrane associated protein, lipid, or small molecule. In some embodiments the cargo is chimeric (e.g. a chimeric protein, or nucleic acid) and comprises a domain that mediates binding or dimerization with a membrane associated protein, lipid, or small molecule. Membrane-associated proteins of interest include, but are not limited to, any protein having a domain that stably associates, e.g., binds to, integrates into, etc., a cell membrane (i.e., a membrane-association domain), where such domains may include myristoylated domains, farnesylated domains,

transmembrane domains, and the like. Specific membrane-associated proteins of interest include, but are not limited to: myristoylated proteins, e.g., p 60 v-src and the like; farnesylated proteins, e.g., Ras, Rheb and CENP-E,F, proteins binding specific lipid bilayer components e.g. AnnexinV, by binding to phosphatidyl-serine, a lipid component of the cell membrane bilayer and the like; membrane anchor proteins; transmembrane proteins, e.g., transferrin receptors and portions thereof; and membrane fusion proteins. In some embodiments, the membrane associated protein contains a first dimerization domain. The first dimerization domain may be, e.g., a domain that directly binds to a second dimerization domain of a cargo or binds to a second dimerization domain via a dimerization mediator. In some embodiments the cargo contains a second dimerization domain. The second dimerization domain may be, e.g., a domain that dimerizes (e.g., stably associates with, such as by non-covalent bonding interaction, either directly or through a mediator) with the first dimerization domain of the membrane associated protein either directly or through a dimerization mediator. With respect to the dimerization domains, these domains are domains that participate in a binding event, either directly or via a dimerization mediator, where the binding event results in production of the desired multimeric, e.g., dimeric, complex of the membrane associated and target proteins. The first and second dimerization domains may be homodimeric, such that they are made up of the same sequence of amino acids, or heterodimeric, such that they are made up of differing sequences of amino acids. Dimerization domains may vary, where domains of interest include, but are not limited to: ligands of target biomolecules, such as ligands that specifically bind to particular proteins of interest (e.g., protein:protein interaction domains), such as SH2 domains, Paz domains, RING domains, transcriptional activator domains, DNA binding domains, enzyme catalytic domains, enzyme regulatory domains, enzyme subunits, domains for localization to a defined cellular location, recognition domains for the localization domain, the domains listed at URL: pawsonlab.mshri.on.ca/index.php?option=com_content&task=view&id=30&Itemid=63/, etc. In some embodiments the first dimerization domain binds nucleic acid (e.g. mRNA, miRNA, siRNA, DNA) and the second dimerization domain is a nucleic acid sequence present on the cargo (e.g. the first dimerization domain is MS2 and the second dimerization domain is the high affinity binding loop of MS2 RNA). Any convenient compound that functions as a dimerization mediator may be employed. A wide variety of compounds, including both naturally occurring and synthetic

substances, can be used as dimerization mediators. Applicable and readily observable or measurable criteria for selecting a dimerization mediator include: (A) the ligand is physiologically acceptable (i.e., lacks undue toxicity towards the cell or animal for which it is to be used); (B) it has a reasonable therapeutic dosage range; (C) it can cross the cellular and other membranes, as necessary (where in some instances it may be able to mediate dimerization from outside of the cell), and (D) binds to the target domains of the chimeric proteins for which it is designed with reasonable affinity for the desired application. A first desirable criterion is that the compound is relatively physiologically inert, but for its dimerization mediator activity. In some instances, the ligands will be non-peptide and non-nucleic acid. Additional dimerization domains are described, e.g., in US20170087087 and US20170130197, each of which is herein incorporated by reference in its entirety.

Payload Agents

[000380] The methods and compositions described herein can be used to target payload agents. For instance, payload agents can be targeted to a cellular membrane, e.g., through the use of a co-translational endoplasmic reticulum (ER) signal. The cellular membrane can be, e.g., an ER membrane, a plasma membrane, membrane of secreted and/or secretory vesicles, or lysosomal membrane. In some embodiments, payload agents are targeted for secretion. In some embodiments, the methods and compositions described herein can be used to target payloads to the lumen of an organelle (e.g. a Golgi apparatus, secretory vesicle, or lysosome) after translation in the ER.

[000381] A protein payload agent (e.g., a membrane protein payload agent or a secreted protein payload agent) may be or comprise, e.g., a protein, or a nucleic acid encoding a protein, selected from: a transmembrane protein, a cell surface protein, a protein associated with the cytosolic side of a membrane, an endoplasmic reticulum protein, a lysosome protein, a Golgi apparatus protein, a secreted protein, a secretory vesicle protein, or an endosomal protein, or a combination thereof. In some embodiments, the membrane protein payload agent is an exogenous version of a protein that is naturally present in or targeted to the target membrane. In some embodiments, the membrane protein payload agent is not naturally present or targeted to the target membrane. In some embodiments, a protein payload agent (e.g., a membrane protein payload agent

or a secreted protein payload agent) may be or comprise, e.g., a protein, or a nucleic acid encoding a protein, selected from: a cell surface receptor protein, a transporter, an ion channel, membrane associated enzyme, a cell adhesion protein, an immunoglobulin, a T cell receptor, an endoplasmic reticulum protein, a lysosome protein, a Golgi apparatus protein, a secreted protein, a secretory vesicle protein, an endosomal protein. A membrane protein payload agent may be, e.g., a recombinant version of a naturally occurring membrane protein, or a synthetic protein, e.g., a protein having a sequence not found in nature or domains not found together in nature, e.g., a chimeric membrane protein, e.g., a transmembrane protein having an extracellular domain derived from a first naturally occurring protein and a transmembrane domain and/or intracellular domain derived from a second naturally occurring protein, e.g., a chimeric antigen receptor.

[000382] In some embodiments, a fusosome comprises a protein payload agent (e.g., a membrane protein payload agent or a secreted protein payload agent). In some embodiments, a protein payload agent is a protein and/or a nucleic acid that encodes it. In some embodiments the protein is expressed in a cell line and then incorporated into a fusosome. A person of ordinary skill will appreciate that to the extent any such protein is expressed by the cell line, the cell line is capable of any post-translational processing necessary to make the protein. In some embodiments post-translational processing comprises one or more of protein splicing, protein cleavage, protein folding, protein glycosylation, dimerization, etc.

[000383] In some embodiments, the protein (e.g., membrane protein or secreted protein) is expressed by the source cell from which a fusosome is derived. A person of ordinary skill will appreciate that to the extent any such protein is expressed by the source cell, the source cell is capable of any post-translational processing necessary to make the protein. In some embodiments post-translational processing comprises one or more of protein splicing, protein cleavage, protein folding, protein glycosylation, dimerization, etc. In some embodiments a protein payload agent is a nucleic acid. In some embodiments the nucleic acid encodes a cell surface protein. In some embodiments the nucleic acid encodes an endoplasmic reticulum protein, a lysosome protein, a Golgi apparatus protein, a secreted protein, a secretory vesicle protein, or an endosomal protein. In some embodiments a protein payload agent, e.g., membrane protein payload agent, is a protein or nucleic acid encoding a protein selected from the cell surface antigens described herein. In

some embodiments the nucleic acid encodes an engineered cell surface protein. In some embodiments an engineered cell surface protein is a chimeric antigen receptor.

[000384] In some embodiments, a protein payload agent (e.g., a membrane protein payload agent or a secreted protein payload agent) comprises a nucleic acid which is expressed by the fused target cell. A person of ordinary skill will appreciate that to the extent any protein produced by expression of a nucleic acid protein membrane payload agent requires post-translational processing, such post-translational processing will be performed in the fused target cell. In some embodiments post-translational may comprise one or more of protein splicing, protein cleavage, protein folding, protein glycosylation, dimerization, etc. In some embodiments, the post-translational modification is a covalent attachment of a lipid, such as a fatty acid, isoprenoid, sterol, phospholipid, glycosylphosphatidyl inositol (GPI), cholesterol, farnesyl, geranylgeranyl, myristoyl, palmitoyl, which in some embodiments targets the protein to the plasma membrane.

[000385] In some embodiments, the protein payload agent (e.g., a membrane protein payload agent or a secreted protein payload agent) comprises a nucleic acid, e.g., RNA or DNA. In some embodiments, the nucleic acid is, comprises, or consists of one or more natural nucleic acid residues. In some embodiments, the nucleic acid is, comprises, or consists of one or more nucleic acid analogs. In some embodiments, the nucleic acid has a nucleotide sequence that encodes a functional gene product such as an RNA or protein. In some embodiments, the nucleic acid includes one or more introns. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, the nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 1 10, 120, 130, 140, 150, 160, 170, 180, 190, 20, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long. In some embodiments, the nucleic acid is partly or wholly single stranded; in some embodiments, the nucleic acid is partly or wholly double stranded. In some embodiments the nucleic acid has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide. The nucleic acid may incude variants, e.g., having an overall sequence identity with a reference nucleic

acid of at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99%. In some embodiments, a variant nucleic acid does not share at least one characteristic sequence element with a reference nucleic acid. In some embodiments, a variant nucleic acid shares one or more of the biological activities of the reference nucleic acid. In some embodiments, a nucleic acid variant has a nucleic acid sequence that is identical to that of the reference but for a small number of sequence alterations at particular positions. In some embodiments, fewer than about 20%, about 15%, about 10%, about 9%, about 8%, about 7%, about 6%, about 5%, about 4%, about 3%, or about 2% of the residues in a variant are substituted, inserted, or deleted, as compared to the reference. In some embodiments, a variant nucleic acid comprises about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, about 2, or about 1 substituted residues as compared to a reference. In some embodiments, a variant nucleic acid comprises a very small number (e.g., fewer than about 5, about 4, about 3, about 2, or about 1) number of substituted, inserted, or deleted, functional residues that participate in a particular biological activity relative to the reference. In some embodiments, a variant nucleic acid comprises not more than about 15, about 12, about 9, about 3, or about 1 addition or deletion, and, in some embodiments, comprises no additions or deletions, as compared to the reference. In some embodiments, a variant nucleic acid comprises fewer than about 27, about 24, about 21, about 18, about 15, about 12, about 9, about 6, about 3, or fewer than about 9, about 6, about 3, or about 2 additions or deletions as compared to the reference.

[000386] In some embodiments, the protein payload agent (e.g., a membrane protein payload agent or a secreted protein payload agent) comprises a protein. The protein may include moieties other than amino acids (*e.g.*, may be glycoproteins, proteoglycans, etc.) and/or may be otherwise processed or modified. The protein can sometimes include more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. The protein may contain L-amino acids, D-amino acids, or both and may contain any of a variety of amino acid modifications or analogs. In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof. A polypeptide may incude its variants, e.g., having an overall sequence identity with a reference polypeptide of at least 85%, 86%, 87%, 88%, 89%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, or 99%. In some embodiments, a variant polypeptide does not share at least one characteristic sequence element with a reference polypeptide. In some embodiments, a variant polypeptide shares one or more of the biological activities of the reference polypeptide. In some embodiments, a polypeptide variant has an amino acid sequence that is identical to that of the reference but for a small number of sequence alterations at particular positions. In some embodiments, fewer than about 20%, about 15%, about 10%, about 9%, about 8%, about 7%, about 6%, about 5%, about 4%, about 3%, or about 2% of the residues in a variant are substituted, inserted, or deleted, as compared to the reference. In some embodiments, a variant polypeptide comprises about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, about 2, or about 1 substituted residues as compared to a reference. In some embodiments, a variant polypeptide comprises a very small number (e.g., fewer than about 5, about 4, about 3, about 2, or about 1) number of substituted, inserted, or deleted, functional that participate in a particular biological activity relative to the reference. In some embodiments, a variant polypeptide comprises not more than about 5, about 4, about 3, about 2, or about 1 addition or deletion, and, in some embodiments, comprises no additions or deletions, as compared to the reference. In some embodiments, a variant polypeptide comprises fewer than about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 10, about 9, about 8, about 7, about 6, and commonly fewer than about 5, about 4, about 3, or about 2 additions or deletions as compared to the reference.

Signal Sequences

[000387] In some embodiments, a protein payload agent (e.g., a membrane protein payload agent or a secreted protein payload agent) is a protein (or nucleic acid encoding it) that includes or included a signal sequence directing the protein to a particular site or location (e.g., to the cell surface). Those skilled in the art will appreciate that, in certain instances, a cell uses "sorting signals" which are amino acid motifs that are at least temporarily part of a protein (e.g., when initially produced), to target the protein to particular subcellular location (e.g., to a particular organelle or surface membrane of a target cell). In some embodiments a sorting signal is a signal sequence, a signal peptide, or a leader sequence, which directs a protein to an organelle called the endoplasmic reticulum (ER); in some such embodiments, the protein is then delivered to the

plasma membrane. See US20160289674A1. In some such embodiments, the protein is then secreted. In some such embodiments, the protein is then trafficked to the lysosome. In some such embodiments, the protein is then trafficked to the Golgi apparatus. In some such embodiments, the protein is then trafficked to a secretory vesicle, and may then be secreted from the cell. In some such embodiments, the protein is then trafficked to an endosome.

[000388] In some embodiments, protein targeting to the ER is cotranslational. In some embodiments protein translocation and membrane insertion are coupled to protein synthesis. In some embodiments a signal sequence may be hydrophobic. In some embodiments a signal sequence may be partially hydrophobic. In some embodiments a signal sequence is recognized by a signal recognition particle (SRP). In some embodiments the SRP recognizing a signal sequence as it emerges from a ribosome. In some embodiments, a nascent peptide chain-ribosome complex is targeted to the ER by binding to an SRP receptor. In some embodiments a signal sequence interacts with an Sec61α subunit of a translocon and initiates translocation of a membrane protein or partial chain of said membrane protein.

[000389] In some embodiments, a membrane protein payload agent comprises an in-frame fusion of a protein of interest to the coding sequence of a transmembrane protein, or an in-frame fusion of a protein of interest to the transmembrane domain or membrane-anchoring domain of a protein (e.g. fusion to the transferrin receptor membrane anchor domain). See, e.g., Winndard, P, et al. Development of novel chimeric transmembrane proteins for multimodality imaging of cancer cells, Cancer Biology & Therapy. 12:1889-1899 (2007).

[000390] In some embodiments a sorting signal or signal peptide is appended to the N or C terminus of a protein (e.g., membrane protein or secreted protein). See Goder, V. & Spiess, M., Topogenesis of membrane proteins: determinants and dynamics. FEBS Letters. 504(3): 87-93 (2001). In some embodiments the protein is a natural protein. In some embodiments the membrane protein is a synthetic protein.

[000391] In some embodiments, a signal emerges from ribosome only after translation of a transcript has reached a stop codon. In some embodiments insertion of a membrane protein is post-translational.

[000392] In some embodiments a signal sequence is selected from Table 4. In some

embodiments a signal sequence comprises a sequence selected from Table 4. In some embodiments a signal sequence of Table 4 may be appended to the N-terminus of a protein, e.g., a membrane protein or secreted protein. In some embodiments a signal sequence of Table 4 may be appended to the C-terminus of a protein, e.g., a membrane protein or secreted protein. A person of ordinary skill will appreciate that the signal sequences below are not limited for use with their respective naturally associated proteins. In some embodiments, the nucleic acid includes one or more regulatory elements that direct expression of sequences encoding the membrane protein by the target cell.

Table 4: Exemplary signal sequences.

SEQ ID NO:	Signal Sequence	Naturally Associated Protein	Location Associated Protein is Directed
2	MRVKEKYQHL WRWGWKWGTM LLGILMICSA TE	HIV-1 gp41	Plasma membrane
3	CAAL	p21ras	Plasma membrane
4	KKKKKK	p21ras	Plasma membrane
5	RRRRR	p21ras	Plasma membrane
6	MRLLLALLGV LLSVPGPPVL S	FGFR4	Plasma membrane
7	CSIMNLMCGS TC	ROP7 GTPase	Plasma membrane
8	GHKSEEKREK MKRTLLKDWK TRLSYFLQNS STPGKPKTGK KSKQQ	RGS2	Plasma membrane
9	RSTLKLTTLQ CQYSTVMD	LHR	Plasma membrane \ basolateral cell surface

SEQ ID NO:	Signal Sequence	Naturally Associated Protein	Location Associated Protein is Directed
10	RQGLHNMEDV YELIENSH	TSHR	Plasma membrane \ basolateral cell surface
11	MDCRKMARFS YSVIWIMAIS KVFELGLVAG	TDGF1	Plasma membrane
12	MPAWGALFLL WATAEA	(GP)IX	Plasma membrane
13	RDYR	VPAC2	Plasma membrane
14	KMALRVALNN KQSGQITVKT SSSDHLSLAI AGLVPIALSI YQKFKPGVSP SYSIY	Toc159	Plasma membrane
15	MGSKIVQVFL MLALFATSAL A	Classical arabinogalactan protein 4	Plasma membrane
16	MNSKAMQALI FLGFLATSCL A	Classical arabinogalactan protein 2	Plasma membrane
17	MGAAASIQTT VN	L1R	Plasma membrane
	SVM	GTP-binding protein Rheb	Plasma membrane
18	FALLGTHGAS G	CD147	Plasma membrane
19	RRRTFLK	PlcH	Plasma membrane
20	MGGKWSKSSV	Nef	Plasma membrane

SEQ ID NO:	Signal Sequence	Naturally Associated Protein	Location Associated Protein is Directed
21	DDPERE	Nef	Plasma membrane
22	EEANTGENNS LLHPMS	HIV-1 NA7	Plasma membrane
23	SRRGLV	DmsA	Plasma membrane
24	SRRRFL	TorA/ TorA-MalE	Plasma membrane
25	SRRQFI	SufI	Plasma membrane \ periplasm
26	QRRDFL	YacK	Plasma membrane \ periplasm
27	MNKIYSIKYS AATGGLIAVS ELAKKVICKT NRKISAALLS LAVISYTNII YA	Pet (Serine protease pet autotransporter)	Plasma membrane
28	MNPNQKIITI GSICMVIGIV SLMLQIGNII SIWVSHSIQT	Influenza A Neuraminidase	Plasma Membrane
29	LRCLACSCFR TPVWPR	prRDH	Plasma Membrane
30	MGCGCSSHPE	Lck	Plasma Membrane
31	MPFVNKQFN	BoNT/ A-LC	Plasma Membrane
32	DEQNAKNAAQ DRNSNKSSKG FFSKLGCC	Yck2p	Plasma Membrane
33	MLCCMRRTKQ	GAP-43	Plasma Membrane
34	VTNGSTYILV PLSH	FSHR	Plasma Membrane
35	AETENFV	M3 mAChR	Plasma Membrane

SEQ ID NO:	Signal Sequence	Naturally Associated Protein	Location Associated Protein is Directed
36	RARHRRNVDR VSIGSYRT	pIgR	Plasma Membrane
37	YEDQ	RhBG	Plasma Membrane
38	LLVTSLLLCELPHPAFL IP	GM-CSF Receptor (GM_CSFR)	Plasma membrane

Membrane Proteins Payload Agents

[000393] In some embodiments a membrane protein payload agent is a protein (or a nucleic acid that encodes it) that is naturally found on a membrane surface of a cell (e.g., on a surface of a plasma membrane).

[000394] Exemplary membrane proteins (and/or nucleic acids encoding them) can be found, for example, in U.S. Patent Publication No. 2016/0289674, the contents of which are hereby incorporated by reference. In some embodiments, a membrane protein payload agent (and/or a nucleic acid that encodes it) has a sequence as set forth in any one of SEQ ID NOs: 8144-16131 of U.S. Patent Publication No. 2016/0289674, or in a functional fragment tereof. In some embodiments, a membrane protein payload agent is a plasma membrane protein (nucleic acid encoding it) as set forth in any one of SEQ ID NOs: 8144-16131 of U.S. Patent Publication No. 2016/0289674, or a fragment, variant, or homolog thereof (or nucleic acid that encodes it) of a plasma membrane protein of.

[000395] In some embodiments, a membrane protein relevant to the present disclosure is a therapeutic membrane protein. In some embodiments, a membrane protein relevant to the present disclosure is or comprises a cell surface receptor, a membrane transport protein (e.g., an active or passive transport protein such as, for example, an ion channel protein, a pore-forming protein [e.g., a toxin protein], etc), a membrane enzyme, and/or a cell adhesion protein).

[000396] In some embodiments a membrane protein is a single spanning membrane protein. In some embodiments a single-spanning membrane protein may assume a final topology with a cytoplasmic N- and an exoplasmic C-terminus (N_{cyt}/C_{exo}) or with the opposite orientation (N_{exo}/C_{cyt}).

[000397] In some embodiments a membrane protein is a Type I membrane protein comprising an N-terminal cleavable signal sequence and stop-transfer sequence (N_{exo}/C_{cyt}). In some embodiments a signal is at the C terminus. In some embodiments the N-terminal cleavable signal sequence targets nascent peptide to the ER. In some embodiments an N-terminal cleavable signal sequence comprises a hydrophobic stretch of typically 7–15 predominantly apolar residues. In some embodiments a Type I membrane protein comprises a stop-transfer sequence which halts the further translocation of the polypeptide and acts as a transmembrane anchor. In some embodiments a stop transfer sequence comprises an amino acid sequence of about 20 hydrophobic residues. In some embodiments the N-terminus of the Type I membrane protein is extracellular and the C-terminus is cytoplasmic. In some embodiments a Type I membrane protein may be a glycophorin or an LDL receptor.

[000398] In some embodiments a membrane protein is a Type II membrane protein comprising a signal-anchor sequence (N_{cyt}/C_{exo}). In some embodiments a signal is at the C terminus. In some embodiments a signal-anchor sequence is responsible for both insertion and anchoring of a Type II membrane protein. In some embodiments a signal-anchor sequence comprises about 18-25 predominantly apolar residues. In some embodiments a signal-anchor sequence lacks a signal peptidase cleavage site. In some embodiments a signal-anchor sequence may be positioned internally within a polypeptide chain. In some embodiments a signal-anchor sequence induces translocation of the C-terminal end of a protein across a cell membrane. In some embodiments the C-terminus of the Type II membrane protein is extracellular and the N-terminus is cytoplasmic. In some embodiments a Type II membrane protein may be a transferrin receptor or a galactosyl transferase receptor.

[000399] In some embodiments a membrane protein is a Type III membrane protein comprising a reverse signal-anchor sequence (N_{exo}/C_{cyt}). In some embodiments a signal is at the N terminus. In some embodiments a reverse signal-anchor sequence is responsible for both

insertion and anchoring of a Type III membrane protein. In some embodiments a reverse signal-anchor sequence comprises about 18-25 predominantly apolar residues. In some embodiments a signal-anchor sequence lacks a signal peptidase cleavage site. In some embodiments a signal-anchor sequence may be positioned internally within a polypeptide chain. In some embodiments a signal-anchor sequence induces translocation of the N-terminal end of a protein across a cell membrane. In some embodiments the N-terminus of the Type III membrane protein is extracellular and the C-terminus is cytoplasmic. In some embodiments a Type I membrane protein may be a synaptogamin, neuregulin, or cytochrome P-450.

[000400] In some embodiments, Type I, Type II, or Type III membrane proteins are inserted into a cell membrane via a cellular pathway comprising SRP, SRP receptor and Sec61 translocon.

[000401] In some embodiments a membrane protein is predominantly exposed to cytosol and anchored to a membrane by a C-terminal signal sequence, but which does not interact with an SRP. In some embodiments a protein is cytochrome b_5, or a SNARE protein (e.g., synaptobrevin).

[000402] In some embodiments a membrane protein payload agent comprises a signal sequence which localizes the payload membrane protein to the cell membrane. In some embodiments a membrane protein payload agent is a nucleic acid wherein the nucleic acid encodes a signal sequence which localizes a payload membrane protein encoded by the nucleic acid to the cell membrane.

(i) Integrin Membrane Protein Payloads

[000403] In some embodiments, a membrane protein payload agent is or compromises an integrin or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises an integrin selected from Table 5, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 5, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 5.

Table 5: Exemplary integrin proteins

UniProt ID	Entry name	Gene names
P05556	ITB1_HUMAN	ITGB1 FNRB MDF2 MSK12
P05106	ITB3_HUMAN	ITGB3 GP3A
P06756	ITAV_HUMAN	ITGAV MSK8 VNRA VTNR
P05107	ITB2_HUMAN	ITGB2 CD18 MFI7
P17301	ITA2_HUMAN	ITGA2 CD49B
P23229	ITA6_HUMAN	ITGA6
P26006	ITA3_HUMAN	ITGA3 MSK18
P16144	ITB4_HUMAN	ITGB4
P08514	ITA2B_HUMAN	ITGA2B GP2B ITGAB
P08648	ITA5_HUMAN	ITGA5 FNRA
P13612	ITA4_HUMAN	ITGA4 CD49D
P20701	ITAL_HUMAN	ITGAL CD11A
P11215	ITAM_HUMAN	ITGAM CD11B CR3A
P26010	ITB7_HUMAN	ITGB7
P20702	ITAX_HUMAN	ITGAX CD11C
P56199	ITA1_HUMAN	ITGA1
Q9UKX5	ITA11_HUMAN	ITGA11 MSTP018
P18084	ITB5_HUMAN	ITGB5
Q13683	ITA7_HUMAN	ITGA7 UNQ406/PRO768
P53708	ITA8_HUMAN	ITGA8
P18564	ITB6_HUMAN	ITGB6
P26012	ITB8_HUMAN	ITGB8
P38570	ITAE_HUMAN	ITGAE
O75578	ITA10_HUMAN	ITGA10 UNQ468/PRO827
Q13349	ITAD_HUMAN	ITGAD
Q13797	ITA9_HUMAN	ITGA9

(ii) Ion Channel Proteins

[000404] In some embodiments, a membrane protein payload agent is or compromises an ion channel protein or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it.

In some embodiments a membrane protein payload agent is or compromises an ion channel protein selected from Table 6, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 6, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 6.

Table 6: Exemplary ion channel proteins

Uniprot ID	Entry name	Gene names
P46098	5HT3A_HUMAN	HTR3A 5HT3R HTR3
O95264	5HT3B_HUMAN	HTR3B
Q8WXA8	5HT3C_HUMAN	HTR3C
Q70Z44	5HT3D_HUMAN	HTR3D
A5X5Y0	5HT3E_HUMAN	HTR3E
P02708	ACHA_HUMAN	CHRNA1 ACHRA CHNRA
P11230	ACHB_HUMAN	CHRNB1 ACHRB CHRNB
Q07001	ACHD_HUMAN	CHRND ACHRD
Q04844	ACHE_HUMAN	CHRNE ACHRE
P07510	ACHG_HUMAN	CHRNG ACHRG
P78348	ASIC1_HUMAN	ASIC1 ACCN2 BNAC2
Q16515	ASIC2_HUMAN	ASIC2 ACCN ACCN1 BNAC1 MDEG
Q9UHC3	ASIC3_HUMAN	ASIC3 ACCN3 SLNAC1 TNAC1
Q96FT7	ASIC4_HUMAN	ASIC4 ACCN4
Q9NY37	ASIC5_HUMAN	ASIC5 ACCN5 HINAC
P37088	SCNNA_HUMAN	SCNN1A SCNN1
P51168	SCNNB_HUMAN	SCNN1B
P51172	SCNND_HUMAN	SCNN1D DNACH
P51170	SCNNG_HUMAN	SCNN1G

Uniprot ID	Entry name	Gene names
P48048	KCNJ1_HUMAN	KCNJ1 ROMK1
P78508	KCJ10_HUMAN	KCNJ10
Q14654	KCJ11_HUMAN	KCNJ11
Q14500	KCJ12_HUMAN	KCNJ12 IRK2 KCNJN1
Q9UNX9	KCJ14_HUMAN	KCNJ14 IRK4
Q99712	KCJ15_HUMAN	KCNJ15 KCNJ14
Q15842	KCNJ8_HUMAN	KCNJ8
Q9UGQ2	FLOWR_HUMAN	CACFD1 C9orf7 PSEC0107 PSEC0248 UNQ3071/PRO9903
Q12791	KCMA1_HUMAN	KCNMA1 KCNMA SLO
Q8NEC5	CTSR1_HUMAN	CATSPER1
Q96P56	CTSR2_HUMAN	CATSPER2
Q86XQ3	CTSR3_HUMAN	CATSPER3
Q7RTX7	CTSR4_HUMAN	CATSPER4
P29973	CNGA1_HUMAN	CNGA1 CNCG CNCG1
Q96S66	CLCC1_HUMAN	CLCC1 KIAA0761 MCLC
P35523	CLCN1_HUMAN	CLCN1 CLC1
P51788	CLCN2_HUMAN	CLCN2
P51800	CLCKA_HUMAN	CLCNKA
P51801	CLCKB_HUMAN	CLCNKB
O00299	CLIC1_HUMAN	CLIC1 G6 NCC27
O15247	CLIC2_HUMAN	CLIC2
O95833	CLIC3_HUMAN	CLIC3
Q9Y696	CLIC4_HUMAN	CLIC4
Q9NZA1	CLIC5_HUMAN	CLIC5
Q96NY7	CLIC6_HUMAN	CLIC6 CLIC1L
P51797	CLCN6_HUMAN	CLCN6 KIAA0046
Q494W8	CRFM7_HUMAN	CHRFAM7A
Q16281	CNGA3_HUMAN	CNGA3 CNCG3
Q8IV77	CNGA4_HUMAN	CNGA4

Uniprot ID	Entry name	Gene names
Q14028	CNGB1_HUMAN	CNGB1 CNCG2 CNCG3L CNCG4 RCNC2
Q9NQW8	CNGB3_HUMAN	CNGB3
Q16280	CNGA2_HUMAN	CNGA2 CNCA CNCA1 CNCG2
P48549	KCNJ3_HUMAN	KCNJ3 GIRK1
P48051	KCNJ6_HUMAN	KCNJ6 GIRK2 KATP2 KCNJ7
Q92806	KCNJ9_HUMAN	KCNJ9 GIRK3
P48544	KCNJ5_HUMAN	KCNJ5 GIRK4
P14867	GBRA1_HUMAN	GABRA1
P47869	GBRA2_HUMAN	GABRA2
P34903	GBRA3_HUMAN	GABRA3
P48169	GBRA4_HUMAN	GABRA4
P31644	GBRA5_HUMAN	GABRA5
Q16445	GBRA6_HUMAN	GABRA6
P18505	GBRB1_HUMAN	GABRB1
P47870	GBRB2_HUMAN	GABRB2
P28472	GBRB3_HUMAN	GABRB3
O14764	GBRD_HUMAN	GABRD
P78334	GBRE_HUMAN	GABRE
Q8N1C3	GBRG1_HUMAN	GABRG1
P18507	GBRG2_HUMAN	GABRG2
Q99928	GBRG3_HUMAN	GABRG3
O00591	GBRP_HUMAN	GABRP
P24046	GBRR1_HUMAN	GABRR1
P28476	GBRR2_HUMAN	GABRR2
A8MPY1	GBRR3_HUMAN	GABRR3
Q9UN88	GBRT_HUMAN	GABRQ
P42261	GRIA1_HUMAN	GRIA1 GLUH1 GLUR1
P42262	GRIA2_HUMAN	GRIA2 GLUR2
P42263	GRIA3_HUMAN	GRIA3 GLUR3 GLURC
P48058	GRIA4_HUMAN	GRIA4 GLUR4
Q9ULK0	GRID1_HUMAN	GRID1 KIAA1220
O43424	GRID2_HUMAN	GRID2 GLURD2

Uniprot ID	Entry name	Gene names
P39086	GRIK1_HUMAN	GRIK1 GLUR5
Q13002	GRIK2_HUMAN	GRIK2 GLUR6
Q13003	GRIK3_HUMAN	GRIK3 GLUR7
Q16099	GRIK4_HUMAN	GRIK4 GRIK
Q16478	GRIK5_HUMAN	GRIK5 GRIK2
Q05586	NMDZ1_HUMAN	GRIN1 NMDAR1
Q12879	NMDE1_HUMAN	GRIN2A NMDAR2A
Q13224	NMDE2_HUMAN	GRIN2B NMDAR2B
Q14957	NMDE3_HUMAN	GRIN2C NMDAR2C
O15399	NMDE4_HUMAN	GRIN2D GluN2D NMDAR2D
Q8TCU5	NMD3A_HUMAN	GRIN3A KIAA1973
O60391	NMD3B_HUMAN	GRIN3B
P23415	GLRA1_HUMAN	GLRA1
P23416	GLRA2_HUMAN	GLRA2
O75311	GLRA3_HUMAN	GLRA3
Q5JXX5	GLRA4_HUMAN	GLRA4
P48167	GLRB_HUMAN	GLRB
P51790	CLCN3_HUMAN	CLCN3
P51793	CLCN4_HUMAN	CLCN4
P51795	CLCN5_HUMAN	CLCN5 CLCK2
P51798	CLCN7_HUMAN	CLCN7
O15554	KCNN4_HUMAN	KCNN4 IK1 IKCA1 KCA4 SK4
O60928	KCJ13_HUMAN	KCNJ13
Q9NPI9	KCJ16_HUMAN	KCNJ16
B7U540	KCJ18_HUMAN	KCNJ18

Uniprot ID	Entry name	Gene names
P63252	KCNJ2_HUMAN	KCNJ2 IRK1
P48050	KCNJ4_HUMAN	KCNJ4 IRK3
Q9GZZ6	ACH10_HUMAN	CHRNA10 NACHRA10
Q15822	ACHA2_HUMAN	CHRNA2
P32297	ACHA3_HUMAN	CHRNA3 NACHRA3
P43681	ACHA4_HUMAN	CHRNA4 NACRA4
P30532	ACHA5_HUMAN	CHRNA5 NACHRA5
Q15825	ACHA6_HUMAN	CHRNA6
P36544	ACHA7_HUMAN	CHRNA7 NACHRA7
Q9UGM1	ACHA9_HUMAN	CHRNA9 NACHRA9
P17787	ACHB2_HUMAN	CHRNB2
Q05901	ACHB3_HUMAN	CHRNB3
P30926	ACHB4_HUMAN	CHRNB4
O00180	KCNK1_HUMAN	KCNK1 HOHO1 KCNO1 TWIK1
P57789	KCNKA_HUMAN	KCNK10 TREK2
Q9HB15	KCNKC_HUMAN	KCNK12
Q9HB14	KCNKD_HUMAN	KCNK13
Q9H427	KCNKF_HUMAN	KCNK15 TASK5
Q96T55	KCNKG_HUMAN	KCNK16 TALK1
Q96T54	KCNKH_HUMAN	KCNK17 TALK2 TASK4 UNQ5816/PRO19634
Q7Z418	KCNKI_HUMAN	KCNK18 TRESK TRIK
O95069	KCNK2_HUMAN	KCNK2 TREK TREK1
O14649	KCNK3_HUMAN	KCNK3 TASK TASK1
Q9NYG8	KCNK4_HUMAN	KCNK4 TRAAK
O95279	KCNK5_HUMAN	KCNK5 TASK2
Q9Y257	KCNK6_HUMAN	KCNK6 TOSS TWIK2

Uniprot ID	Entry name	Gene names
Q9Y2U2	KCNK7_HUMAN	KCNK7
Q9NPC2	KCNK9_HUMAN	KCNK9 TASK3
Q5JUK3	KCNT1_HUMAN	KCNT1 KIAA1422
Q6UVM3	KCNT2_HUMAN	KCNT2 SLICK
A8MYU2	KCNU1_HUMAN	KCNU1 KCNMA3 KCNMC1 SLO3
Q09470	KCNA1_HUMAN	KCNA1
Q16322	KCA10_HUMAN	KCNA10
P16389	KCNA2_HUMAN	KCNA2
P22001	KCNA3_HUMAN	KCNA3 HGK5
P22459	KCNA4_HUMAN	KCNA4 KCNA4L
P22460	KCNA5_HUMAN	KCNA5
P17658	KCNA6_HUMAN	KCNA6
Q96RP8	KCNA7_HUMAN	KCNA7
Q14721	KCNB1_HUMAN	KCNB1
Q92953	KCNB2_HUMAN	KCNB2
P48547	KCNC1_HUMAN	KCNC1
Q96PR1	KCNC2_HUMAN	KCNC2
Q14003	KCNC3_HUMAN	KCNC3
Q03721	KCNC4_HUMAN	KCNC4 C1orf30
Q9NSA2	KCND1_HUMAN	KCND1
Q9NZV8	KCND2_HUMAN	KCND2 KIAA1044
Q9UK17	KCND3_HUMAN	KCND3
P15382	KCNE1_HUMAN	KCNE1
A0A087WTH5	KCE1B_HUMAN	KCNE1B
Q9Y6J6	KCNE2_HUMAN	KCNE2
Q9Y6H6	KCNE3_HUMAN	KCNE3
Q8WWG9	KCNE4_HUMAN	KCNE4
Q9UJ90	KCNE5_HUMAN	KCNE5 AMMECR2 KCNE1L
Q9H3M0	KCNF1_HUMAN	KCNF1
Q9UIX4	KCNG1_HUMAN	KCNG1
Q9UJ96	KCNG2_HUMAN	KCNG2 KCNF2

Uniprot ID	Entry name	Gene names
Q8TAE7	KCNG3_HUMAN	KCNG3
Q8TDN1	KCNG4_HUMAN	KCNG4 KCNG3
O95259	KCNH1_HUMAN	KCNH1 EAG EAG1
Q12809	KCNH2_HUMAN	KCNH2 ERG ERG1 HERG
Q9ULD8	KCNH3_HUMAN	KCNH3 KIAA1282
Q9UQ05	KCNH4_HUMAN	KCNH4
Q8NCM2	KCNH5_HUMAN	KCNH5 EAG2
Q9H252	KCNH6_HUMAN	KCNH6 ERG2
Q9NS40	KCNH7_HUMAN	KCNH7 ERG3
Q96L42	KCNH8_HUMAN	KCNH8
P51787	KCNQ1_HUMAN	KCNQ1 KCNA8 KCNA9 KVLQT1
O43526	KCNQ2_HUMAN	KCNQ2
O43525	KCNQ3_HUMAN	KCNQ3
P56696	KCNQ4_HUMAN	KCNQ4
Q9NR82	KCNQ5_HUMAN	KCNQ5
Q96KK3	KCNS1_HUMAN	KCNS1
Q9ULS6	KCNS2_HUMAN	KCNS2 KIAA1144
Q9BQ31	KCNS3_HUMAN	KCNS3
Q6PIU1	KCNV1_HUMAN	KCNV1
Q8TDN2	KCNV2_HUMAN	KCNV2
O60741	HCN1_HUMAN	HCN1 BCNG1
Q9UL51	HCN2_HUMAN	HCN2 BCNG2
Q9P1Z3	HCN3_HUMAN	HCN3 KIAA1535
Q9Y3Q4	HCN4_HUMAN	HCN4
Q92952	KCNN1_HUMAN	KCNN1 SK
Q9H2S1	KCNN2_HUMAN	KCNN2
Q9UGI6	KCNN3_HUMAN	KCNN3 K3
P35498	SCN1A_HUMAN	SCN1A NAC1 SCN1
Q9Y5Y9	SCNAA_HUMAN	SCN10A
Q9UI33	SCNBA_HUMAN	SCN11A SCN12A SNS2
Q99250	SCN2A_HUMAN	SCN2A NAC2 SCN2A1 SCN2A2

Uniprot ID	Entry name	Gene names
Q9NY46	SCN3A_HUMAN	SCN3A KIAA1356 NAC3
P35499	SCN4A_HUMAN	SCN4A
Q14524	SCN5A_HUMAN	SCN5A
Q01118	SCN7A_HUMAN	SCN7A SCN6A
Q9UQD0	SCN8A_HUMAN	SCN8A MED
Q15858	SCN9A_HUMAN	SCN9A NENA
Q07699	SCN1B_HUMAN	SCN1B
O60939	SCN2B_HUMAN	SCN2B UNQ326/PRO386
Q9NY72	SCN3B_HUMAN	SCN3B KIAA1158
Q8IWT1	SCN4B_HUMAN	SCN4B
Q8IZF0	NALCN_HUMAN	NALCN VGCNL1
Q9ULQ1	TPC1_HUMAN	TPCN1 KIAA1169 TPC1
Q8NHX9	TPC2_HUMAN	TPCN2 TPC2
P54289	CA2D1_HUMAN	CACNA2D1 CACNL2A CCHL2A MHS3
Q9NY47	CA2D2_HUMAN	CACNA2D2 KIAA0558
Q8IZS8	CA2D3_HUMAN	CACNA2D3
Q7Z3S7	CA2D4_HUMAN	CACNA2D4
Q13936	CAC1C_HUMAN	CACNA1C CACH2 CACN2 CACNL1A1 CCHL1A1
Q01668	CAC1D_HUMAN	CACNA1D CACH3 CACN4 CACNL1A2 CCHL1A2
O60840	CAC1F_HUMAN	CACNA1F CACNAF1
Q13698	CAC1S_HUMAN	CACNA1S CACH1 CACN1 CACNL1A3
Q02641	CACB1_HUMAN	CACNB1 CACNLB1
Q08289	CACB2_HUMAN	CACNB2 CACNLB2 MYSB
P54284	CACB3_HUMAN	CACNB3 CACNLB3
O00305	CACB4_HUMAN	CACNB4 CACNLB4
Q00975	CAC1B_HUMAN	CACNA1B CACH5 CACNL1A5
O00555	CAC1A_HUMAN	CACNA1A CACH4 CACN3 CACNL1A4
Q15878	CAC1E_HUMAN	CACNA1E CACH6 CACNL1A6
O43497	CAC1G_HUMAN	CACNA1G KIAA1123
O95180	CAC1H_HUMAN	CACNA1H
Q9P0X4	CAC1I_HUMAN	CACNA1I KIAA1120

Uniprot ID	Entry name	Gene names
Q96D96	HVCN1_HUMAN	HVCN1 VSOP UNQ578/PRO1140
Q14722	KCAB1_HUMAN	KCNAB1 KCNA1B
Q13303	KCAB2_HUMAN	KCNAB2 KCNA2B KCNK2
O43448	KCAB3_HUMAN	KCNAB3 KCNA3B
Q5VU97	CAHD1_HUMAN	CACHD1 KIAA1573 VWCD1
Q401N2	ZACN_HUMAN	ZACN L2 LGICZ LGICZ1 ZAC

(iii) Pore Forming Proteins

[000405] In some embodiments, a membrane protein payload agent is or compromises a pore forming protein or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments, a pore forming protein may be a hemolysin or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a hemolysin selected from Table 7, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments, a pore forming protein may be a colicin or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a colicin selected from Table 8, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it.

[000406] In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 7, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 7.

[000407] In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 8, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or

99% identical to the nucleic acid sequence of a gene of Table 8.

Table 7: Exemplary hemolysin proteins

Uniprot ID	Entry name	Gene names
P19247	VVHA_VIBVU	vvhA VV2_0404
P09545	HLYA_VIBCH	hlyA VC_A0219
Q08677	HLY4_AERSA	ash4
P55870	HLY1_AERHH	ahh1 AHA_1512
Q4UK99	HLYC_RICFE	tlyC RF_1185
Q68W10	HLYC_RICTY	tlyC RT0725
O05961	HLYC_RICPR	tlyC RP740
A8GTI4	HLYC_RICRS	tlyC A1G_06280
Q92GI2	HLYC_RICCN	tlyC RC1141
A8F2M1	HLYC_RICM5	tlyC RMA_1168
Q93RR6	HLYE_SALPA	hlyE clyA sheA SPA1306
Q9REB3	HLYE_ECO57	hlyE clyA sheA Z1944 ECs1677
Q8Z727	HLYE_SALTI	hlyE clyA sheA STY1498 t1477
P77335	HLYE_ECOLI	hlyE clyA hpr sheA ycgD b1182 JW5181
P14711	HLYT_GRIHO	
A8GUH1	HLYC_RICB8	tlyC A1I_00305
A8EZU0	HLYC_RICCK	tlyC A1E_04760
A8GPR9	HLYC_RICAH	tlyC A1C_05795
Q1RGX2	HLYC_RICBR	tlyC RBE_1311
Q9RCT3	HLYEL_SHIFL	SF1171 S1259
Q8FI27	HLYEL_ECOL6	c1630
P28030	HLY_VIBMI	tdh
P28031	HLY1_GRIHO	tdh
P19249	HLY1_VIBPA	tdh1 tdh VPA1378
P19250	HLY2_VIBPA	tdh2 tdh trh VPA1314
P28029	HLY3_VIBPH	tdh3 tdh/I tdhX

Table 8: Exemplary colicin proteins

Uniprot ID	Entry name	Gene names
Q47500	CE05_ECOLX	cfa
Q47125	CE10_ECOLX	cta
P04480	CEA_CITFR	caa
Q47108	CEA_ECOLX	caa
P02978	CEA1_ECOLX	cea
P21178	CEA1_SHISO	cea
P04419	CEA2_ECOLX	col ceaB
P00646	CEA3_ECOLX	ceaC
P18000	CEA5_ECOLX	col
P17999	CEA6_ECOLX	
Q47112	CEA7_ECOLX	colE7 cea
P09882	CEA8_ECOLX	col
P09883	CEA9_ECOLX	col cei
P05819	CEAB_ECOLX	cba
P00645	CEAC_ECOLX	ccl
P17998	CEAD_ECOLX	cda
Q47502	CEAK_ECOLX	cka
P08083	CEAN_ECOLX	cna
P06716	CEIA_ECOLX	cia
P04479	CEIB_ECOLX	cib
P22520	CVAB_ECOLX	cvaB
Q06583	PYS1_PSEAI	pys1
Q06584	PYS2_PSEAE	pys2 PA1150

(iv) Toll-Like Receptors

[000408] In some embodiments, a membrane protein payload agent is or compromises a toll-like receptor (TLR) or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a toll-like receptor selected from Table 9, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In embodiments, a membrane protein payload agent comprises a protein having a

sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 9, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 9.

Table 9: Exemplary toll-like receptors

UniProt ID	Entry name	Gene names (primary)
Q86XR7	TCAM2_HUMAN	TICAM2
Q9BXR5	TLR10_HUMAN	TLR10
Q15399	TLR1_HUMAN	TLR1
O60603	TLR2_HUMAN	TLR2
O15455	TLR3_HUMAN	TLR3
O00206	TLR4_HUMAN	TLR4
O60602	TLR5_HUMAN	TLR5
Q9Y2C9	TLR6_HUMAN	TLR6
Q9NYK1	TLR7_HUMAN	TLR7
Q9NR97	TLR8_HUMAN	TLR8
Q9NR96	TLR9_HUMAN	TLR9

[000409] In some embodiments, a membrane protein payload agent is or compromises an interleukin receptor or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises an interleukin receptor selected from Table 10 or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 10, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 10.

(v) Interleukin Receptor Payloads

Table 10: Exemplary interleukin receptors

Uniprot ID	Entry name	Gene names (primary)
Q5EG05	CAR16_HUMAN	CARD16
Q92851	CASPA_HUMAN	CASP10
P25024	CXCR1_HUMAN	CXCR1
P25025	CXCR2_HUMAN	CXCR2
P31785	IL2RG_HUMAN	IL2RG
Q01167	FOXK2_HUMAN	FOXK2
Q8IU57	INLR1_HUMAN	IFNLR1
Q8IU54	IFNL1_HUMAN	IFNL1
Q8IZJ0	IFNL2_HUMAN	IFNL2
Q8IZI9	IFNL3_HUMAN	IFNL3
Q12905	ILF2_HUMAN	ILF2
Q12906	ILF3_HUMAN	ILF3
P01583	IL1A_HUMAN	IL1A
P01584	IL1B_HUMAN	IL1B
Q8WWZ1	IL1FA_HUMAN	IL1F10
Q9NPH3	IL1AP_HUMAN	IL1RAP
Q9NZN1	IRPL1_HUMAN	IL1RAPL1
P18510	IL1RA_HUMAN	IL1RN
P14778	IL1R1_HUMAN	IL1R1
P27930	IL1R2_HUMAN	IL1R2
P51617	IRAK1_HUMAN	IRAK1
Q5VVH5	IKBP1_HUMAN	IRAK1BP1
Q9Y616	IRAK3_HUMAN	IRAK3
Q9NWZ3	IRAK4_HUMAN	IRAK4
O43187	IRAK2_HUMAN	IRAK2
Q01638	ILRL1_HUMAN	IL1RL1
Q9HB29	ILRL2_HUMAN	IL1RL2
P22301	IL10_HUMAN	IL10
Q13651	I10R1_HUMAN	IL10RA

Uniprot ID	Entry name	Gene names (primary)
Q08334	I10R2_HUMAN	IL10RB
P20809	IL11_HUMAN	IL11
Q14626	I11RA_HUMAN	IL11RA
P42701	I12R1_HUMAN	IL12RB1
Q99665	I12R2_HUMAN	IL12RB2
P29459	IL12A_HUMAN	IL12A
P29460	IL12B_HUMAN	IL12B
P35225	IL13_HUMAN	IL13
P78552	I13R1_HUMAN	IL13RA1
Q14627	I13R2_HUMAN	IL13RA2
P40933	IL15_HUMAN	IL15
Q13261	I15RA_HUMAN	IL15RA
Q96F46	I17RA_HUMAN	IL17RA
Q9NRM6	I17RB_HUMAN	IL17RB
Q8NAC3	I17RC_HUMAN	IL17RC
Q8NFM7	I17RD_HUMAN	IL17RD
Q8NFR9	I17RE_HUMAN	IL17RE
Q16552	IL17_HUMAN	IL17A
Q9UHF5	IL17B_HUMAN	IL17B
Q9P0M4	IL17C_HUMAN	IL17C
Q8TAD2	IL17D_HUMAN	IL17D
Q96PD4	IL17F_HUMAN	IL17F
Q13478	IL18R_HUMAN	IL18R1
O95256	I18RA_HUMAN	IL18RAP
O95998	I18BP_HUMAN	IL18BP
P60568	IL2_HUMAN	IL2
P01589	IL2RA_HUMAN	IL2RA
P14784	IL2RB_HUMAN	IL2RB
Q9NYY1	IL20_HUMAN	IL20
Q9UHF4	I20RA_HUMAN	IL20RA
Q6UXL0	I20RB_HUMAN	IL20RB
Q9HBE4	IL21_HUMAN	IL21

Uniprot ID	Entry name	Gene names (primary)
Q9HBE5	IL21R_HUMAN	IL21R
Q9GZX6	IL22_HUMAN	IL22
Q8N6P7	I22R1_HUMAN	IL22RA1
Q969J5	I22R2_HUMAN	IL22RA2
Q5VWK5	IL23R_HUMAN	IL23R
Q9NPF7	IL23A_HUMAN	IL23A
Q9H293	IL25_HUMAN	IL25
Q9NPH9	IL26_HUMAN	IL26
Q6UWB1	I27RA_HUMAN	IL27RA
Q8NEV9	IL27A_HUMAN	IL27
Q14213	IL27B_HUMAN	EBI3
P08700	IL3_HUMAN	IL3
P26951	IL3RA_HUMAN	IL3RA
Q6EBC2	IL31_HUMAN	IL31
Q8NI17	IL31R_HUMAN	IL31RA
O95760	IL33_HUMAN	IL33
Q6ZMJ4	IL34_HUMAN	IL34
Q9UHA7	IL36A_HUMAN	IL36A
Q9NZH7	IL36B_HUMAN	IL36B
Q9NZH8	IL36G_HUMAN	IL36G
Q9UBH0	I36RA_HUMAN	IL36RN
Q9NZH6	IL37_HUMAN	IL37
P05112	IL4_HUMAN	IL4
P24394	IL4RA_HUMAN	IL4R
P05113	IL5_HUMAN	IL5
Q01344	IL5RA_HUMAN	IL5RA
P05231	IL6_HUMAN	IL6
P08887	IL6RA_HUMAN	IL6R
P40189	IL6RB_HUMAN	IL6ST
P13232	IL7_HUMAN	IL7
P16871	IL7RA_HUMAN	IL7R
P10145	IL8_HUMAN	CXCL8

Uniprot ID	Entry name	Gene names (primary)
P15248	IL9_HUMAN	IL9
Q01113	IL9R_HUMAN	IL9R
Q16649	NFIL3_HUMAN	NFIL3
Q99650	OSMR_HUMAN	OSMR
Q8NDX1	PSD4_HUMAN	PSD4
Q14005	IL16_HUMAN	IL16
Q6ZVW7	I17EL_HUMAN	IL17REL
O43353	RIPK2_HUMAN	RIPK2
Q6IA17	SIGIR_HUMAN	SIGIRR
Q8IUC6	TCAM1_HUMAN	TICAM1
Q86XR7	TCAM2_HUMAN	TICAM2
Q9Y4K3	TRAF6_HUMAN	TRAF6
P58753	TIRAP_HUMAN	TIRAP
Q15399	TLR1_HUMAN	TLR1
O60603	TLR2_HUMAN	TLR2
O60602	TLR5_HUMAN	TLR5
Q8TDR0	MIPT3_HUMAN	TRAF3IP1
Q13445	TMED1_HUMAN	TMED1
Q08881	ITK_HUMAN	ITK
Q9NP60	IRPL2_HUMAN	IL1RAPL2

(vi) Cell Adhesion Protein Payloads

[000410] In some embodiments, a membrane protein payload agent is or compromises a cell adhesion protein or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a cell adhesion protein selected from Table 11, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments, a cell adhesion protein may be a cadherin or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a cadherin selected from Table 12, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments, a cell adhesion protein may be a selectin or functional fragment, variant, or homolog

thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a selectin selected from Table 13, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments, a cell adhesion protein may be a mucin or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a mucin selected from Table 14, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it.

[000411] In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 11, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 11.

[000412] In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 12, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 12.

[000413] In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 13, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 13.

[000414] In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 14, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or

99% identical to the nucleic acid sequence of a gene of Table 14.

Table 11: Exemplary intercellular adhesion molecule proteins

UniProt ID	Entry name	Gene names (primary)
P05362	ICAM1_HUMAN	ICAM1
P13598	ICAM2_HUMAN	ICAM2
P32942	ICAM3_HUMAN	ICAM3
Q14773	ICAM4_HUMAN	ICAM4
Q9UMF0	ICAM5_HUMAN	ICAM5

Table 12: Exemplary cadherin proteins

Uniprot ID	Entry name	Gene names (primary)
P07949	RET_HUMAN	RET
P12830	CADH1_HUMAN	CDH1
P55290	CAD13_HUMAN	CDH13
Q9H251	CAD23_HUMAN	CDH23
P19022	CADH2_HUMAN	CDH2
O60716	CTND1_HUMAN	CTNND1
P35221	CTNA1_HUMAN	CTNNA1
P22223	CADH3_HUMAN	CDH3
P33151	CADH5_HUMAN	CDH5
Q9NYQ6	CELR1_HUMAN	CELSR1
Q14126	DSG2_HUMAN	DSG2
Q9HBB8	CDHR5_HUMAN	CDHR5
Q96JQ0	PCD16_HUMAN	DCHS1
O94985	CSTN1_HUMAN	CLSTN1
Q02487	DSC2_HUMAN	DSC2
Q6V0I7	FAT4_HUMAN	FAT4
Q02413	DSG1_HUMAN	DSG1
Q14517	FAT1_HUMAN	FAT1
Q9BYE9	CDHR2_HUMAN	CDHR2
P55291	CAD15_HUMAN	CDH15
A7KAX9	RHG32_HUMAN	ARHGAP32
Q12864	CAD17_HUMAN	CDH17

Uniprot ID	Entry name	Gene names (primary)
P55287	CAD11_HUMAN	CDH11
Q9NYQ7	CELR3_HUMAN	CELSR3
Q14574	DSC3_HUMAN	DSC3
Q9NYQ8	FAT2_HUMAN	FAT2
P55283	CADH4_HUMAN	CDH4
P55285	CADH6_HUMAN	CDH6
P55286	CADH8_HUMAN	CDH8
Q9Y6N8	CAD10_HUMAN	CDH10
P55289	CAD12_HUMAN	CDH12
Q13634	CAD18_HUMAN	CDH18
Q9UJ99	CAD22_HUMAN	CDH22
O75309	CAD16_HUMAN	CDH16
Q9H159	CAD19_HUMAN	CDH19
Q9HBT6	CAD20_HUMAN	CDH20
Q8IXH8	CAD26_HUMAN	CDH26
Q9ULB5	CADH7_HUMAN	CDH7
Q9ULB4	CADH9_HUMAN	CDH9
Q6ZTQ4	CDHR3_HUMAN	CDHR3
Q9HCU4	CELR2_HUMAN	CELSR2
Q86UP0	CAD24_HUMAN	CDH24
Q96JP9	CDHR1_HUMAN	CDHR1
A6H8M9	CDHR4_HUMAN	CDHR4
Q9UI47	CTNA3_HUMAN	CTNNA3
A4D0V7	CPED1_HUMAN	CPED1
Q08554	DSC1_HUMAN	DSC1
P32926	DSG3_HUMAN	DSG3
Q86SJ6	DSG4_HUMAN	DSG4
Q8TDW7	FAT3_HUMAN	FAT3
Q9NPG4	PCD12_HUMAN	PCDH12
O60330	PCDGC_HUMAN	PCDHGA12
Q6V1P9	PCD23_HUMAN	DCHS2
Q9UN71	PCDGG_HUMAN	PCDHGB4

Uniprot ID	Entry name	Gene names (primary)
Q08174	PCDH1_HUMAN	PCDH1

Table 13: Exemplary selectin proteins

UniProt ID	Entry name	Gene names (primary)
P16109	LYAM3_HUMAN	SELP
P16581	LYAM2_HUMAN	SELE
Q14242	SELPL_HUMAN	SELPLG
P14151	LYAM1_HUMAN	SELL

Table 14: Exemplary mucin proteins

UniProt ID	Entry name	Gene names (primary)
P15941	MUC1_HUMAN	MUC1
Q99102	MUC4_HUMAN	MUC4
Q8TDQ0	HAVR2_HUMAN	HAVCR2
Q9HC84	MUC5B_HUMAN	MUC5B
P98088	MUC5A_HUMAN	MUC5AC
Q685J3	MUC17_HUMAN	MUC17
Q9UJU6	DBNL_HUMAN	DBNL
Q9HBB8	CDHR5_HUMAN	CDHR5
Q8WXI7	MUC16_HUMAN	MUC16
Q9UHX3	AGRE2_HUMAN	ADGRE2
Q02817	MUC2_HUMAN	MUC2
Q8TAX7	MUC7_HUMAN	MUC7
Q96D42	HAVR1_HUMAN	HAVCR1
Q9H3R2	MUC13_HUMAN	MUC13
Q8N307	MUC20_HUMAN	MUC20
Q6W4X9	MUC6_HUMAN	MUC6
Q02505	MUC3A_HUMAN	MUC3A
Q7L513	FCRLA_HUMAN	FCRLA
Q14246	AGRE1_HUMAN	ADGRE1
Q86WA6	BPHL_HUMAN	BPHL
Q7Z5P9	MUC19_HUMAN	MUC19
Q9UKN1	MUC12_HUMAN	MUC12

UniProt ID	Entry name	Gene names (primary)
Q5SSG8	MUC21_HUMAN	MUC21
Q9BY15	AGRE3_HUMAN	ADGRE3
Q6UWI2	PARM1_HUMAN	PARM1
O95395	GCNT3_HUMAN	GCNT3
Q86SQ3	AGRE4_HUMAN	ADGRE4P
Q6BAA4	FCRLB_HUMAN	FCRLB
Q96DR8	MUCL1_HUMAN	MUCL1
E2RYF6	MUC22_HUMAN	MUC22
Q9H195	MUC3B_HUMAN	MUC3B
Q9ULC0	MUCEN_HUMAN	EMCN
Q8N387	MUC15_HUMAN	MUC15
Q12889	OVGP1_HUMAN	OVGP1
E2RYF7	PBMU2_HUMAN	HCG22
Q96H15	TIMD4_HUMAN	TIMD4

(vii) Transport Protein Payloads

In some embodiments, a membrane protein payload agent is or compromises a transport protein or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In some embodiments a membrane protein payload agent is or compromises a transport protein selected from Table 15, or functional fragment, variant, or homolog thereof, or a nucleic acid encoding it. In embodiments, a membrane protein payload agent comprises a protein having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the polypeptide sequence of a protein of Table 15, or a nucleic acid encoding the same. In embodiments, the membrane protein payload agent comprises a nucleic acid having a sequence at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99% identical to the nucleic acid sequence of a gene of Table 15.

Table 15: Exemplary transport proteins

UniProt ID	Entry name	Gene names (primary)
Q9NRA8	4ET_HUMAN	EIF4ENIF1

UniProt ID	Entry name	Gene names (primary)
Q9NS82	AAA1_HUMAN	SLC7A10
Q15758	AAAT_HUMAN	SLC1A5
O95477	ABCA1_HUMAN	ABCA1
Q9BZC7	ABCA2_HUMAN	ABCA2
Q99758	ABCA3_HUMAN	ABCA3
P78363	ABCA4_HUMAN	ABCA4
Q8IZY2	ABCA7_HUMAN	ABCA7
Q86UK0	ABCAC_HUMAN	ABCA12
Q9NP58	ABCB6_HUMAN	ABCB6
O75027	ABCB7_HUMAN	ABCB7
Q9NP78	ABCB9_HUMAN	ABCB9
Q9NRK6	ABCBA_HUMAN	ABCB10
Q9UG63	ABCF2_HUMAN	ABCF2
P45844	ABCG1_HUMAN	ABCG1
Q9UNQ0	ABCG2_HUMAN	ABCG2
O00400	ACATN_HUMAN	SLC33A1
P82251	BAT1_HUMAN	SLC7A9
Q8N1D0	BWR1B_HUMAN	SLC22A18AS
Q12864	CAD17_HUMAN	CDH17
Q53S99	CB083_HUMAN	C2orf83
P51790	CLCN3_HUMAN	CLCN3
P51793	CLCN4_HUMAN	CLCN4
P51795	CLCN5_HUMAN	CLCN5
P51798	CLCN7_HUMAN	CLCN7
Q9NRU3	CNNM1_HUMAN	CNNM1
Q9H8M5	CNNM2_HUMAN	CNNM2
Q8NE01	CNNM3_HUMAN	CNNM3
Q6P4Q7	CNNM4_HUMAN	CNNM4
O15431	COPT1_HUMAN	SLC31A1
O15432	COPT2_HUMAN	SLC31A2
Q8WWI5	CTL1_HUMAN	SLC44A1
Q8IWA5	CTL2_HUMAN	SLC44A2

UniProt ID	Entry name	Gene names (primary)
Q8N4M1	CTL3_HUMAN	SLC44A3
Q53GD3	CTL4_HUMAN	SLC44A4
Q8NCS7	CTL5_HUMAN	SLC44A5
P30825	CTR1_HUMAN	SLC7A1
P52569	CTR2_HUMAN	SLC7A2
Q8WY07	CTR3_HUMAN	SLC7A3
O43246	CTR4_HUMAN	SLC7A4
P43003	EAA1_HUMAN	SLC1A3
P43004	EAA2_HUMAN	SLC1A2
P43005	EAA3_HUMAN	SLC1A1
P48664	EAA4_HUMAN	SLC1A6
O00341	EAA5_HUMAN	SLC1A7
P55899	FCGRN_HUMAN	FCGRT
Q9UPI3	FLVC2_HUMAN	FLVCR2
Q96A29	FUCT1_HUMAN	SLC35C1
O43826	G6PT1_HUMAN	SLC37A4
O95528	GTR10_HUMAN	SLC2A10
Q9BYW1	GTR11_HUMAN	SLC2A11
Q8TD20	GTR12_HUMAN	SLC2A12
Q8TDB8	GTR14_HUMAN	SLC2A14
P11166	GTR1_HUMAN	SLC2A1
P11168	GTR2_HUMAN	SLC2A2
P11169	GTR3_HUMAN	SLC2A3
P14672	GTR4_HUMAN	SLC2A4
P22732	GTR5_HUMAN	SLC2A5
Q9UGQ3	GTR6_HUMAN	SLC2A6
Q6PXP3	GTR7_HUMAN	SLC2A7
Q9NY64	GTR8_HUMAN	SLC2A8
Q9NRM0	GTR9_HUMAN	SLC2A9
Q9HCP6	HHATL_HUMAN	HHATL
Q6P1K1	HRG1_HUMAN	SLC48A1
Q12756	KIF1A_HUMAN	KIF1A

UniProt ID	Entry name	Gene names (primary)
Q6ZP29	LAAT1_HUMAN	PQLC2
Q15012	LAP4A_HUMAN	LAPTM4A
Q01650	LAT1_HUMAN	SLC7A5
Q9GIP4	LAT1L_HUMAN	SLC7A5P2
Q8MH63	LAT1N_HUMAN	SLC7A5P1
Q9UHI5	LAT2_HUMAN	SLC7A8
O75387	LAT3_HUMAN	SLC43A1
Q8N370	LAT4_HUMAN	SLC43A2
Q9NUN5	LMBD1_HUMAN	LMBRD1
Q9H0U3	MAGT1_HUMAN	MAGT1
Q8N8R3	MCATL_HUMAN	SLC25A29
Q96MC6	MF14A_HUMAN	MFSD14A
Q9NYZ2	MFRN1_HUMAN	SLC25A37
Q96A46	MFRN2_HUMAN	SLC25A28
Q14728	MFS10_HUMAN	MFSD10
Q5TF39	MFS4B_HUMAN	MFSD4B
Q6N075	MFSD5_HUMAN	MFSD5
Q9H2D1	MFTC_HUMAN	SLC25A32
Q8N4V1	MMGT1_HUMAN	MMGT1
Q8TF71	MOT10_HUMAN	SLC16A10
Q8NCK7	MOT11_HUMAN	SLC16A11
Q6ZSM3	MOT12_HUMAN	SLC16A12
Q7RTY0	MOT13_HUMAN	SLC16A13
Q7RTX9	MOT14_HUMAN	SLC16A14
P53985	MOT1_HUMAN	SLC16A1
O60669	MOT2_HUMAN	SLC16A7
O95907	MOT3_HUMAN	SLC16A8
O15427	MOT4_HUMAN	SLC16A3
O15374	MOT5_HUMAN	SLC16A4
O15375	MOT6_HUMAN	SLC16A5
O15403	MOT7_HUMAN	SLC16A6
P36021	MOT8_HUMAN	SLC16A2

UniProt ID	Entry name	Gene names (primary)
Q7RTY1	MOT9_HUMAN	SLC16A9
P33527	MRP1_HUMAN	ABCC1
Q92887	MRP2_HUMAN	ABCC2
O15438	MRP3_HUMAN	ABCC3
O15439	MRP4_HUMAN	ABCC4
O15440	MRP5_HUMAN	ABCC5
O95255	MRP6_HUMAN	ABCC6
Q9HD23	MRS2_HUMAN	MRS2
Q7RTP0	NIPA1_HUMAN	NIPA1
Q8N8Q9	NIPA2_HUMAN	NIPA2
Q6NVV3	NIPA3_HUMAN	NIPAL1
Q0D2K0	NIPA4_HUMAN	NIPAL4
P49281	NRAM2_HUMAN	SLC11A2
Q12908	NTCP2_HUMAN	SLC10A2
Q9Y619	ORNT1_HUMAN	SLC25A15
Q9BXI2	ORNT2_HUMAN	SLC25A2
Q86UW1	OSTA_HUMAN	SLC51A
Q86UW2	OSTB_HUMAN	SLC51B
Q04671	P_HUMAN	OCA2
Q96NT5	PCFT_HUMAN	SLC46A1
O75915	PRAF3_HUMAN	ARL6IP5
Q02094	RHAG_HUMAN	RHAG
Q9H310	RHBG_HUMAN	RHBG
Q9UBD6	RHCG_HUMAN	RHCG
Q92681	RSCA1_HUMAN	RSC1A1
Q9BXP2	S12A9_HUMAN	SLC12A9
Q8WWT9	S13A3_HUMAN	SLC13A3
Q86YT5	S13A5_HUMAN	SLC13A5
P46059	S15A1_HUMAN	SLC15A1
Q16348	S15A2_HUMAN	SLC15A2
Q8IY34	S15A3_HUMAN	SLC15A3
Q8N697	S15A4_HUMAN	SLC15A4

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Q6NT16	S18B1_HUMAN	SLC18B1
P41440	S19A1_HUMAN	SLC19A1
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Q9BZV2	S19A3_HUMAN	SLC19A3
Q8WUM9	S20A1_HUMAN	SLC20A1
Q08357	S20A2_HUMAN	SLC20A2
O15245	S22A1_HUMAN	SLC22A1
O15244	S22A2_HUMAN	SLC22A2
O75751	S22A3_HUMAN	SLC22A3
Q9H015	S22A4_HUMAN	SLC22A4
O76082	S22A5_HUMAN	SLC22A5
Q4U2R8	S22A6_HUMAN	SLC22A6
Q9Y694	S22A7_HUMAN	SLC22A7
Q8TCC7	S22A8_HUMAN	SLC22A8
Q8IVM8	S22A9_HUMAN	SLC22A9
Q63ZE4	S22AA_HUMAN	SLC22A10
Q9NSA0	S22AB_HUMAN	SLC22A11
Q96S37	S22AC_HUMAN	SLC22A12
Q9Y226	S22AD_HUMAN	SLC22A13
Q9Y267	S22AE_HUMAN	SLC22A14
Q8IZD6	S22AF_HUMAN	SLC22A15
Q86VW1	S22AG_HUMAN	SLC22A16
Q8WUG5	S22AH_HUMAN	SLC22A17
Q96BI1	S22AI_HUMAN	SLC22A18
A6NK97	S22AK_HUMAN	SLC22A20
Q6T423	S22AP_HUMAN	SLC22A25
Q9UHI7	S23A1_HUMAN	SLC23A1
Q9UGH3	S23A2_HUMAN	SLC23A2
Q6PIS1	S23A3_HUMAN	SLC23A3
Q86VD7	S2542_HUMAN	SLC25A42
Q86WA9	S2611_HUMAN	SLC26A11
Q9H2B4	S26A1_HUMAN	SLC26A1

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Q8TE54	S26A7_HUMAN	SLC26A7
Q96RN1	S26A8_HUMAN	SLC26A8
Q7LBE3	S26A9_HUMAN	SLC26A9
O00337	S28A1_HUMAN	SLC28A1
O43868	S28A2_HUMAN	SLC28A2
Q99808	S29A1_HUMAN	SLC29A1
Q14542	S29A2_HUMAN	SLC29A2
Q9BZD2	S29A3_HUMAN	SLC29A3
Q7RTT9	S29A4_HUMAN	SLC29A4
P78382	S35A1_HUMAN	SLC35A1
P78381	S35A2_HUMAN	SLC35A2
Q9Y2D2	S35A3_HUMAN	SLC35A3
Q96G79	S35A4_HUMAN	SLC35A4
Q9BS91	S35A5_HUMAN	SLC35A5
P78383	S35B1_HUMAN	SLC35B1
Q8TB61	S35B2_HUMAN	SLC35B2
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Q8IY50	S35F3_HUMAN	SLC35F3
Q7Z2H8	S36A1_HUMAN	SLC36A1
Q495M3	S36A2_HUMAN	SLC36A2
Q495N2	S36A3_HUMAN	SLC36A3
Q6YBV0	S36A4_HUMAN	SLC36A4
Q9H2H9	S38A1_HUMAN	SLC38A1
Q96QD8	S38A2_HUMAN	SLC38A2
Q99624	S38A3_HUMAN	SLC38A3
Q969I6	S38A4_HUMAN	SLC38A4

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A6NNN8	S38A8_HUMAN	SLC38A8
Q8NBW4	S38A9_HUMAN	SLC38A9
Q9HBR0	S38AA_HUMAN	SLC38A10
Q08AI6	S38AB_HUMAN	SLC38A11
Q9NY26	S39A1_HUMAN	SLC39A1
Q9NP94	S39A2_HUMAN	SLC39A2
Q9BRY0	S39A3_HUMAN	SLC39A3
Q6P5W5	S39A4_HUMAN	SLC39A4
Q6ZMH5	S39A5_HUMAN	SLC39A5
Q13433	S39A6_HUMAN	SLC39A6
Q92504	S39A7_HUMAN	SLC39A7
Q9C0K1	S39A8_HUMAN	SLC39A8
Q9NUM3	S39A9_HUMAN	SLC39A9
Q9ULF5	S39AA_HUMAN	SLC39A10
Q8N1S5	S39AB_HUMAN	SLC39A11
Q504Y0	S39AC_HUMAN	SLC39A12
Q96H72	S39AD_HUMAN	SLC39A13
Q15043	S39AE_HUMAN	SLC39A14
Q9NP59	S40A1_HUMAN	SLC40A1
Q9Y2W3	S45A1_HUMAN	SLC45A1
Q9UMX9	S45A2_HUMAN	SLC45A2
Q8NBS3	S4A11_HUMAN	SLC4A11
Q9Y6M7	S4A7_HUMAN	SLC4A7
Q9NWF4	S52A1_HUMAN	SLC52A1
Q9HAB3	S52A2_HUMAN	SLC52A2
Q9NQ40	S52A3_HUMAN	SLC52A3
P48066	S6A11_HUMAN	SLC6A11
P48065	S6A12_HUMAN	SLC6A12
Q9NSD5	S6A13_HUMAN	SLC6A13

UniProt ID	Entry name	Gene names (primary)
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Q9H2J7	S6A15_HUMAN	SLC6A15
Q9GZN6	S6A16_HUMAN	SLC6A16
Q9H1V8	S6A17_HUMAN	SLC6A17
Q96N87	S6A18_HUMAN	SLC6A18
Q695T7	S6A19_HUMAN	SLC6A19
Q9NP91	S6A20_HUMAN	SLC6A20
Q8TCU3	S7A13_HUMAN	SLC7A13
Q8TBB6	S7A14_HUMAN	SLC7A14
Q70HW3	SAMC_HUMAN	SLC25A26
P43007	SATT_HUMAN	SLC1A4
P53794	SC5A3_HUMAN	SLC5A3
Q9Y289	SC5A6_HUMAN	SLC5A6
Q9GZV3	SC5A7_HUMAN	SLC5A7
Q8N695	SC5A8_HUMAN	SLC5A8
Q8WWX8	SC5AB_HUMAN	SLC5A11
Q1EHB4	SC5AC_HUMAN	SLC5A12
P30531	SC6A1_HUMAN	SLC6A1
P23975	SC6A2_HUMAN	SLC6A2
Q01959	SC6A3_HUMAN	SLC6A3
P31645	SC6A4_HUMAN	SLC6A4
Q9Y345	SC6A5_HUMAN	SLC6A5
P31641	SC6A6_HUMAN	SLC6A6
Q99884	SC6A7_HUMAN	SLC6A7
P48029	SC6A8_HUMAN	SLC6A8
P48067	SC6A9_HUMAN	SLC6A9
P46721	SO1A2_HUMAN	SLCO1A2
Q9Y6L6	SO1B1_HUMAN	SLCO1B1
Q9NPD5	SO1B3_HUMAN	SLCO1B3
G3V0H7	SO1B7_HUMAN	SLCO1B7
Q9NYB5	SO1C1_HUMAN	SLCO1C1
Q92959	SO2A1_HUMAN	SLCO2A1

UniProt ID	Entry name	Gene names (primary)
O94956	SO2B1_HUMAN	SLCO2B1
Q9UIG8	SO3A1_HUMAN	SLCO3A1
Q96BD0	SO4A1_HUMAN	SLCO4A1
Q6ZQN7	SO4C1_HUMAN	SLCO4C1
Q9H2Y9	SO5A1_HUMAN	SLCO5A1
Q86UG4	SO6A1_HUMAN	SLCO6A1
Q3KNW5	SOAT_HUMAN	SLC10A6
O95149	SPN1_HUMAN	SNUPN
Q8N434	SVOPL_HUMAN	SVOPL
Q9BRV3	SWET1_HUMAN	SLC50A1
Q03518	TAP1_HUMAN	TAP1
Q03519	TAP2_HUMAN	TAP2
Q13454	TUSC3_HUMAN	TUSC3
Q13336	UT1_HUMAN	SLC14A1
Q15849	UT2_HUMAN	SLC14A2
Q16572	VACHT_HUMAN	SLC18A3
Q9P2U7	VGLU1_HUMAN	SLC17A7
Q9P2U8	VGLU2_HUMAN	SLC17A6
Q8NDX2	VGLU3_HUMAN	SLC17A8
Q9H598	VIAAT_HUMAN	SLC32A1
P54219	VMAT1_HUMAN	SLC18A1
Q05940	VMAT2_HUMAN	SLC18A2
Q9UPY5	XCT_HUMAN	SLC7A11
Q9UM01	YLAT1_HUMAN	SLC7A7
Q92536	YLAT2_HUMAN	SLC7A6
Q6XR72	ZNT10_HUMAN	SLC30A10
Q9Y6M5	ZNT1_HUMAN	SLC30A1
Q9BRI3	ZNT2_HUMAN	SLC30A2
Q99726	ZNT3_HUMAN	SLC30A3
O14863	ZNT4_HUMAN	SLC30A4
Q8TAD4	ZNT5_HUMAN	SLC30A5
Q6NXT4	ZNT6_HUMAN	SLC30A6

UniProt ID	Entry name	Gene names (primary)
Q8NEW0	ZNT7_HUMAN	SLC30A7
Q8IWU4	ZNT8_HUMAN	SLC30A8
Q6PML9	ZNT9_HUMAN	SLC30A9

(viii) Chimeric Antigen Receptor Payloads

[000415] In some embodiments a membrane protein payload agent is or comprises a CAR, e.g., a first generation CAR or a nucleic acid encoding a first generation CAR. In some embodiments, a first generation CAR comprises an antigen binding domain, a transmembrane domain, and signaling domain. In some embodiments a signaling domain mediates downstream signaling during T-cell activation.

[000416] In some embodiments a membrane protein payload agent is or comprises a second generation CAR or a nucleic acid encoding a second generation CAR. In some embodiments a second generation CAR comprises an antigen binding domain, a transmembrane domain, and two signaling domains. In some embodiments a signaling domain mediates downstream signaling during T-cell activation. In some embodiments a signaling domain is a costimulatory domain. In some embodiments, a costimulatory domain enhances cytokine production, CAR T-cell proliferation, and or CAR T-cell persistence during T cell activation.

[000417] In some embodiments a membrane protein payload agent is or comprises a third generation CAR or a nucleic acid encoding a third generation CAR. In some embodiments, a third generation CAR comprises an antigen binding domain, a transmembrane domain, and at least three signaling domains. In some embodiments a signaling domain mediates downstream signaling during T-cell activation. In some embodiments a signaling domain is a costimulatory domain. In some embodiments, a costimulatory domain enhances cytokine production, CAR T-cell proliferation, and or CAR T-cell persistence during T cell activation. In some embodiments, a third generation CAR comprises at least two costimulatory domains. In some embodiments, the at least two costimulatory domains are not the same.

[000418] In some embodiments a membrane protein payload agent is or comprises a fourth generation CAR or a nucleic acid encoding a fourth generation CAR. In some embodiments a

fourth generation CAR comprises an antigen binding domain, a transmembrane domain, and at least two, three, or four signaling domains. In some embodiments a signaling domain mediates downstream signaling during T-cell activation. In some embodiments a signaling domain is a costimulatory domain. In some embodiments, a costimulatory domain enhances cytokine production, CAR T-cell proliferation, and or CAR T-cell persistence during T cell activation.

[000419] In some embodiments, a first, second, third, or fourth generation CAR further comprises a domain which upon successful signaling of the CAR induces expression of a cytokine gene. In some embodiments, a cytokine gene is endogenous or exogenous to a target cell comprising a CAR which comprises a domain which upon successful signaling of the CAR induces expression of a cytokine gene. In some embodiments a cytokine gene encodes a pro-inflammatory cytokine. In some embodiments a cytokine gene encodes IL-1, IL-2, IL-9, IL-12, IL-18, TNF, or IFN-gamma, or functional fragment thereof. In some embodiments a domain which upon successful signaling of the CAR induces expression of a cytokine gene is or comprises a transcription factor or functional domain or fragment thereof. In some embodiments a domain which upon successful signaling of the CAR induces expression of a cytokine gene is or comprises a transcription factor or functional domain or fragment thereof. In some embodiments a transcription factor or functional domain or fragment thereof is or comprises a nuclear factor of activated T cells (NFAT), an NF-kB, or functional domain or fragment thereof. See, e.g., Zhang. C. et al., Engineering CAR-T cells. Biomarker Research. 5:22 (2017); WO 2016126608; Sha, H. et al. Chimaeric antigen receptor T-cell therapy for tumour immunotherapy. Bioscience Reports Jan 27, 2017, 37 (1).

(a) CAR Antigen Binding Domains

[000420] In some embodiments, a CAR antigen binding domain is or comprises an antibody or antigen-binding portion thereof. In some embodiments, a CAR antigen binding domain is or comprises an scFv or Fab. In some embodiments a CAR antigen binding domain comprises an scFv or Fab fragment of a T-cell alpha chain antibody; T-cell β chain antibody; T-cell γ chain antibody; T-cell δ chain antibody; CCR7 antibody; CD3 antibody; CD4 antibody; CD5 antibody; CD7 antibody; CD8 antibody; CD11b antibody; CD11c antibody; CD16 antibody; CD19 antibody; CD20 antibody; CD21 antibody; CD22 antibody; CD25 antibody; CD28 antibody;

CD34 antibody; CD35 antibody; CD40 antibody; CD45RA antibody; CD45RO antibody; CD52 antibody; CD56 antibody; CD62L antibody; CD68 antibody; CD80 antibody; CD95 antibody; CD117 antibody; CD127 antibody; CD133 antibody; CD137 (4-1 BB) antibody; CD163 antibody; F4/80 antibody; IL-4Ra antibody; Sca-1 antibody; CTLA-4 antibody; GITR antibody GARP antibody; LAP antibody; granzyme B antibody; LFA-1 antibody; or transferrin receptor antibody.

[000421] In some embodiments, an antigen binding domain binds to a cell surface antigen of a cell. In some embodiments, a cell surface antigen is characteristic of one type of cell. In some embodiments, a cell surface antigen is characteristic of more than one type of cell.

[000422] In some embodiments a CAR antigen binding domain binds a cell surface antigen characteristic of a T-cell. In some embodiments, an antigen characteristic of a T-cell may be a cell surface receptor, a membrane transport protein (e.g., an active or passive transport protein such as, for example, an ion channel protein, a pore-forming protein, etc.), a transmembrane receptor, a membrane enzyme, and/or a cell adhesion protein characteristic of a T-cell. In some embodiments, an antigen characteristic of a T-cell may be a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, or histidine kinase associated receptor.

[000423] In some embodiments, an antigen characteristic of a T-cell may be a T-cell receptor. In some embodiments, a T-cell receptor may be AKT1; AKT2; AKT3; ATF2; BCL10; CALM1; CD3D (CD3δ); CD3E (CD3ε); CD3G (CD3γ); CD4; CD8; CD28; CD45; CD80 (B7-1); CD86 (B7-2); CD247 (CD3ζ); CTLA4 (CD152); ELK1; ERK1 (MAPK3); ERK2; FOS; FYN; GRAP2 (GADS); GRB2; HLA-DRA; HLA-DRB1; HLA-DRB3; HLA-DRB4; HLA-DRB5; HRAS; IKBKA (CHUK); IKBKB; IKBKE; IKBKG (NEMO); IL2; ITPR1; ITK; JUN; KRAS2; LAT; LCK; MAP2K1 (MEK1); MAP2K2 (MEK2); MAP2K3 (MKK3); MAP2K4 (MKK4); MAP2K6 (MKK6); MAP2K7 (MKK7); MAP3K1 (MEKK1); MAP3K3; MAP3K4; MAP3K5; MAP3K8; MAP3K14 (NIK); MAPK8 (JNK1); MAPK9 (JNK2); MAPK10 (JNK3); MAPK11 (p38β); MAPK12 (p38γ); MAPK13 (p38δ); MAPK14 (p38α); NCK; NFAT1; NFAT2; NFKB1; NFKB2; NFKBIA; NRAS; PAK1; PAK2; PAK3; PAK4; PIK3C2B; PIK3C3 (VPS34); PIK3CA; PIK3CB; PIK3CD; PIK3R1; PKCA; PKCB; PKCM; PKCQ; PLCY1; PRF1 (Perforin); PTEN; RAC1; RAF1; RELA; SDF1; SHP2; SLP76; SOS; SRC; TBK1; TCRA; TEC; TRAF6; VAV1; VAV2; or

ZAP70.

[000424] In some embodiments a CAR antigen binding domain binds an antigen characteristic of a cancer. In some embodiments an antigen characteristic of a cancer is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor, Epidermal Growth Factor Receptors (EGFR) (including ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4), Fibroblast Growth Factor Receptors (FGFR) (including FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF18, and FGF21) Vascular Endothelial Growth Factor Receptors (VEGFR) (including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF), RET Receptor and the Eph Receptor Family (including EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA9, EphA10, EphB1, EphB2. EphB3, EphB4, and EphB6), CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CFTR, CIC-1, CIC-2, CIC-4, CIC-5, CIC-7, CIC-Ka, CIC-Kb, Bestrophins, TMEM16A, GABA receptor, glycin receptor, ABC transporters, NAV1.1, NAV1.2, NAV1.3, NAV1.4, NAV1.5, NAV1.6, NAV1.7, NAV1.8, NAV1.9, sphingosin-1-phosphate receptor (S1P1R), NMDA channel, transmembrane protein, multispan transmembrane protein, T-cell receptor motifs; T-cell alpha chains; T-cell β chains; T-cell γ chains; T-cell δ chains; CCR7; CD3; CD4; CD5; CD7; CD8; CD11b; CD11c; CD16; CD19; CD20; CD21 ; CD22; CD25; CD28; CD34; CD35; CD40; CD45RA; CD45RO; CD52; CD56; CD62L; CD68; CD80; CD95; CD117; CD127; CD133; CD137 (4-1 BB); CD163; F4/80; IL-4Ra; Sca-1 ; CTLA-4; GITR; GARP; LAP; granzyme B; LFA-1 ; transferrin receptor; NKp46, perforin, CD4+; Th1; Th2; Th17; Th40; Th22; Th9; Tfh, Canonical Treg. FoxP3+; Tr1; Th3; Treg17; $T_{RE}G$; CDCP1, NT5E, EpCAM, CEA, gpA33, Mucins, TAG-72, Carbonic anhydrase IX, PSMA, Folate binding protein, Gangliosides (e.g., CD2, CD3, GM2), Lewis-γ^2, VEGF, VEGFR 1/2/3, αVβ3, α5β1, ErbB1/EGFR, ErbB1/HER2, ErB3, c-MET, IGF1R, EphA3, TRAIL-R1, TRAIL-R2, RANKL, FAP, Tenascin, PDL-1, BAFF, HDAC, ABL, FLT3, KIT, MET, RET, IL-1β, ALK, RANKL, mTOR, CTLA-4, IL-6, IL-6R, JAK3, BRAF, PTCH, Smoothened, PIGF, ANPEP, TIMP1, PLAUR, PTPRJ, LTBR, or ANTXR1, Folate receptor alpha (FRa), ERBB2 (Her2/neu), EphA2, IL-13Ra2, epidermal growth factor receptor (EGFR), Mesothelin, TSHR, CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1, CD33,

EGFRvIII , GD2, GD3, BCMA, MUC16 (CA125), L1CAM, LeY, MSLN, IL13Rα1, L1-CAM, Tn Ag, prostate specific membrane antigen (PSMA), ROR1, FLT3, FAP, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, interleukin-11 receptor a (IL-11Ra), PSCA, PRSS21, VEGFR2, LewisY, CD24, platelet-derived growth factor receptor-beta (PDGFR-beta), SSEA-4, CD20, MUC1, NCAM, Prostase, PAP, ELF2M, Ephrin B2, IGF-1 receptor, CAIX, LMP2, gplOO, bcr-abl, tyrosinase, Fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-A1, legumain, HPV E6, E7, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, prostein, survivin, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin B1, MYCN, RhoC, TRP-2, CYPIB I, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, a neoantigen, CD133, CD15, CD184, CD24, CD56, CD26, CD29, CD44, HLA-A, HLA-B, HLA-C, (HLA-A,B,C) CD49f, CD151 CD340, CD200, tkrA, trkB, or trkC, or an antigenic fragment or antigenic portion thereof.

[000425] In some embodiments a CAR antigen binding domain binds an antigen characteristic of an infectious disease (e.g. a viral infection or a bacterial infection). In some embodiments an antigen is characteristic of an infectious disease selected from HIV, hepatitis B virus, hepatitis C virus, Human herpes virus, Human herpes virus 8 (HHV-8, Kaposi sarcoma-associated herpes virus (KSHV)), Human T-lymphotrophic virus-1 (HTLV-1), Merkel cell polyomavirus (MCV), Simian virus 40 (SV40), Eptstein-Barr virus, CMV, human papillomavirus. In some embodiments an antigen characteristic of an infectious disease is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, or histidine kinase associated receptor. In some embodiments, a CAR antigen binding domain binds an antigen characteristic of an infectious disease, wherein the antigen is selected from HIV Env, gpl20, or

CD4-induced epitope on HIV-1 Env. See, e.g., WO2015/077789, the contents of which are herein incorporated by reference. In some embodiments, a CAR antigen binding domain comprises CD4 or an HIV binding fragment thereof.

[000426] In some embodiments a CAR antigen binding domain binds an antigen characteristic of an autoimmune or inflammatory disorder. In some embodiments the antigen is characteristic of an autoimmune or inflammatory disorder selected from chronic graft-vs-host disease (GVHD), lupus, arthritis, immune complex glomerulonephritis, goodpasture, uveitis, hepatitis, systemic sclerosis or scleroderma, type I diabetes, multiple sclerosis, cold agglutinin disease, Pemphigus vulgaris, Grave's disease, autoimmune hemolytic anemia, Hemophilia A, Primary Sjogren's Syndrome, thrombotic thrombocytopenia purrpura, neuromyelits optica, Evan's syndrome, IgM mediated neuropathy, cyroglobulinemia, dermatomyositis, idiopathic thrombocytopenia, ankylosing spondylitis, bullous pemphigoid, acquired angioedema, chronic urticarial, antiphospholipid demyelinating polyneuropathy, and autoimmune thrombocytopenia or neutropenia or pure red cell aplasias, while exemplary non-limiting examples of alloimmune diseases include allosensitization (see, for example, Blazar et al., 2015, Am. J. Transplant, 15(4):931-41) or xenosensitization from hematopoietic or solid organ transplantation, blood transfusions, pregnancy with fetal allosensitization, neonatal alloimmune thrombocytopenia, hemolytic disease of the newborn, sensitization to foreign antigens such as can occur with replacement of inherited or acquired deficiency disorders treated with enzyme or protein replacement therapy, blood products, and gene therapy. In some embodiments an antigen characteristic of an autoimmune or inflammatory disorder is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, or histidine kinase associated receptor. In some embodiments, a CAR antigen binding domain binds to a ligand expressed on B cells, plasma cells, or plasmablasts. In some embodiments, a CAR antigen binding domain binds an antigen characteristic of an autoimmune or inflammatory disorder, wherein the antigen is selected from CD10, CD19, CD20, CD22, CD24, CD27, CD38, CD45R, CD138, CD319, BCMA, CD28, TNF, interferon receptors, GM-CSF, ZAP-70, LFA-1, CD3 gamma, CD5 or CD2. See US 2003/0077249; WO 2017/058753; WO 2017/058850, the contents of which are herein

incorporated by reference.

(b) CAR Transmembrane Domains

[000427] In some embodiments a CAR comprises a transmembrane domain. In some embodiments a CAR comprises at least a transmembrane region of the alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, or functional variant thereof. In some embodiments a CAR comprises at least a transmembrane region of CD8α, CD8β, 4-1BB/CD137, CD28, CD34, CD4, FcεRIγ, CD16, OX40/CD134, CD3ζ, CD3ε, CD3γ, CD3δ, TCRα, TCRβ, TCRζ, CD32, CD64, CD64, CD45, CD5, CD9, CD22, CD37, CD80, CD86, CD40, CD40L/CD154, VEGFR2, FAS, and FGFR2B, or functional variant thereof.

(c) CAR Signaling Domains

[000428] In some embodiments a CAR comprises a signaling domain of one or more of B7-1/CD80; B7-2/CD86; B7-H1/PD-L1; B7-H2; B7-H3; B7-H4; B7-H6; B7-H7; BTLA/CD272; CD28; CTLA-4; Gi24/VISTA/B7-H5; ICOS/CD278; PD-1; PD-L2/B7-DC; PDCD6); 4-1BB/TNFSF9/CD137; 4-1BB Ligand/TNFSF9; BAFF/BLyS/TNFSF13B; BAFF R/TNFRSF13C; CD27/TNFRSF7; CD27 Ligand/TNFSF7; CD30/TNFRSF8; CD30 Ligand/TNFSF8; CD40/TNFRSF5; CD40/TNFSF5; CD40 Ligand/TNFSF5; DR3/TNFRSF25; GITR/TNFRSF18; GITR Ligand/TNFSF18; HVEM/TNFRSF14; LIGHT/TNFSF14; Lymphotoxin-alpha/TNF-beta; OX40/TNFRSF4; OX40 Ligand/TNFSF4; RELT/TNFRSF19L; TACI/TNFRSF13B; TL1A/TNFSF15; TNF-alpha; TNF RII/TNFRSF1B); 2B4/CD244/SLAMF4; BLAME/SLAMF8; CD2; CD2F-10/SLAMF9; CD48/SLAMF2; CD58/LFA-3; CD84/SLAMF5; CD229/SLAMF3; CRACC/SLAMF7; NTB-A/SLAMF6; SLAM/CD150); CD2; CD7; CD53; CD82/Kai-1; CD90/Thy1; CD96; CD160; CD200; CD300a/LMIR1; HLA Class I; HLA-DR; Ikaros; Integrin alpha 4/CD49d; Integrin alpha 4 beta 1; Integrin alpha 4 beta 7/LPAM-1; LAG-3; TCL1A; TCL1B; CRTAM; DAP12; Dectin-1/CLEC7A; DPPIV/CD26; EphB6; TIM-1/KIM-1/HAVCR; TIM-4; TSLP; TSLP R; lymphocyte function associated antigen-1 (LFA-1); NKG2C, a CD3 zeta domain, an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof.

[000429] In some embodiments a CAR comprises a signaling domain which is a

costimulatory domain. In some embodiments a CAR comprises a second costimulatory domain. In some embodiments a CAR comprises at least two costimulatory domains. In some embodiments a CAR comprises at least three costimulatory domains. In some embodiments a CAR comprises a costimulatory domain selected from one or more of CD27, CD28, 4-1BB, CD134/OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

[000430] In some embodiments, a CAR comprises a CD3 zeta domain or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof. In some embodiments, a CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; and (ii) a CD28 domain, or a 4-1BB domain, or functional variant thereof. In some embodiments, a CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain or functional variant thereof; and (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof. In some embodiments, a CAR comprises a (i) a CD3 zeta domain, or an immunoreceptor tyrosine-based activation motif (ITAM), or functional variant thereof; (ii) a CD28 domain or functional variant thereof; (iii) a 4-1BB domain, or a CD134 domain, or functional variant thereof; and (iv) a cytokine or costimulatory ligand transgene.

(d) CAR Spacers

[000431] In some embodiments a CAR comprises one or more spacers. In some embodiments a CAR comprises a spacer between the antigen binding domain and the transmembrane domain. In some embodiments a CAR comprises a spacer between the transmembrane domain and the intracellular signaling domain.

(e) CAR Membrane Protein Payload Agents

[000432] In addition to the CARs described herein, various chimeric antigen receptors and nucleotide sequences encoding the same are known in the art and would be suitable for fusosomal delivery and reprogramming of target cells in vivo and in vitro as described herein. See, e.g., WO2013040557; WO2012079000; WO2016030414; Smith T, et al., Nature Nanotechnology. 2017. DOI: 10.1038/NNANO.2017.57, the disclosures of which are herein incorporated by

reference.

[000433] In some embodiments a fusosome comprising a membrane protein payload agent which is or comprises a CAR or a nucleic acid encoding a CAR (e.g., a DNA, a gDNA, a cDNA, an RNA, a pre-MRNA, an mRNA, an miRNA, an siRNA, etc.) is delivered to a target cell. In some embodiments the target cell is an effector cell, e.g., a cell of the immune system that expresses one or more Fc receptors and mediates one or more effector functions. In some embodiments, a target cell may include, but may not be limited to, one or more of a monocyte, macrophage, neutrophil, dendritic cell, eosinophil, mast cell, platelet, large granular lymphocyte, Langerhans' cell, natural killer (NK) cell, T-lymphocyte (e.g., T-cell), a Gamma delta T cell, B-lymphocyte (e.g., B-cell) and may be from any organism including but not limited to humans, mice, rats, rabbits, and monkeys.

(ix) T cell receptor Payloads

[000434] In some embodiments, a membrane protein payload agent described herein comprises or encodes a polypeptide comprising a T cell receptor, e.g., a T-cell receptor fusion protein (TFP). In some embodiments, the TFP comprises a recombinant polypeptide derived from the various polypeptides comprising the TCR that is generally capable of i) binding to a surface antigen on target cells and ii) interacting with other polypeptide components of the intact TCR complex, typically when co-located in or on the surface of a T-cell. In some embodiments, the TFP incorporates into a TCR when expressed in a T-cell. In some embodiments the membrane protein payload agent comprises or encodes (i) an antigen binding domain operatively linked to (ii) a TCR domain.

[000435] The antigen-binding domain may comprise, e.g., an scFv, e.g., an scFv that binds an antigen comprised by a cancer cell, e.g., an antigen at the surface of a cancer cell. The antigen-binding domain may be human or humanized. In some embodiments, the antigen-binding domain is an antigen-binding domain described herein, e.g., in the section entitled "CAR Antigen Binding Domains".

[000436] In some embodiments, the antigen-binding domain binds an Fc domain of an antibody. In some embodiments, the antigen-binding domain selectively binding an IgG1 antibody. In some embodiments, the antigen-binding domain binds to a cell surface antigen, e.g., a cell

surface antigen on the surface of a tumor cell. In some embodiments, the antigen-binding domain comprises a monomer, a dimer, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an octomer, a nonamer, or a decamer. In some embodiments, the antigen-binding domain does not comprise an antibody or fragment thereof. In some embodiments, the antigen-binding domain comprises a CD16 polypeptide or fragment thereof. In some embodiments, the antigen-binding domain comprises a CD16-binding polypeptide.

[000437] In some embodiments, the TFP includes an extracellular domain of a TCR subunit that comprises an extracellular domain or portion thereof of a protein selected from the group consisting of the alpha or beta chain of the T cell receptor, CD3 delta, CD3 epsilon, or CD3 gamma, or a functional fragment or variant thereof. In some embodiments, the TCR domain includes a transmembrane domain, e.g., at least a transmembrane region of a transmembrane domain of a TCR alpha chain, a TCR beta chain, a CD3 epsilon TCR subunit, a CD3 gamma TCR subunit, a CD3 delta TCR subunit, or a CD3 zeta TCR subunit, or a functional fragment or variant thereof.

[000438] In further embodiments, the TCR domain comprises a TCR intracellular domain comprising a stimulatory domain selected from an intracellular signaling domain of CD3 epsilon, CD3 gamma, or CD3 delta, or a variant thereof.

[000439] In further embodiments, the TCR domain comprises (i) a TCR extracellular domain, (ii) a TCR transmembrane domain, and (iii) a TCR intracellular domain, wherein at least two or all three of (i), (ii), and (iii) are from the same TCR subunit.

[000440] In some embodiments, the TCR domain comprises CD3ε or a functional fragment or variant thereof. In some embodiments, the TCR domain (e.g., CD3ε-based TCR domain) binds endogenous CD3ζ. In some embodiments, the TCR domain (e.g., CD3ε-based TCR domain) binds endogenous CD3γ and/or endogenous CD3δ. In some embodiments, the TCR domain comprises CD3α or a functional fragment or variant thereof. In some embodiments, the TCR domain comprises CD3β or a functional fragment or variant thereof. In some embodiments, the TCR domain (e.g., CD3α-based or CD3β-based TCR domain) binds endogenous CD3ζ. In some embodiments, the TCR domain (e.g., CD3α-based TCR domain) binds endogenous CD3β. In some embodiments, the TCR domain (e.g., CD3β-based TCR domain) binds endogenous CD3α. In some embodiments, the TCR domain (e.g., CD3α-based or CD3β-based TCR domain) binds

endogenous CD3δ.

[000441] In some embodiments, a TFP comprises a TCR subunit comprising at least a portion of a TCR extracellular domain, and a TCR intracellular domain comprising a stimulatory domain from an intracellular signaling domain of CD3 (e.g., CD3 epsilon, CD3 gamma, CD3 delta, TCR alpha, or TCR beta); and a human or humanized antigen binding domain, wherein the TCR subunit and the antigen binding domain are operatively linked, and wherein the TFP incorporates into a TCR when expressed in a T-cell. In some embodiments, a TFP comprises a TCR subunit and a human or humanized antibody domain comprising an antigen binding domain that is an anti-CD19 binding domain or an anti-B-cell maturation antigen (BCMA) binding domain.

[000442] Exemplary TFPs are described, e.g., in WO2016187349, WO2018026953, WO2018067993, WO2018098365, WO2018119298, and WO2018232020, each of which is incorporated herein by reference in its entirety.

Secreted Payload Agents, e.g., Secreted Protein Payload Agents

[000443] Payload agents, e.g., protein payload agents, can also be targeted for secretion. In some embodiments, the methods and compositions described herein can be used to target payloads to the lumen of an organelle (e.g. a Golgi apparatus, secretory vesicle) after translation in the ER. In some embodiments, a secreted protein payload agent comprises a secreted protein or a nucleic acid encoding it.

Chondrisomes

[000444] In some embodiments, a fusosome or fusosome composition further comprises a chondriosome or chondrisome preparation. In some embodiments, a fusosome or fusosome composition comprises a modified chondrisome preparation derived from a cellular source of mitochondria. In some embodiments, a fusosome or fusosome composition comprises a chondrisome preparation expressing an exogenous protein. In some embodiments, the exogenous protein is exogenous to said mitochondria. In some embodiments, the exogenous protein is exogenous to said cellular source of mitochondria. Additional features and embodiments including chondrisomes, chondrisome preparations, methods, and uses are contemplated by the invention, e.g., as described in international application, PCT/US16/64251.

Immunogenicity

[000445] In some embodiments of any of the aspects described herein, the fusosome composition is substantially non-immunogenic. Immunogenicity can be quantified, e.g., as described herein.

[000446] In some embodiments, the fusosome composition has membrane symmetry of a cell which is, or is known to be, substantially non-immunogenic, e.g., a stem cell, mesenchymal stem cell, induced pluripotent stem cell, embryonic stem cell, sertoli cell, or retinal pigment epithelial cell. In some embodiments, the fusosome has an immunogenicity no more than 5%, 10%, 20%, 30%, 40%, or 50% greater than the immunogenicity of a stem cell, mesenchymal stem cell, induced pluripotent stem cell, embryonic stem cell, sertoli cell, or retinal pigment epithelial cell as measured by an assay described herein.

[000447] In some embodiments, the fusosome composition comprises elevated levels of an immunosuppressive agent as compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell. In some embodiments, the elevated level is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold. In some embodiments, the fusosome composition comprises an immunosuppressive agent that is absent from the reference cell. In some embodiments, the fusosome composition comprises reduced levels of an immune activating agent as compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell. In some embodiments, the reduced level is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% compared to the reference cell. In some embodiments, the immune activating agent is substantially absent from the fusosome.

[000448] In some embodiments, the fusosome composition comprises a membrane with composition substantially similar, e.g., as measured by proteomics, to that of a source cell, e.g., a substantially non-immunogenic source cell. In some embodiments, the fusosome composition comprises a membrane comprising at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or 100% of the membrane proteins of the source cell. In some embodiments, the fusosome composition comprises a membrane comprising membrane proteins expressed at, at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%,

90%,95%, 99%, or 100% of the level of expression of the membrane proteins on a membrane of the source cell.

[000449] In some embodiments, the fusosome composition, or the source cell from which the fusosome composition is derived from, has one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, or more of the following characteristics:

a. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of MHC class I or MHC class II, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a HeLa cell;

b. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of one or more co-stimulatory proteins including but not limited to: LAG3, ICOS-L, ICOS, Ox40L, OX40, CD28, B7, CD30, CD30L 4-1BB, 4-1BBL, SLAM, CD27, CD70, HVEM, LIGHT, B7-H3, or B7-H4, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a reference cell described herein;

c. expression of surface proteins which suppress macrophage engulfment e.g., CD47, e.g., detectable expression by a method described herein, e.g., more than 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, or more expression of the surface protein which suppresses macrophage engulfment, e.g., CD47, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

d. expression of soluble immunosuppressive cytokines, e.g., IL-10, e.g., detectable expression by a method described herein, e.g., more than 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, or more expression of soluble immunosuppressive cytokines, e.g., IL-10, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

e. expression of soluble immunosuppressive proteins, e.g., PD-L1, e.g., detectable expression by a method described herein, e.g., more than 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, or more expression of soluble immunosuppressive proteins, e.g., PD-L1, compared to a reference cell e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

f. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of soluble immune stimulating cytokines, e.g., IFN-gamma or TNF-a, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a U-266 cell;

g. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of endogenous immune-stimulatory antigen, e.g., Zg16 or Hormad1, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or an A549 cell or a SK-BR-3 cell;

h. expression of, e.g., detectable expression by a method described herein, HLA-E or HLA-G, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

i. surface glycosylation profile, e.g., containing sialic acid, which acts to, e.g., suppress NK cell activation;

j. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of TCRα/β, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

k. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of ABO blood groups, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a HeLa cell;

l. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of Minor Histocompatibility Antigen (MHA), compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell; or

m. has less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, of mitochondrial MHAs, compared to a reference cell e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell, or has no detectable mitochondrial MHAs.

[000450] In embodiments, the co-stimulatory protein is 4-1BB, B7, SLAM, LAG3, HVEM, or LIGHT, and the ref cell is HDLM-2. In some embodiments, the co-stimulatory protein is BY-H3 and the reference cell is HeLa. In some embodiments, the co-stimulatory protein is ICOSL or B7-H4, and the reference cell is SK-BR-3. In some embodiments, the co-stimulatory protein is ICOS or OX40, and the reference cell is MOLT-4. In some embodiments, the co-stimulatory protein is CD28, and the reference cell is U-266. In some embodiments, the co-stimulatory protein is CD30L or CD27, and the reference cell is Daudi.

[000451] In some embodiments, the fusosome composition does not substantially elicit an immunogenic response by the immune system, e.g., innate immune system. In embodiments, an

immunogenic response can be quantified, e.g., as described herein. In some embodiments, the an immunogenic response by the innate immune system comprises a response by innate immune cells including, but not limited to NK cells, macrophages, neutrophils, basophils, eosinophils, dendritic cells, mast cells, or gamma/delta T cells. In some embodiments, an immunogenic response by the innate immune system comprises a response by the complement system which includes soluble blood components and membrane bound components.

[000452] In some embodiments, the fusosome composition does not substantially elicit an immunogenic response by the immune system, e.g., adaptive immune system. In embodiments, an immunogenic response can be quantified, e.g., as described herein. In some embodiments, an immunogenic response by the adaptive immune system comprises an immunogenic response by an adaptive immune cell including, but not limited to a change, e.g., increase, in number or activity of T lymphocytes (e.g., CD4 T cells, CD8 T cells, and or gamma-delta T cells), or B lymphocytes. In some embodiments, an immunogenic response by the adaptive immune system includes increased levels of soluble blood components including, but not limited to a change, e.g., increase, in number or activity of cytokines or antibodies (e.g., IgG, IgM, IgE, IgA, or IgD).

[000453] In some embodiments, the fusosome composition is modified to have reduced immunogenicity. Immunogenicity can be quantified, e.g., as described herein. In some embodiments, the fusosome composition has an immunogenicity less than 5%, 10%, 20%, 30%, 40%, or 50% lesser than the immunogenicity of a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell.

[000454] In some embodiments of any of the aspects described herein, the fusosome composition is derived from a source cell, e.g., a mammalian cell, having a modified genome, e.g., modified using a method described herein, to reduce, e.g., lessen, immunogenicity. Immunogenicity can be quantified, e.g., as described herein.

[000455] In some embodiments, the fusosome composition is derived from a mammalian cell depleted of, e.g., with a knock out of, one, two, three, four, five, six, seven or more of the following:

a. MHC class I, MHC class II or MHA;
b. one or more co-stimulatory proteins including but not limited to: LAG3, ICOS-L, ICOS, Ox40L, OX40, CD28, B7, CD30, CD30L 4-1BB, 4-1BBL, SLAM, CD27,

CD70, HVEM, LIGHT, B7-H3, or B7-H4;

c. soluble immune-stimulating cytokines e.g., IFN-gamma or TNF-a;

d. endogenous immune-stimulatory antigen, e.g., Zg16 or Hormad1;

e. T-cell receptors (TCR);

f. The genes encoding ABO blood groups, e.g., *ABO* gene;

g. transcription factors which drive immune activation, e.g., NFkB;

h. transcription factors that control MHC expression e.g., class II trans-activator (CIITA), regulatory factor of the Xbox 5 (RFX5), RFX-associated protein (RFXAP), or RFX ankyrin repeats (RFXANK; also known as RFXB); or

i. TAP proteins, e.g., TAP2, TAP1, or TAPBP, which reduce MHC class I expression.

[000456] In some embodiments, the fusosome is derived from a source cell with a genetic modification which results in increased expression of an immunosuppressive agent, e.g., one, two, three or more of the following (e.g., wherein before the genetic modification the cell did not express the factor):

a. surface proteins which suppress macrophage engulfment, e.g., CD47; e.g., increased expression of CD47 compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

b. soluble immunosuppressive cytokines, e.g., IL-10, e.g., increased expression of IL-10 compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

c. soluble immunosuppressive proteins, e.g., PD-1, PD-L1, CTLA4, or BTLA; e.g., increased expression of immunosuppressive proteins compared to a reference cell, e.g., an unmodified cell otherwise similar to the cell source, or a Jurkat cell; or

d. a tolerogenic protein, e.g., an ILT-2 or ILT-4 agonist, e.g., HLA-E or HLA-G or any other endogenous ILT-2 or ILT-4 agonist, e.g., increased expression of HLA-E, HLA-G, ILT-2 or ILT-4 compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell.

In some embodiments, the increased expression level is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold higher as

compared to a reference cell.

In some embodiments, the fusosome is derived from a source cell modified to have decreased expression of an immune activating agent, e.g., one, two, three, four, five, six, seven, eight or more of the following:

a. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of MHC class I or MHC class II, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a HeLa cell;

b. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of one or more co-stimulatory proteins including but not limited to: LAG3, ICOS-L, ICOS, Ox40L, OX40, CD28, B7, CD30, CD30L 4-1BB, 4-1BBL, SLAM, CD27, CD70, HVEM, LIGHT, B7-H3, or B7-H4, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a reference cell described herein;

c. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of soluble immune stimulating cytokines, e.g., IFN-gamma or TNF-a, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a U-266 cell;

d. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of endogenous immune-stimulatory antigen, e.g., Zg16 or Hormad1, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or an A549 cell or a SK-BR-3 cell;

e. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of T-cell receptors (TCR) compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell;

f. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of ABO blood groups, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a HeLa cell;

g. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of transcription factors which drive immune activation, e.g., NFkB; compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell

h. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of transcription factors that control MHC expression, e.g., class II trans-activator (CIITA), regulatory factor of the Xbox 5 (RFX5), RFX-associated protein (RFXAP), or RFX ankyrin repeats

(RFXANK; also known as RFXB) compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a Jurkat cell; or

i. less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% or lesser expression of TAP proteins, e.g., TAP2, TAP1, or TAPBP, which reduce MHC class I expression compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, or a HeLa cell.

[000457] In some embodiments, a fusosome composition derived from a mammalian cell, e.g., a mesenchymal stem cell, modified using shRNA expressing lentivirus to decrease MHC Class I expression, has lesser expression of MHC Class I compared to an unmodified cell, e.g., a mesenchymal stem cell that has not been modified. In some embodiments, a fusosome composition derived from a mammalian cell, e.g., a mesenchymal stem cell, modified using lentivirus expressing HLA-G to increase expression of HLA-G, has increased expression of HLA-G compared to an unmodified cell, e.g., a mesenchymal stem cell that has not been modified.

[000458] In some embodiments, the fusosome composition is derived from a source cell, e.g., a mammalian cell, which is not substantially immunogenic, wherein the source cells stimulate, e.g., induce, T-cell IFN-gamma secretion, at a level of 0 pg/mL to >0 pg/mL, e.g., as assayed *in vitro,* by IFN-gamma ELISPOT assay.

[000459] In some embodiments, the fusosome composition is derived from a source cell, e.g., a mammalian cell, wherein the mammalian cell is from a cell culture treated with an immunosuppressive agent, e.g., a glucocorticoid (e.g., dexamethasone), cytostatic (e.g., methotrexate), antibody (e.g., Muromonab-CD3), or immunophilin modulator (e.g., Ciclosporin or rapamycin).

[000460] In some embodiments, the fusosome composition is derived from a source cell, e.g., a mammalian cell, wherein the mammalian cell comprises an exogenous agent, e.g., a therapeutic agent.

[000461] In some embodiments, the fusosome composition is derived from a source cell, e.g., a mammalian cell, wherein the mammalian cell is a recombinant cell.

[000462] In some embodiments, the fusosome is derived from a mammalian cell genetically modified to express viral immunoevasins, e.g., hCMV US2, or US11.

[000463] In some embodiments, the surface of the fusosome, or the surface of the mammalian cell the fusosome is derived from, is covalently or non-covalently modified with a polymer, e.g., a biocompatible polymer that reduces immunogenicity and immune-mediated clearance, e.g., PEG.

[000464] In some embodiments, the surface of the fusosome, or the surface of the mammalian cell the fusosome is derived from is covalently or non-covalently modified with a sialic acid, e.g., a sialic acid comprising glycopolymers, which contain NK-suppressive glycan epitopes.

[000465] In some embodiments, the surface of the fusosome, or the surface of the mammalian cell the fusosome is derived from is enzymatically treated, e.g., with glycosidase enzymes, e.g., α-N-acetylgalactosaminidases, to remove ABO blood groups

[000466] In some embodiments, the surface of the fusosome, or the surface of the mammalian cell the fusosome is derived from is enzymatically treated, to give rise to, e.g., induce expression of, ABO blood groups which match the recipient's blood type.

Parameters for assessing immunogenicity

[000467] In some embodiments, the fusosome composition is derived from a source cell, e.g., a mammalian cell which is not substantially immunogenic, or modified, e.g., modified using a method described herein, to have a reduction in immunogenicity. Immunogenicity of the source cell and the fusosome composition can be determined by any of the assays described herein.

[000468] In some embodiments, the fusosome composition has an increase, e.g., an increase of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more, in in vivo graft survival compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell. In some embodiments, graft survival is determined by an assay measuring in vivo graft survival as described herein, in an appropriate animal model, e.g., an animal model described herein.

[000469] In some embodiments, the fusosome composition has an increase, e.g., an increase of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in teratoma formation compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell. In some embodiments, teratroma formation is determined by an assay measuring teratoma formation as described herein, in an appropriate animal model, e.g., in an animal model described herein.

[000470] In some embodiments, the fusosome composition has an increase, e.g., an increase of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in teratoma survival compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell. In some embodiments, the fusosome composition survives for one or more days in an assay of teratoma survival. In some embodiments, teratroma survival is determined by an assay measuring teratoma survival as described herein, in an appropriate animal model, e.g., in an animal model described herein. In an embodiments, teratoma formation is measured by imaging analysis, e.g., IHC staining, fluorescent staining or H&E, of fixed tissue, e.g., frozen or formalin fixed, as described in the Examples. In some embodiments, fixed tissue can be stained with any one or all of the following antibodies: anti-human CD3, anti-human CD4, or anti-human CD8.

[000471] In some embodiments, the fusosome composition has a reduction, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in CD8+ T cell infiltration into a graft or teratoma compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell. In some embodiments, CD8 T cell infiltration is determined by an assay measuring CD8+ T cell infiltration as described herein, e.g., histological analysis, in an appropriate animal model, e.g., an animal model described herein. In some embodiments, teratomas derived from the fusosome composition have CD8+ T cell infiltration in 0%, 0.1%, 1% 5%, 10%, 20%, 30%, 40% 50%, 60%, 70%, 80%, 90%, or 100% of 50x image fields of a histology tissue section.

[000472] In some embodiments, a fusosome composition has a reduction, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in CD4+ T cell infiltration into a graft or teratoma compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell. In some embodiments, CD4 T cell infiltration is determined by an assay measuring CD4+ T cell infiltration as described herein, , e.g., histological analysis, in an appropriate animal model, e.g., an animal model described herein. In some embodiments, teratomas derived from the fusosome composition have CD4+ T cell infiltration in 0%, 0.1%, 1% 5%, 10%, 20%, 30%, 40% 50%, 60%, 70%, 80%, 90%, or 100% of 50x image fields of a histology tissue section.

[000473] In some embodiments, a fusosome composition has a reduction, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in CD3+ NK cell infiltration into a graft or teratoma compared to a reference cell, e.g., an unmodified cell otherwise similar to

the source cell. In some embodiments, CD3+ NK cell infiltration is determined by an assay measuring CD3+ NK cell infiltration as described herein, e.g., histological analysis, in an appropriate animal model, e.g., an animal model described herein. In some embodiments, teratomas derived from the fusosome composition have CD3+ NK T cell infiltration in 0%, 0.1%, 1% 5%, 10%, 20%, 30%, 40% 50%, 60%, 70%, 80%, 90%, or 100% of 50x image fields of a histology tissue section.

[000474] In some embodiments, the fusosome composition has a reduction in immunogenicity as measured by a reduction in humoral response following one or more implantation of the fusosome derived into an appropriate animal model, e.g., an animal model described herein, compared to a humoral response following one or more implantation of a reference cell, e.g., an unmodified cell otherwise similar to the source cell, into an appropriate animal model, e.g., an animal model described herein. In some embodiments, the reduction in humoral response is measured in a serum sample by an anti-cell antibody titre, e.g., anti-fusosome antibody titre, e.g., by ELISA. In some embodiments, the serum sample from animals administered the fusosome composition has a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of an anti-cell antibody titer compared to the serum sample from animals administered an unmodified cell. In some embodiments, the serum sample from animals administered the fusosome composition has an increased anti-cell antibody titre, e.g., increased by 1%, 2%, 5%, 10%, 20%, 30%, or.40% from baseline, e.g., wherein baseline refers to serum sample from the same animals before administration of the fusosome composition.

[000475] In some embodiments, the fusosome composition has a reduction in macrophage phagocytosis, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in macrophage phagocytosis compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein the reduction in macrophage phagocytosis is determined by assaying the phagocytosis index *in vitro*, e.g., as described in Example 82. In some embodiments, the fusosome composition has a phagocytosis index of 0, 1, 10, 100, or more, e.g., as measured by an assay of Example 82, when incubated with macrophages in an *in vitro* assay of macrophage phagocytosis.

[000476] In some embodiments, the source cell has a reduction in cytotoxicity mediated cell

lysis by PBMCs, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in cell lysis compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell or a mesenchymal stem cells, e.g., using an assay of Example 83. In embodiments, the source cell expresses exogenous HLA-G.

[000477] In some embodiments, the fusosome composition has a reduction in NK-mediated cell lysis, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in NK-mediated cell lysis compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein NK-mediated cell lysis is assayed *in vitro,* by a chromium release assay or europium release assay.

[000478] In some embodiments, the fusosome composition has a reduction in CD8+ T-cell mediated cell lysis, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in CD8 T cell mediated cell lysis compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein CD8 T cell mediated cell lysis is assayed *in vitro,* by a chromium release assay or europium release assay. In embodiments, activation and/or proliferation is measured as described in Example 85.

[000479] In some embodiments, the fusosome composition has a reduction in CD4+ T-cell proliferation and/or activation, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein CD4 T cell proliferation is assayed *in vitro* (*e.g.* co-culture assay of modified or unmodified mammalian source cell, and CD4+T-cells with CD3/CD28 Dynabeads), e.g., as described in Example 86.

[000480] In some embodiments, the fusosome composition has a reduction in T-cell IFN-gamma secretion, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in T-cell IFN-gamma secretion compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein T-cell IFN-gamma secretion is assayed *in vitro, e.g.,* by IFN-gamma ELISPOT.

[000481] In some embodiments, the fusosome composition has a reduction in secretion of immunogenic cytokines, e.g., a reduction of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in secretion of immunogenic cytokines compared to a reference cell, e.g., an

unmodified cell otherwise similar to the source cell, wherein secretion of immunogenic cytokines is assayed i*n vitro* using ELISA or ELISPOT.

[000482] In some embodiments, the fusosome composition results in increased secretion of an immunosuppressive cytokine, e.g., an increase of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in secretion of an immunosuppressive cytokine compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein secretion of the immunosuppressive cytokine is assayed i*n vitro* using ELISA or ELISPOT.

[000483] In some embodiments, the fusosome composition has an increase in expression of HLA-G or HLA-E, e.g., an increase in expression of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of HLA-G or HLA-E, compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein expression of HLA-G or HLA-E is assayed i*n vitro* using flow cytometry, e.g., FACS. In some embodiments, the fusosome composition is derived from a source cell which is modified to have an increased expression of HLA-G or HLA-E, e.g., compared to an unmodified cell, e.g., an increased expression of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of HLA-G or HLA-E, wherein expression of HLA-G or HLA-E is assayed *in vitro* using flow cytometry, e.g., FACS. In some embodiments, the fusosome composition derived from a modified cell with increased HLA-G expression demonstrates reduced immunogenicity, e.g., as measured by reduced immune cell infiltration, in a teratoma formation assay, e.g., a teratoma formation assay as described herein.

[000484] In some embodiments, the fusosome composition has an increase in expression of T cell inhibitor ligands (e.g. CTLA4, PD1, PD-L1), e.g., an increase in expression of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of T cell inhibitor ligands as compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein expression of T cell inhibitor ligands is assayed *in vitro* using flow cytometry, e.g., FACS.

[000485] In some embodiments, the fusosome composition has a decrease in expression of co-stimulatory ligands, e.g., a decrease of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more in expression of co-stimulatory ligands compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell, wherein expression of co-stimulatory ligands is assayed i*n vitro* using flow cytometry, e.g., FACS.

[000486] In some embodiments, the fusosome composition has a decrease in expression of MHC class I or MHC class II, e.g., a decrease in expression of 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of MHC Class I or MHC Class II compared to a reference cell, e.g., an unmodified cell otherwise similar to the source cell or a HeLa cell, wherein expression of MHC Class I or II is assayed *in vitro* using flow cytometry, e.g., FACS.

[000487] In some embodiments, the fusosome composition is derived from a cell source, e.g., a mammalian cell source, which is substantially non-immunogenic. In some embodiments, immunogenicity can be quantified, e.g., as described herein. In some embodiments, the mammalian cell source comprises any one, all or a combination of the following features:

a. wherein the source cell is obtained from an autologous cell source; e.g., a cell obtained from a recipient who will be receiving, e.g., administered, the fusosome composition;

b. wherein the source cell is obtained from an allogeneic cell source which is of matched, e.g., similar, gender to a recipient, e.g., a recipient described herein who will be receiving, e.g., administered; the fusosome composition;

c. wherein the source cell is obtained is from an allogeneic cell source is which is HLA matched with a recipient's HLA, e.g., at one or more alleles;

d. wherein the source cell is obtained is from an allogeneic cell source which is an HLA homozygote;

e. wherein the source cell is obtained is from an allogeneic cell source which lacks (or has reduced levels compared to a reference cell) MHC class I and II; or

f. wherein the source cell is obtained is from a cell source which is known to be substantially non-immunogenic including but not limited to a stem cell, a mesenchymal stem cell, an induced pluripotent stem cell, an embryonic stem cell, a sertoli cell, or a retinal pigment epithelial cell .

[000488] In some embodiments, the subject to be administered the fusosome composition has, or is known to have, or is tested for, a pre-existing antibody (e.g., IgG or IgM) reactive with a fusosome. In some embodiments, the subject to be administered the fusosome composition does not have detectable levels of a pre-existing antibody reactive with the fusosome. Tests for the antibody are described, e.g., in Example 78.

[000489] In some embodiments, a subject that has received the fusosome composition has, or is known to have, or is tested for, an antibody (e.g., IgG or IgM) reactive with a fusosome. In some embodiments, the subject that received the fusosome composition (e.g., at least once, twice, three times, four times, five times, or more) does not have detectable levels of antibody reactive with the fusosome. In embodiments, levels of antibody do not rise more than 1%, 2%, 5%, 10%, 20%, or 50% between two timepoints, the first timepoint being before the first administration of the fusosome, and the second timepoint being after one or more administrations of the fusosome. Tests for the antibody are described, e.g., in Example 79.

Additional therapeutic agents

[000490] In some embodiments, the fusosome composition is co-administered with an additional agent, e.g., a therapeutic agent, to a subject, e.g., a recipient, e.g., a recipient described herein. In some embodiments, the co-administered therapeutic agent is an immunosuppressive agent, e.g., a glucocorticoid (e.g., dexamethasone), cytostatic (e.g., methotrexate), antibody (e.g., Muromonab-CD3), or immunophilin modulator (e.g., Ciclosporin or rapamycin). In embodiments, the immunosuppressive agent decreases immune mediated clearance of fusosomes. In some embodiments the fusosome composition is co-administered with an immunostimulatory agent, e.g., an adjuvant, an interleukin, a cytokine, or a chemokine.

[000491] In some embodiments, the fusosome composition and the immunosuppressive agent are administered at the same time, e.g., contemporaneously administered. In some embodiments, the fusosome composition is administered before administration of the immunosuppressive agent. In some embodiments, the fusosome composition is administered after administration of the immunosuppressive agent.

[000492] In some embodiments, the immunosuppressive agent is a small molecule such as ibuprofen, acetaminophen, cyclosporine, tacrolimus, rapamycin, mycophenolate, cyclophosphamide, glucocorticoids, sirolimus, azathriopine, or methotrexate.

[000493] In some embodiments, the immunosuppressive agent is an antibody molecule, including but not limited to: muronomab (anti-CD3), Daclizumab (anti-IL12), Basiliximab, Infliximab (Anti-TNFa), or rituximab (Anti-CD20).

[000494] In some embodiments, co-administration of the fusosome composition with the immunosuppressive agent results in enhanced persistence of the fusosome composition in the subject compared to administration of the fusosome composition alone. In some embodiments, the enhanced persistence of the fusosome composition in the co-administration is at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or longer, compared to persistence of the fusosome composition when administered alone. In some embodiments, the enhanced persistence of the fusosome composition in the co-administration is at least 1, 2, 3, 4, 5, 6, 7, 10, 15, 20, 25, or 30 days or longer, compared to survival of the fusosome composition when administered alone.

Delivery

[000495] In some embodiments, a fusogen (e.g., protein, lipid, or chemical fusogen) or a fusogen binding partner is delivered to a target cell or tissue prior to, at the same time, or after the delivery of a fusosome.

[000496] In some embodiments, a fusogen (e.g., protein, lipid, or chemical fusogen) or a fusogen binding partner is delivered to a non-target cell or tissue prior to, at the same time, or after the delivery of a fusosome.

[000497] In some embodiments, a nucleic acid that encodes a fusogen (e.g., protein or lipid fusogen) or a fusogen binding partner is delivered to a target cell or tissue prior to, at the same time, or after the delivery of a fusosome.

[000498] In some embodiments, a polypeptide, nucleic acid, ribonucleoprotein, or small-molecule that upregulates or downregulates expression of a fusogen (e.g., protein, lipid, or chemical fusogen) or a fusogen binding partner is delivered to a target cell or tissue prior to, at the same time, or after the delivery of a fusosome.

[000499] In some embodiments, a polypeptide, nucleic acid, ribonucleoprotein, or small-molecule that upregulates or downregulates expression of a fusogen (e.g., protein, lipid, or chemical fusogen) or a fusogen binding partner is delivered to a non-target cell or tissue prior to, at the same time, or after the delivery of a fusosome.

[000500] In some embodiments, the target cell or tissue is modified by (e.g., inducing stress or cell division) to increase the rate of fusion prior to, at the same time, or after the delivery of a

fusosome. Some nonlimiting examples include, inducing ischemia, treatment with chemotherapy, antibiotic, irradiation, toxin, inflammation, inflammatory molecules, anti-inflammatory molecules, acid injury, basic injury, burn, polyethylene glycol, neurotransmitters, myelotoxic drugs, growth factors, or hormones, tissue resection, starvation, and/or exercise.

[000501] In some embodiments, the target cell or tissue is treated with a vasodilator (e.g. nitric oxide (NO), carbon monoxide, prostacyclin (PGI2), nitroglycerine, phentolamine) or vasoconstrictors (e.g. angiotensin (AGT), endothelin (EDN), norepinephrine)) to increase the rate of fusosome transport to the target tissue.

[000502] In some embodiments, the target cell or tissue is treated with a chemical agent, e.g., a chemotherapeutic. In such embodiments, the chemotherapeutic induces damage to the target cell or tissue that enhances fusogenic activity of target cells or tissue.

[000503] In some embodiments, the target cell or tissue is treated with a physical stress, e.g., electrofusion. In such embodiments, the physical stress destabilizes the membranes of the target cell or tissue to enhance fusogenic activity of target cells or tissue.

[000504] In some embodiments, the target cell or tissue may be treated with an agent to enhance fusion with a fusosome. For example, specific neuronal receptors may be stimulated with an anti-depressant to enhance fusogenic properties.

[000505] Compositions comprising the fusosomes described herein may be administered or targeted to the circulatory system, hepatic system, renal system, cardio-pulmonary system, central nervous system, peripheral nervous system, musculoskeletal system, lymphatic system, immune system, sensory nervous systems (sight, hearing, smell, touch, taste), digestive system, endocrine systems (including adipose tissue metabolic regulation), and reproductive system.

[000506] In embodiments, a fusosome composition described herein is delivered ex-vivo to a cell or tissue, e.g., a human cell or tissue. In some embodiments, the composition is delivered to an ex vivo tissue that is in an injured state (e.g., from trauma, disease, hypoxia, ischemia or other damage).

[000507] In some embodiments, the fusosome composition is delivered to an ex-vivo transplant (e.g., a tissue explant or tissue for transplantation, e.g., a human vein, a musculoskeletal

graft such as bone or tendon, cornea, skin, heart valves, nerves; or an isolated or cultured organ, e.g., an organ to be transplanted into a human, e.g., a human heart, liver, lung, kidney, pancreas, intestine, thymus, eye). The composition improves viability, respiration, or other function of the transplant. The composition can be delivered to the tissue or organ before, during and/or after transplantation.

[000508] In some embodiments, a fusosome composition described herein is delivered ex-vivo to a cell or tissue derived from a subject. In some embodiments the cell or tissue is readministered to the subject (i.e., the cell or tissue is autologous).

[000509] The fusosomes may fuse with a cell from any mammalian (e.g., human) tissue, e.g., from epithelial, connective, muscular, or nervous tissue or cells, and combinations thereof. The fusosomes can be delivered to any eukaryotic (e.g., mammalian) organ system, for example, from the cardiovascular system (heart, vasculature); digestive system (esophagus, stomach, liver, gallbladder, pancreas, intestines, colon, rectum and anus); endocrine system (hypothalamus, pituitary gland, pineal body or pineal gland, thyroid, parathyroids, adrenal glands); excretory system (kidneys, ureters, bladder); lymphatic system (lymph, lymph nodes, lymph vessels, tonsils, adenoids, thymus, spleen); integumentary system (skin, hair, nails); muscular system (e.g., skeletal muscle); nervous system (brain, spinal cord, nerves)'; reproductive system (ovaries, uterus, mammary glands, testes, vas deferens, seminal vesicles, prostate); respiratory system (pharynx, larynx, trachea, bronchi, lungs, diaphragm); skeletal system (bone, cartilage), and combinations thereof.

[000510] In embodiments, the fusosome targets a tissue, e.g., liver, lungs, heart, spleen, pancreas, gastrointestinal tract, kidney, testes, ovaries, brain, reproductive organs, central nervous system, peripheral nervous system, skeletal muscle, endothelium, inner ear, adipose tissue (e.g., brown adipose tissue or white adipose tissue) or eye, when administered to a subject, e.g., wherein at least 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the fusosomes in a population of administered fusosomes are present in the target tissue after 24, 48, or 72 hours, e.g., by an assay of Example 87 or 100.

[000511] In embodiments, the fusosomes may fuse with a cell from a source of stem cells or progenitor cells, e.g., bone marrow stromal cells, marrow-derived adult progenitor cells (MAPCs),

endothelial progenitor cells (EPC), blast cells, intermediate progenitor cells formed in the subventricular zone, neural stem cells, muscle stem cells, satellite cells, liver stem cells, hematopoietic stem cells, bone marrow stromal cells, epidermal stem cells, embryonic stem cells, mesenchymal stem cells, umbilical cord stem cells, precursor cells, muscle precursor cells, myoblast, cardiomyoblast, neural precursor cells, glial precursor cells, neuronal precursor cells, hepatoblasts.

Fusogen Binding Partners, e.g., for landing pad embodiments

[000512] In certain aspects, the disclosure provides a method of delivering a fusosome to a target cell in a subject. In some embodiments, the method comprises administering to a subject a fusosome comprising a nucleic acid encoding a fusogen, e.g., a myomaker protein, wherein the nucleic acid is not present or is not expressed (e.g., is present but is not transcribed or not translated) within a cell, under conditions that allow the fusogen to be expressed on the surface of the fusosome in the subject. In some embodiments, the method further comprises administering to the subject a composition comprising an agent, e.g., a therapeutic agent, and a fusogen binding partner, optionally, comprising a carrier, e.g., a membrane, under conditions that allow fusion of the fusogen on the fusosome, and the fusogen binding partner. In some embodiments, the carrier comprises a membrane, e.g., a lipid bilayer, e.g., the agent is disposed within a lipid bilayer. In some embodiments, the lipid bilayer fuses with the target cell, thereby delivering the agent to the target cell in the subject.

[000513] In some embodiments, a fusogen binding partner is a moiety, e.g., a protein molecule, disposed in a membrane (e.g., a lipid bilayer), of a target cell, e.g., a target cell disclosed herein. In some embodiments, the membrane can be a cell surface membrane, or a subcellular membrane of an organelle, e.g., a mitochondrion, lysosome, or Golgi apparatus. In some embodiments, a fusogen binding partner can be endogenously expressed, overexpressed, or exogenously expressed (e.g., by a method described herein). In some embodiments, the fusogen binding partner can cluster with other fusogen binding partners at the membrane.

[000514] In some embodiments, the presence of a fusogen binding partner, or a plurality of fusogen binding partners, in a membrane of a target cell, creates an interface that can facilitate the interaction, e.g., binding, between a fusogen binding partner on a target cell (e.g., a cell described

herein), and a fusogen on a fusosome (e.g., a fusosome described herein). In some embodiments, the fusogen on a fusosome interacts with, e.g., binds to, a fusogen binding partner on target cell, e.g., on the membrane (e.g., lipid bilayer), of a target cell, to induce fusion of the fusosome with the target membrane. In some embodiments, the fusogen interacts with, e.g., binds to, a fusogen binding partner on a landing pad on a subcellular organelle, including a mitochondrion, to induce fusion of the fusosome with the subcellular organelle.

[000515] A fusogen binding partner can be introduced in a target cell, e.g., a target cell disclosed herein, by any of the methods discussed below.

[000516] In some embodiments, a method of introducing a fusogen binding partner to a target cell comprises removal, e.g., extraction, of a target cell (e.g., via apheresis or biopsy), from a subject (e.g., a subject described herein), and administration of, e.g., exposure to, a fusogen binding partner under conditions that allow the fusogen binding partner to be expressed on a membrane of the target cell. In some embodiments, a method comprises contacting the target cell expressing a fusogen binding partner *ex vivo* with a fusosome comprising a fusogen to induce fusion of the fusosome with the target cell membrane. In some embodiments, a target cell fused to the fusosome is reintroduced into the subject, e.g., intravenously.

[000517] In some embodiments, a target cell expressing a fusogen binding partner is reintroduced into the subject, e.g., intravenously. In some embodiments, a method comprises administering to the subject a fusosome comprising a fusogen to allow interaction, e.g., binding, of the fusogen on the fusosome with the fusogen binding partner on the target cell, and fusion of the fusosome with the target cell membrane.

[000518] In some embodiments, the target cells are treated with an epigenetic modifier, e.g., a small molecule epigenetic modifier, to increase or decrease expression of an endogenous cell surface molecule (e.g.,in some embodiments, endogenous relative to the target cell), e.g., a fusogen binding partner, e.g., an organ, tissue, or cell targeting molecule, where the cell surface molecule is a protein, glycan, lipid or low molecular weight molecule. In some embodiments, a target cell is genetically modified to increase the expression of an endogenous cell surface molecule, e.g., a fusogen binding partner, e.g., an organ, tissue, or cell targeting molecule, where the cell surface molecule is a protein, glycan, lipid or low molecular weight molecule. In some embodiments, a

genetic modification may decrease expression of a transcriptional activator of the endogenous cell surface molecule, e.g., a fusogen binding partner.

[000519] In some embodiments, a target cell is genetically modified to express, e.g., overexpress, an exogenous cell surface molecule, e.g., a fusogen binding partner, where the cell surface molecule is a protein, glycan, lipid or low molecular weight molecule.

[000520] In some embodiments, the target cell is genetically modified to increase the expression of an exogenous fusogen in the cell, e.g., delivery of a transgene. In some embodiments, a nucleic acid, e.g., DNA, mRNA or siRNA, is transferred to the target cell, e.g., to increase or decrease the expression of a cell surface molecule (protein, glycan, lipid or low molecular weight molecule). In some embodiments, the nucleic acid targets a repressor of a fusogen binding partner, e.g., an shRNA, or siRNA construct. In some embodiments, the nucleic acid encodes an inhibitor of a fusogen binding partner repressor.

Methods of Use

[000521] The administration of a pharmaceutical composition described herein may be by way of oral, inhaled, transdermal or parenteral (including intravenous, intratumoral, intraperitoneal, intramuscular, intracavity, and subcutaneous) administration. The fusosomes may be administered alone or formulated as a pharmaceutical composition.

[000522] The fusosomes may be administered in the form of a unit-dose composition, such as a unit dose oral, parenteral, transdermal or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for oral, inhaled, transdermal or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusable solutions or suspensions or suppositories or aerosols.

[000523] In some embodiments, delivery of a membrane protein payload agent via a fusosome composition described herein may induce or block cellular differentiation, de-differentiation, or trans-differentiation. The target mammalian cell may be a precursor cell. Alternatively, the target mammalian cell may be a differentiated cell, and the cell fate alteration

includes driving de-differentiation into a pluripotent precursor cell, or blocking such de-differentiation. In situations where a change in cell fate is desired, effective amounts of a fusosome described herein encoding a cell fate inductive molecule or signal is introduced into a target cell under conditions such that an alteration in cell fate is induced. In some embodiments, a fusosome described herein is useful to reprogram a subpopulation of cells from a first phenotype to a second phenotype. Such a reprogramming may be temporary or permanent. Optionally, the reprogramming induces a target cell to adopt an intermediate phenotype.

[000524] Also provided are methods of reducing cellular differentiation in a target cell population. For example, a target cell population containing one or more precursor cell types is contacted with a fusosome composition described herein, under conditions such that the composition reduces the differentiation of the precursor cell. In certain embodiments, the target cell population contains injured tissue in a mammalian subject or tissue affected by a surgical procedure. The precursor cell is, e.g., a stromal precursor cell, a neural precursor cell, or a mesenchymal precursor cell.

[000525] A fusosome composition described herein, comprising a membrane protein payload agent may be used to deliver such agent to a cell tissue or subject. Delivery of a membrane protein payload agent by administration of a fusosome composition described herein may modify cellular protein expression levels. In certain embodiments, the administered directs upregulation of (via expression in the cell, delivery in the cell, or induction within the cell) of one or more membrane protein payload agent (e.g., a polypeptide or nucleic acid) that provide a functional activity which is substantially absent or reduced in the cell in which the membrane protein payload agent is delivered. For example, the missing functional activity may be enzymatic, structural, signaling or regulatory in nature. In related embodiments, the administered composition directs up-regulation of one or more membrane protein payload agent that increases (e.g., synergistically) a functional activity which is present but substantially deficient in the cell in which the membrane protein payload agent is upregulated. In related embodiments, the administered composition directs down-regulation of one or more polypeptides that decreases (e.g., synergistically) a functional activity which is present or upregulated in the cell in which the polypeptide is downregulated. In certain embodiments, the administered composition directs upregulation of certain functional activities

and downregulation of other functional activities.

[000526] In embodiments, the fusosome composition mediates an effect on a target cell, and the effect lasts for at least 1, 2, 3, 4, 5, 6, or 7 days, 2, 3, or 4 weeks, or 1, 2, 3, 6, or 12 months. In some embodiments (e.g., wherein the fusosome composition comprises an exogenous protein), the effect lasts for less than 1, 2, 3, 4, 5, 6, or 7 days, 2, 3, or 4 weeks, or 1, 2, 3, 6, or 12 months.

Ex-vivo Applications

[000527] In embodiments, the fusosome composition described herein is delivered ex-vivo to a cell or tissue, e.g., a human cell or tissue. In embodiments, the composition improves function of a cell or tissue ex-vivo, e.g., improves cell viability, signaling, respiration, or other function (e.g., another function described herein).

[000528] In some embodiments, the composition is delivered to an ex vivo tissue that is in an injured state (e.g., from trauma, disease, hypoxia, ischemia or other damage).

[000529] In some embodiments, the composition is delivered to an ex-vivo transplant (e.g., a tissue explant or tissue for transplantation, e.g., a human vein, a musculoskeletal graft such as bone or tendon, cornea, skin, heart valves, nerves; or an isolated or cultured organ, e.g., an organ to be transplanted into a human, e.g., a human heart, liver, lung, kidney, pancreas, intestine, thymus, eye). The composition can be delivered to the tissue or organ before, during and/or after transplantation.

[000530] In some embodiments, the composition is delivered, administered or contacted with a cell, e.g., a cell preparation. The cell preparation may be a cell therapy preparation (a cell preparation intended for administration to a human subject). In embodiments, the cell preparation comprises cells expressing a chimeric antigen receptor (CAR), e.g., expressing a recombinant CAR. The cells expressing the CAR may be, e.g., T cells, Natural Killer (NK) cells, cytotoxic T lymphocytes (CTL), regulatory T cells. In embodiments, the cell preparation is a neural stem cell preparation. In embodiments, the cell preparation is a mesenchymal stem cell (MSC) preparation. In embodiments, the cell preparation is a hematopoietic stem cell (HSC) preparation. In embodiments, the cell preparation is an islet cell preparation.

In Vivo Uses

[000531] The fusosome compositions described herein can be administered to a subject, e.g., a mammal, e.g., a human. In such embodiments, the subject may be at risk of, may have a symptom of, or may be diagnosed with or identified as having, a particular disease or condition (e.g., a disease or condition described herein). In one embodiment, the subject has cancer. In one embodiment, the subject has an infectious disease.

[000532] In some embodiments, the source of fusosomes is from the same subject that is administered a fusosome composition. In other embodiments, they are different. For example, the source of fusosomes and recipient tissue may be autologous (from the same subject) or heterologous (from different subjects). In either case, the donor tissue for fusosome compositions described herein may be a different tissue type than the recipient tissue. For example, the donor tissue may be muscular tissue and the recipient tissue may be connective tissue (e.g., adipose tissue). In other embodiments, the donor tissue and recipient tissue may be of the same or different type, but from different organ systems.

[000533] A fusosome composition described herein may be administered to a subject having a cancer, an autoimmune disease, an infectious disease, a metabolic disease, a neurodegenerative disease, or a genetic disease (e.g., enzyme deficiency). In some embodiments, a tissue of the subject is in need of regeneration.

[000534] In some embodiments, a therapeutically effective amount of a fusosome composition described herein is administered to a subject. In some embodiments, a therapeutically effective amount of a substance is an amount that is sufficient, when administered to a subject who has or is susceptible to a disease, disorder, and/or condition, to treat, and/or delay the onset of the disease, disorder, and/or condition. For example, in embodiments the effective amount of a fusosome in a formulation to treat a disease, disorder, and/or condition is the amount that alleviates, ameliorates, relieves, inhibits, delays onset of, reduces severity of and/or reduces incidence of one or more symptoms or features of the disease, disorder, and/or condition.

[000535] In some embodiments, a subject is treated with a fusosome composition. In some embodimetns, the treatment partially or completely alleviates, ameliorates, relieves, inhibits, delays onset of, reduces severity of, and/or reduces incidence of one or more symptoms, features,

and/or causes of a particular disease, disorder, and/or condition. In some embodiments, treatment may be of a subject who has been diagnosed as suffering from the relevant disease, disorder, and/or condition. In some embodiments, treatment may be of a subject known to have one or more susceptibility factors that are statistically correlated with increased risk of development of the relevant disease, disorder, and/or condition. In some embodiments, treatment partially or completely ameliorates the root cause of the relevant disease, disorder, and/or condition.

[000536] In some embodiments, the fusosome composition is effective to treat the disease, e.g., cancer. In some embodiments, the fusosome composition is effective to reduce the number of cancer cells in the subject compared to the number of cancer cells in the subject before administration. In some embodiments, the fusosome composition is effective to reduce the number of cancer cells in the subject compared to the expected course of disease without treatment. In some embodiments, the subject experiences a complete response or partial response after administration of the fusosome composition.

[000537] In some embodiments, the fusosome is co-administered with an inhibitor of a protein that inhibits membrane fusion. For example, Suppressyn is a human protein that inhibits cell-cell fusion (Sugimoto et al., "A novel human endogenous retroviral protein inhibits cell-cell fusion" Scientific Reports 3:1462 DOI: 10.1038/srep01462). Thus, in some embodiments, the fusosome is co-administered with an inhibitor of sypressyn, e.g., a siRNA or inhibitory antibody.

Non-human Applications

[000538] Compositions described herein may also be used to similarly modulate the cell or tissue function or physiology of a variety of other organisms including but not limited to: farm or working animals (horses, cows, pigs, chickens etc.), pet or zoo animals (cats, dogs, lizards, birds, lions, tigers and bears etc.), aquaculture animals (fish, crabs, shrimp, oysters etc.), plants species (trees, crops, ornamentals flowers etc), fermentation species (saccharomyces etc.). Fusosome compositions described herein can be made from such non-human sources and administered to a non-human target cell or tissue or subject.

[000539] Fusosome compositions can be autologous, allogeneic or xenogeneic to the target.

[000540] All references and publications cited herein are hereby incorporated by reference.

[000541] The following examples are provided to further illustrate some embodiments of the present invention, but are not intended to limit the scope of the invention; it will be understood by their exemplary nature that other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLES

Example 1. Generating enucleated fusogenic cells via chemical treatment (PEG)

[000542] Mito-DsRed (a mitochondrial specific targeted dye) expressing donor HeLa cells were trypsinized with 0.25% trypsin, collected, spun at 500xg for 5min, washed once in PBS and counted. $10x10^6$ cells were subsequently resuspended in 3ml of 12.5% ficoll in complete MEM-alpha (+10% FBS, + 1% penicillin/streptomycin, + glutamine) supplemented with 10ug/mL cytochalasin-B for 15 min. To enucleate cells, they were transferred to a discontinuous ficoll gradient consisting of the following ficoll fractions (from top to bottom): 2 mL 12.5% ficoll, 0.5 mL 15% ficoll, 0.5 mL 16% ficoll, 2 mL 17% ficoll gradient, 2 mL 25% ficoll. All ficoll gradient fractions were made in complete DMEM supplemented with 10ug/mL cytochalasin-B. Gradients were spun on a Beckman SW-40 ultracentrifuge, Ti-70 rotor at 107971xg for 1h at 37°C. Following centrifugation, enucleated HeLa cells were collected from the 12.5%, 15%, 16%, and 1/2 of the 17% ficoll fractions and resuspended in complete DMEM (+10% FBS, + 1% penicillin/streptomycin, + glutamine), and spun at 500xg for 5min to pellet. Enucleated Mito-DsRed donor cells were washed 2x in DMEM. Simultaneously, Mito-GFP (a mitochondrial specific targeted dye) expressing recipient HeLa cells were trypsinized, counted, and prepared for fusion.

[000543] For fusion, enucleated Mito-DsRed donor HeLa cells were combined at a 1:1 ratio with Mito-GFP recipient HeLa cells (200,000 each) in a 50% polyethylene glycol solution (50% PEG by w/v prepared in DMEM complete w/10% DMSO) for 1 minute at 37°C. Cells were subsequently washed 3x in 10mL complete DMEM and plated on 35mm glass-bottom quadrant imaging dishes at density of 50k cells/quadrant, with each quadrant having an area of 1.9 cm^2.

Example 2. Generating nucleated fusogenic cells via chemical treatment (PEG)

[000544] Mito-DsRed (a mitochondrial specific targeted dye) expressing donor HeLa cells were trypsinized with 0.25% trypsin, collected, spun at 500xg for 5min, washed once in PBS and counted. $2x10^6$ cells were subsequently resuspended in complete DMEM (+10% FBS, + 1% penicillin/streptomycin, + glutamine), counted, and prepared for fusion.

[000545] Mito-DsRed donor cells were washed 3x in DMEM. Simultaneously, Mito-GFP (a mitochondrial specific targeted dye) expressing recipient HeLa cells were trypsinized, counted, and prepared for fusion.

[000546] For fusion, Mito-DsRed donor HeLa cells were combined at a 1:1 ratio with Mito-GFP recipient HeLa cells (200,000 each) in a 50% polyethylene glycol solution (50% PEG by w/v prepared in DMEM complete w/10% DMSO) with for 1 minute at 37°C. Cells were subsequently washed 3X in 10ml complete DMEM and plated on 35 mm glass-bottom quadrant imaging dishes at density of 50k cells/quadrant, with each quadrant having an area of 1.9 cm^2.

Example 3. Creation of HeLa cells expressing exogenous fusogens

[000547] This example describes the creation of tissue culture cells expressing an exogenous fusogen. The following example is equally applicable to any protein based fusogen and is equally applicable to production in primary cells (in suspension or adherent) and tissue. In certain cases, a fusogen pair can be used to induce fusion (delineated as fusogen and a fusogen binding partner).

[000548] The fusogen gene, fusion failure 1 (EFF-1), is cloned into pIRES2-AcGFP1 vector (Clontech), and this construct is then transfected into HeLa cells (CCL-2™, ATCC) using the Lipofectamine 2000 transfection reagent (Invitrogen). The fusogen binding partner gene, anchor-cell fusion failure 1 (AFF-1), is cloned into pIRES2 DsRed-Express 2 vector (Clontech), and this construct is then transfected into HeLa cells (CCL-2™, ATCC) using the Lipofectamine 2000 transfection reagent (Invitrogen). Transfected HeLa cells are kept at 37 °C, 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX (GIBCO), 10% fetal calf serum (GIBCO) and 500 mg/mL zeocin. EFF-1 expressing cells are isolated by sorting fluorescent activated cell sorting (FACS) to get a pure population of GFP+ Hela cells expressing EFF-1 fusogen. AFF-1 expressing cells are isolated by sorting fluorescent activated cell sorting (FACS)

to get a pure population of DSRED+ Hela cells expressing AFF-1 fusogen binding partner.

Example 4. Organelle delivery via chemically enhanced fusogenic enucleated cells

[000549] Fusogenic cells (Mito-DsRed donor enucleated cells and Mito-GFP recipient HeLa cells) produced and fused as described in Example 1 were imaged on a Zeiss LSM 780 inverted confocal microscope at 63X magnification 24h following deposition in the imaging dish. Cells expressing only Mito-DsRed alone and Mito-GFP alone were imaged separately to configure acquisition settings in such a way as to ensure no signal overlap between the two channels in conditions where both Mito-DsRed and Mito-GFP were both present and acquired simultaneously. Ten regions of interest were chosen in a completely unbiased manner, with the only criteria being that a minimum of 10 cells be contained within each ROI, such that a minimum of 100 cells were available for downstream analysis. A given pixel in these images was determined to be positive for mitochondria if its intensity for either channel (mito-DsRed and mito-GFP) was greater than 10% of the maximum intensity value for each respective channel across all three ROIs.

[000550] Fusion events with organelle delivery were identified based on the criteria that >50% of the mitochondria (identified by all pixels that are either mito-GFP+ or mito-Ds-Red+) in a cell were positive for both mitoDs-Red and mito-GFP based on the above indicated threshold, indicating that organelles (in this case mitochondria) containing these proteins have been delivered, fused and their contents intermingled. At the 24-hour time point multiple cells exhibited positive organelle delivery via fusion as indicated in Figure 7. This is the image of a positive organelle delivery via fusion between donor and recipient HeLa cells. The intracellular areas indicated in white indicate overlap between donor and recipient mitochondria. The intracellular regions in grey indicate where donor and recipient organelles do not overlap.

Example 5. Organelle delivery via chemically enhanced fusogenic nucleated cells

[000551] Fusogenic cells (Mito-DsRed donor cells and Mito-GFP recipient HeLa cells) produced and combined as described in example 2 were imaged on a Zeiss LSM 780 inverted confocal microscope at 63X magnification 24h following deposition in the imaging dish. Cells expressing only Mito-DsRed alone and Mito-GFP alone were imaged separately to configure acquisition settings in such a way as to ensure no signal overlap between the two channels in conditions where both Mito-DsRed and Mito-GFP were both present and acquired simultaneously.

Ten regions of interest were chosen in a completely unbiased manner, with the only criteria being that a minimum of 10 cells be contained within each ROI, such that a minimum of 100 cells were available for downstream analysis. A given pixel in these images was determined to be positive for mitochondria if it's intensity for either channel (mito-DsRed and mito-GFP) was greater than 20% of the maximum intensity value for each respective channel across all three ROIs.

[000552] Fusion events with organelle delivery were identified based on the criteria that >50% of the mitochondria (identified by all pixels that are either mito-GFP+ or mito-Ds-Red+) in a cell were positive for both mitoDs-Red and mito-GFP based on the above indicated threshold, indicating that organelles (in this case mitochondria) containing these proteins have been delivered, fused and their contents intermingled. At the 24-hour time point multiple cells exhibited positive organelle delivery via fusion as indicated in Figure 8. This is the image of a positive organelle delivery via fusion between donor and recipient HeLa cells. The intracellular areas indicated in white indicate overlap between donor and recipient mitochondria. The intracellular regions in grey indicate where donor and recipient organelles do not overlap.

Example 6. Delivery of mitochondria via protein enhanced fusogenic enucleated cells

[000553] Fusogenic cells produced and combined as described in Example 3 are imaged on a Zeiss LSM 780 inverted confocal microscope at 63X magnification 24h following deposition in the imaging dish. Cells expressing only Mito-DsRed alone and Mito-GFP alone are imaged separately to configure acquisition settings in such a way as to ensure no signal overlap between the two channels in conditions where both Mito-DsRed and Mito-GFP are both present and acquired simultaneously. Ten regions of interest are chosen in a completely unbiased manner, with the only criteria being that a minimum of 10 cells be contained within each ROI, such that a minimum number of cells are available for downstream analysis. A given pixel in these images is determined to be positive for mitochondria if it's intensity for either channel (mito-DsRed and mito-GFP) is greater than 10% of the maximum intensity value for each respective channel across all three ROIs.

[000554] Fusion events with organelle delivery will be identified based on the criteria that >50% of the mitochondria (identified by all pixels that are either mito-GFP+ or mito-Ds-Red+) in a cell are positive for both mitoDs-Red and mito-GFP based on the above indicated threshold,

which will indicate that organelles (in this case mitochondria) containing these proteins are delivered, fused and their contents intermingled. At the 24-hour time point multiple cells are expected to exhibit positive organelle delivery via fusion.

Example 7: Generation of fusosomes through nucleic acid electroporation

[000555] This example describes fusosome generation through electroporation of cells or vesicles with nucleic acids (e.g., mRNA or DNA) that encode a fusogen.

[000556] Transposase vectors (System Biosciences, Inc.) that include the open reading frame of the Puromycin resistance gene together with an open reading frame of a cloned fragment (e.g. Glycoprotein from Vesicular stomatitis virus [VSV-G], Oxford Genetics # OG592) are electroporated into 293Ts using an electroporator (Amaxa) and a 293T cell line specific nuclear transfection kit (Lonza).

[000557] Following selection with 1 μg/μL puromycin for 3-5 days in DMEM containing 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin, the cells are then washed with 1xPBS, ice-cold lysis buffer (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0 and protease inhibitor cocktail (Abcam, ab201117)), sonicated 3 times, 10-15 secs per time and centrifuged at 16,000 x g for 20min. A western blot is conducted on the recovered supernatant fraction with a probe specific to VSV-G to determine the non-membrane specific concentration of VSV-G from the fusosomes prepared from stably transfected cells or control cells and compared to the standard of VSV-G protein.

[000558] In embodiments, the fusosomes from stably transfected cells will have more VSV-G than fusosomes generated from cells that were not stably transfected.

Example 8: Generation of fusosomes through protein electroporation

[000559] This example describes electroporation of fusogens to generate fusosomes.

[000560] Approximately 5×10^6 cells or vesicles are used for electroporation using an electroporation transfection system (Thermo Fisher Scientific). To set up a master mix, 24 μg of purified protein fusogens is added to resuspension buffer (provided in the kit). The mixture is incubated at room temperature for 10 min. Meanwhile, the cells or vesicles are transferred to a sterile test tube and centrifuged at $500 \times g$ for 5 min. The supernatant is aspirated and the pellet is

resuspended in 1 mL of PBS without Ca^{2+} and Mg^{2+}. The buffer with the fusogens is then used to resuspend the pellet of cells or vesicles. A cell or vesicle suspension is also used for optimization conditions, which vary in pulse voltage, pulse width and the number of pulses. After electroporation, the electroporated cells or vesicles with fusogens are washed with PBS, resuspended in PBS, and kept on ice.

[000561] See, for example, Liang et al., Rapid and highly efficiency mammalian cell engineering via Cas9 protein transfection, *Journal of Biotechnology* 208: 44-53, 2015.

Example 9: Generating and isolating fusosomes through vesicle formation and centrifugation

[000562] This example describes fusosome generation and isolation via vesiculation and centrifugation. This is one of the methods by which fusosomes may be isolated.

[000563] Fusosomes are prepared as follows. Approximately 4 x 106 HEK-293T cells are seeded in a 10 cm dish in complete media (DMEM + 10% FBS + Pen/Strep). One day after seeding, 15 μg of fusogen expressing plasmid or virus is delivered to cells. After a sufficient period of time for fusogen expression, medium is carefully replaced by fresh medium supplemented with 100 μM ATP. Supernatants are harvested 48-72 hours after fusogen expression, clarified by filtration through a 0.45 μm filter, and ultracentrifuged at 150,000 x g for 1 h. Pelleted material is resuspended overnight in ice cold PBS. Fusosomes are resuspended in desired buffer for experimentation.

[000564] See for example, Mangeot et al., Molecular Therapy, vol. 19 no. 9, 1656–1666, Sept. 2011

Example 10: Generating and isolating giant plasma membrane fusosomes

[000565] This example describes fusosome generation and isolation via vesiculation and centrifugation. This is one of the methods by which fusosomes may be isolated. Fusosomes are prepared as follows.

[000566] Briefly, HeLa cells that express a fusogen are washed twice in buffer (10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4), resuspended in a solution (1 mM DTT, 12.5 mM

Paraformaldehyde, and 1 mM N-ethylmaleimide in GPMV buffer), and incubated at 37 °C for 1 h. Fusosomes are clarified from cells by first removing cells by centrifugation at 100 x g for 10 minutes, and then harvesting fusosomes at 20,000 x g for 1 h at 4 °C. The fusosomes are resuspended in desired buffer for experimentation.

[000567] See for example, Sezgin E *et al.* Elucidating membrane structure and protein behavior using giant membrane plasma vesicles. *Nat. Protocols*. 7(6):1042-51 2012.

Example 11: Generating and isolating fusosome ghosts

[000568] This example describes fusosome generation and isolation via hypotonic treatment and centrifugation. This is one of the methods by which fusosomes may be produced.

[000569] First, fusosomes are isolated from mesenchymal stem cells expressing fusogens (10^9 cells) primarily by using hypotonic treatment such that the cell ruptures and fusosomes are formed. Cells are resuspended in hypotonic solution, Tris-magnesium buffer (TM, e.g., pH 7.4 or pH 8.6 at 4^0C, pH adjustment made with HCl). Cell swelling is monitored by phase-contrast microscopy. Once the cells swell and fusosomes are formed, the suspension is placed in a homogenizer. Typically, about 95% cell rupture is sufficient as measured through cell counting and standard AOPI staining. The membranes/fusosomes are then placed in sucrose (0.25 M or higher) for preservation. Alternatively, fusosomes can be formed by other approaches known in the art to lyse cells, such as mild sonication (Arkhiv anatomii, gistologii i embriologii; 1979, Aug, 77(8) 5-13; PMID: 496657), freeze-thaw (Nature. 1999, Dec 2;402(6761):551-5; PMID: 10591218), French-press (Methods in Enzymology, Volume 541, 2014, Pages 169-176; PMID: 24423265), needle-passaging (www.sigmaaldrich.com/technical-documents/protocols/biology/nuclear-protein-extraction.html) or solublization in detergent-containing solutions (www.thermofisher.com/order/catalog/product/89900).

[000570] To avoid adherence, the fusosomes are placed in plastic tubes and centrifuged. A laminated pellet is produced in which the topmost lighter gray lamina includes mostly fusosomes. However, the entire pellet is processed, to increase yields. Centrifugation (e.g., 3,000 rpm for 15 min at 4°C) and washing (e.g., 20 volumes of Tris magnesium/TM-sucrose pH 7.4) may be repeated.

[000571] In the next step, the fusosome fraction is separated by floatation in a discontinuous sucrose density gradient. A small excess of supernatant is left remaining with the washed pellet, which now includes fusosomes, nuclei, and incompletely ruptured whole cells. An additional 60% w/w sucrose in TM, pH 8.6, is added to the suspension to give a reading of 45% sucrose on a refractometer. After this step, all solutions are TM pH 8.6. 15 mL of suspension are placed in SW-25.2 cellulose nitrate tubes and a discontinuous gradient is formed over the suspension by adding 15 mL layers, respectively, of 40% and 35% w/w sucrose, and then adding 5 mL of TM-sucrose (0.25 M). The samples are then centrifuged at 20,000 rpm for 10 min, 4°C. The nuclei sediment form a pellet, the incompletely ruptured whole cells are collected at the 40%-45% interface, and the fusosomes are collected at the 35%-40% interface. The fusosomes from multiple tubes are collected and pooled. See for example, International patent publication, WO2011024172A2.

Example 12: Generating fusosomes through extrusion

[000572] This example describes fusosome manufacturing by extrusion through a membrane.

[000573] Briefly, hematopoietic stem cells that express fusogens are in a 37°C suspension at a density of 1 x 10^6 cells/mL in serum-free media containing protease inhibitor cocktail (Set V, Calbiochem 539137-1ML). The cells are aspirated with a luer lock syringe and passed once through a disposable 5 mm syringe filter into a clean tube. If the membrane fouls and becomes clogged, it is set aside and a new filter is attached. After the entire cell suspension has passed through the filter, 5 mL of serum-free media is passed through all filters used in the process to wash any remaining material through the filter(s). The solution is then combined with the extruded fusosomes in the filtrate.

[000574] Fusosomes may be further reduced in diameter by continued extrusion following the same method with increasingly smaller filter pore sizes, ranging from 5 mm to 0.2 mm. When the final extrusion is complete, suspensions are pelleted by centrifugation (time and speed required vary by size) and resuspended in media.

[000575] Additionally, this process can be supplemented with the use of an actin cytoskeleton inhibitor in order to decrease the influence of the existing cytoskeletal structure on extrusion. Briefly, a 1 x 10^6 cell/mL suspension is incubated in serum-free media with 500 nM Latrunculin B (ab144291, Abcam, Cambridge, MA) and incubated for 30 minutes at 37 °C in the presence of

5% CO_2. After incubation, protease inhibitor cocktail is added and cells are aspirated into a luer lock syringe, with the extrusion carried out as previously described.

[000576] Fusosomes are pelleted and washed once in PBS to remove the cytoskeleton inhibitor before being resuspended in media.

Example 13: Generation of fusosomes through chemical treatment with protein

[000577] This example describes chemical-mediated delivery of fusogens to generate fusosomes. Approximately 5×10^6 cells or vesicles are used for chemical-mediated delivery of fusogens. The cells or vesicles are suspended in 50 µL of Opti-MEM medium. To set up a master mix, 24 µg of purified protein fusogens is mixed with 25 µL of Opti-MEM medium, followed by the addition of 25 µL of Opti-MEM containing 2 µL of lipid transfection reagent 3000. The cells or vesicles and fusogen solutions are mixed by gently swirling the plate and incubating at 37 C for 6 hours, such that the fusogen will be incorporated into the cell or vesicle membrane. Fusosomes are then washed with PBS, resuspended in PBS, and kept on ice.

[000578] See, also, Liang et al., Rapid and highly efficiency mammalian cell engineering via Cas9 protein transfection, *Journal of Biotechnology* 208: 44-53, 2015.

Example 14: Generation of fusosomes through treatment with fusogen-containing liposomes

[000579] This example describes liposome-mediated delivery of fusogens to a source cell to generate fusosomes. Approximately 5×10^6 cells or vesicles are used for liposome-mediated delivery of fusogens. The cells or vesicles are suspended in 50 µL of Opti-MEM medium. The fusogen protein is purified from cells in the presence of n-octyl b-D-glucopyranoside. n-octyl b-D-glucopyranoside is a mild detergent used to solubilize integral membrane proteins. The fusogen protein is then reconstituted into large (400nm diameter) unilamellar vesicles (LUVs) by mixing n-octyl b-D-glucopyranoside -suspended protein with LUVs presaturated with n-octyl b-D-glucopyranoside, followed by removal of n-octyl b-D-glucopyranoside, as described in Top et al., *EMBO* 24: 2980-2988, 2005. To set up a master mix, a mass of liposomes that contains 24 µg of total fusogen protein is mixed with 50 µL of Opti-MEM medium. The solutions of liposomes and source cells or vesicles are then combined, and the entire solution is mixed by gently swirling the

plate and incubating at 37 C for 6 hours under conditions that allow fusion of the fusogen-containing liposomes and the source cells or vesicle, such that the fusogen protein will be incorporated into the source cell or vesicle membrane. Fusosomes are then washed with PBS, resuspended in PBS, and kept on ice. See, also, Liang et al., Rapid and highly efficiency mammalian cell engineering via Cas9 protein transfection, *Journal of Biotechnology* 208: 44-53, 2015.

Example 15: Isolating fusogenic microvesicles freely released from cells

[000580] This example describes isolation of fusosomes via centrifugation. This is one of the methods by which fusosomes may be isolated.

[000581] Fusosomes are isolated from cells expressing fusogens by differential centrifugation. Culture media (DMEM + 10% fetal bovine serum) is first clarified of small particles by ultracentrifugation at >100,000 *x g* for 1 h. Clarified culture media is then used to grow Mouse Embryonic Fibroblasts expressing fusogens. The cells are separated from culture media by centrifugation at 200 *x g* for 10 minutes. Supernatants are collected and centrifuged sequentially twice at 500 x g for 10 minutes, once at 2,000 x g for 15 minutes, once at 10,000 x g for 30 min, and once at 70,000 x g for 60 minutes. Freely released fusosomes are pelleted during the final centrifugation step, resuspended in PBS and repelleted at 70,000 x g. The final pellet is resuspended in PBS.

[000582] See also, Wubbolts R *et al*. Proteomic and Biochemical Analyses of Human B Cell-derived Exosomes: Potential Implications for their Function and Multivesicular Body Formation. *J. Biol. Chem*. 278:10963-10972 2003.

Example 16: Physical enucleation of fusosomes

[000583] This example describes enucleation of fusosomes via cytoskeletal inactivation and centrifugation. This is one of the methods by which fusosomes may be modified.

[000584] Fusosomes are isolated from mammalian primary or immortalized cell lines that express a fusogen. The cells are enucleated by treatment with an actin skeleton inhibitor and ultracentrifugation. Briefly, C2C12 cells are collected, pelleted, and resuspended in DMEM containing 12.5% Ficoll 400 (F2637, Sigma, St. Louis MO) and 500 nM Latrunculin B (ab144291,

Abcam, Cambridge, MA) and incubated for 30 minutes at 37 °C + 5% CO_2. Suspensions are carefully layered into ultracentrifuge tubes containing increasing concentrations of Ficoll 400 dissolved in DMEM (15%, 16%, 17%, 18%, 19%, 20%, 3 mL per layer) that have been equilibrated overnight at 37 °C in the presence of 5% CO_2. Ficoll gradients are spun in a Ti-70 rotor (Beckman-Coulter, Brea, CA) at 32,300 RPM for 60 minutes at 37 C. After ultracentrifugation, fusosomes found between 16 – 18% Ficoll are removed, washed with DMEM, and resuspended in DMEM.

[000585] Staining for nuclear content with Hoechst 33342 as described in Example 35 followed by the use of flow cytometry and/or imaging will be performed to confirm the ejection of the nucleus.

Example 17: Modifying fusosomes via irradiation

[000586] The following example describes modifying fusosomes with gamma irradiation. Without being bound by theory, gamma irradiation may cause double stranded breaks in the DNA and drive cells to undergo apoptosis.

[000587] First, cells expressing fusogens are cultured in a monolayer on tissue culture flasks or plates below a confluent density (e.g. by culturing or plating cells). Then the medium is removed from confluent flasks, cells are rinsed with Ca2+ and Mg2+ free HBSS, and trypsinized to remove the cells from the culture matrix. The cell pellet is then resuspended in 10ml of tissue-culture medium without penicillin/streptomycin and transferred to a 100 mm Petri dish. The number of cells in the pellet should be equivalent to what would be obtained from 10-15 confluent MEF cultures on 150 cm^2 flasks. The cells are then exposed to 4000 rads from a γ-radiation source to generate fusosomes. The fusosomes are then washed and resuspended in the final buffer or media to be used.

Example 18: Modifying fusosomes via chemical treatment

[000588] The following example describes modifying fusosomes with mitomycin C treatment. Without being bound by any particular theory, mitomycin C treatment modifies fusosomes by inactivating the cell cycle.

[000589] First, cells expressing fusogens are cultured from a monolayer in tissue culture

flasks or plates at a confluent density (e.g. by culturing or plating cells). One mg/mL mitomycin C stock solution is added to the medium to a final concentration of 10 μg/mL. The plates are then returned to the incubator for 2 to 3 hours. Then the medium is removed from confluent flasks, cells are rinsed with Ca2+ and Mg2+ free HBSS, and trypsinized to remove the cells from the culture matrix. The cells are then washed and resuspended in the final buffer or media to be used.

[000590] See for example, Mouse Embryo Fibroblast (MEF) Feeder Cell Preparation, Current Protocols in Molecular Biology. David A. Conner 2001.

Example 19: Lack of transcriptional activity in fusosomes

[000591] This Example quantifies transcriptional activity in fusosomes compared to parent cells, e.g., source cells, used for fusosome generation. In some embodiments, transcriptional activity will be low or absent in fusosomes compared to the parent cells, e.g., source cells.

[000592] Fusosomes are a chassis for the delivery of therapeutic agent. Therapeutic agents, such as miRNA, mRNAs, proteins and/or organelles that can be delivered to cells or local tissue environments with high efficiency could be used to modulate pathways that are not normally active or active at pathological low or high levels in recipient tissue. In some embodiments, observation that fusosomes are not capable of transcription, or that fusosomes have transcriptional activity of less than their parent cell, will demonstrate that removal of nuclear material has sufficiently occurred.

[000593] Fusosomes are prepared by any one of the methods described in previous Examples. A sufficient number of fusosomes and parent cells used to generate the fusosomes are then plated into a 6 well low-attachment multiwell plate in DMEM containing 20% Fetal Bovine Serum, 1x Penicillin/Streptomycin and the fluorescent-taggable alkyne–nucleoside EU for 1hr at 37°C and 5% CO_2. For negative controls, a sufficient number of fusosomes and parent cells are also plated in multiwell plate in DMEM containing 20% Fetal Bovine Serum, 1x Penicillin/Streptomycin but with no alkyne–nucleoside EU.

[000594] After the 1 hour incubation the samples are processed following the manufacturer's instructions for an imaging kit (ThermoFisher Scientific). The cell and fusosome samples including the negative controls are washed thrice with 1xPBS buffer and resuspended in 1xPBS

buffer and analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA) using a 488nm argon laser for excitation, and the 530+/-30nm emission. BD FACSDiva software was used for acquisition and analysis. The light scatter channels are set on linear gains, and the fluorescence channels on a logarithmic scale, with a minimum of 10,000 cells analyzed in each condition.

[000595] In some embodiments, transcriptional activity as measured by 530+/-30nm emission in the negative controls will be null due to the omission of the alkyne–nucleoside EU. In some embodiments, the fusosomes will have less than about 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or less transcriptional activity than the parental cells.

[000596] See also, Proc Natl Acad Sci U S A, 2008, Oct 14;105(41):15779-84. doi: 10.1073/pnas.0808480105. Epub 2008 Oct 7.

Example 20: Lack of DNA replication or replication activity

[000597] This Example quantifies DNA replication in fusosomes. In some embodiments, fusosomes will replicate DNA at a low rate compared to cells.

[000598] Fusosomes are prepared by any one of the methods described in previous Examples. Fusosome and parental cell DNA replication activity is assessed by incorporation of a fluorescent-taggable nucleotide (ThermoFisher Scientific # C10632). Fusosomes and an equivalent number of cells are incubated with EdU at a final concentration of 10 μM for 2hr, after preparation of an EdU stock solution with in dimethylsulfoxide. The samples are then fixed for 15 min using 3.7% PFA, washed with 1xPBS buffer, pH 7.4 and permeabilized for 15 min in 0.5% detergent solution in 1xPBS buffer, pH 7.4.

[000599] After permeabilization, fusosomes and cells in suspension in PBS buffer containing 0.5% detergent are washed with 1xPBS buffer, pH 7.4 and incubated for 30 min at 21°C in reaction cocktail, 1xPBS buffer, CuSO4 (Component F), azide-fluor 488, 1x reaction buffer additive.

[000600] A negative control for fusosome and cell DNA replication activity is made with samples treated the same as above but with no azide-fluor 488 in the 1x reaction cocktail.

[000601] The cell and fusosome samples are then washed and resuspended in 1xPBS buffer and analyzed by flow cytometry. Flow cytometry is done with a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 488nm argon laser excitation, and a 530+/-30nm emission

spectrum is collected. FACS analysis software is used for acquisition and analysis. The light scatter channels are set on linear gains, and the fluorescence channels on a logarithmic scale, with a minimum of 10,000 cells analyzed in each condition. The relative DNA replication activity is calculated based on the median intensity of azide-fluor 488 in each sample. All events are captured in the forward and side scatter channels (alternatively, a gate can be applied to select only the fusosome population). The normalized fluorescence intensity value for the fusosomes is determined by subtracting from the median fluorescence intensity value of the fusosome the median fluorescence intensity value of the respective negative control sample. Then the normalized relative DNA replication activity for the fusosomes samples is normalized to the respective nucleated cell samples in order to generate quantitative measurements for DNA replication activity.

[000602] In some embodiments, fusosomes have less DNA replication activity than parental cells.

[000603] See, also, Salic, 2415–2420, doi: 10.1073/pnas.0712168105.

Example 21: Electroporation to modify fusosome with nucleic acid cargo

This example describes electroporation of fusosomes with nucleic acid cargo.

[000604] Fusosomes are prepared by any one of the methods described in a previous Example. Approximately 10^9 fusosomes and 1 μg of nucleic acids, e.g., RNA, are mixed in electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride, 60% iodixanol w/v in water). The fusosomes are electroporated using a single 4 mm cuvette using an electroporation system (BioRad, 165-2081). The fusosomes and nucleic acids are electroporated at 400 V, 125 μF and ∞ ohms, and the cuvette is immediately transferred to ice. After electroporation, fusosomes are washed with PBS, resuspended in PBS, and kept on ice.

[000605] See, for example, Kamerkar et al., Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer, *Nature*, 2017

[000606]

[000607] Example 22: Electroporation to modify fusosome with protein cargo

[000608] This example describes electroporation of fusosomes with protein cargo.

[000609] Fusosomes are prepared by any one of the methods described in a previous Example. Approximately 5×10^6 fusosomes are used for electroporation using an electroporation transfection system (Thermo Fisher Scientific). To set up a master mix, 24 µg of purified protein cargo is added to resuspension buffer (provided in the kit). The mixture is incubated at room temperature for 10 min. Meanwhile, fusosomes are transferred to a sterile test tube and centrifuged at $500 \times g$ for 5 min. The supernatant is aspirated and the pellet is resuspended in 1 mL of PBS without Ca^{2+} and Mg^{2+}. The buffer with the protein cargo is then used to resuspend the pellet of fusosomes. A fusosome suspension is then used for optimization conditions, which vary in pulse voltage, pulse width and the number of pulses. After electroporation, fusosomes are washed with PBS, resuspended in PBS, and kept on ice.

[000610] See, for example, Liang et al., Rapid and highly efficiency mammalian cell engineering via Cas9 protein transfection, *Journal of Biotechnology* 208: 44-53, 2015.

Example 23: Chemical treatment of fusosomes to modify with nucleic acid cargo

[000611] This example describes loading of nucleic acid cargo into a fusosome via chemical treatments.

[000612] Fusosomes are prepared by any one of the methods described in previous Examples. Approximately 10^6 fusosomes are pelleted by centrifugation at 10,000g for 5min at 4°C. The pelleted fusosomes are then resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) with 20µg DNA. The fusosome:DNA solution is treated with a mild detergent to increase DNA permeability across the fusosome membrane (Reagent B, Cosmo Bio Co., LTD, Cat# ISK-GN-001-EX). The solution is centrifuged again and the pellet is resuspended in buffer with a positively-charged peptide, such as protamine sulfate, to increase affinity between the DNA loaded fusosomes and the target recipient cells (Reagent C, Cosmo Bio Co., LTD, Cat# ISK-GN-001-EX). After DNA loading, the loaded fusosomes are kept on ice before use.

See, also, Kaneda, Y., et al., New vector innovation for drug delivery: development of fusigenic non-viral particles. *Curr. Drug Targets*, 2003

Example 24: Chemical treatment of fusosomes to modify with protein cargo

[000613] This example describes loading of protein cargo into a fusosome via chemical

treatments.

[000614] Fusosomes are prepared by any one of the methods described in previous Examples. Approximately 10^6 fusosomes are pelleted by centrifugation at 10,000g for 5min at 4°C. The pelleted fusosomes are then resuspended in buffer with positively-charged peptides, such as protamine sulfate, to increase the affinity between the fusosomes and the cargo proteins (Reagent A, Cosmo Bio Co., LTD, Cat# ISK-GN-001-EX). Next 10µg of cargo protein is added to the fusosome solution followed by addition of a mild detergent to increase protein permeability across the fusosome membrane (Reagent B, Cosmo Bio Co., LTD, Cat# ISK-GN-001-EX). The solution is centrifuged again and the pellet is resuspended in buffer with the positively-charged peptide, such as protamine sulfate, to increase affinity between the protein loaded fusosomes and the target recipient cells (Reagent C, Cosmo Bio Co., LTD, Cat# ISK-GN-001-EX). After protein loading, the loaded fusosomes are kept on ice before use.

[000615] See, also, Yasouka, E., et al., Needleless intranasal administration of HVJ-E containing allergen attenuates experimental allergic rhinitis. *J. Mol. Med.*, 2007

Example 25: Transfection of fusosomes to modify with nucleic acid cargo

[000616] This example describes transfection of nucleic acid cargo into a fusosome. Fusosomes are prepared by any one of the methods described in previous Examples.

[000617] 5×10^6 fusosomes are maintained in Opti-Mem. 0.5 µg of nucleic acid is mixed with 25 µL of Opti-MEM medium, followed by the addition of 25 µL of Opti-MEM containing 2 µL of lipid transfection reagent 2000. The mixture of nucleic acids, Opti-MEM, and lipid transfection reagent is maintained at room temperature for 15 minutes, then is added to the fusosomes. The entire solution is mixed by gently swirling the plate and incubating at 37 C for 6 hours. Fusosomes are then washed with PBS, resuspended in PBS, and kept on ice.

[000618] See, also, Liang et al., Rapid and highly efficiency mammalian cell engineering via Cas9 protein transfection, *Journal of Biotechnology* 208: 44-53, 2015.

Example 26: Transfection of fusosomes to modify with protein cargo

[000619] This example describes transfection of protein cargo into a fusosome.

[000620] Fusosomes are prepared by any one of the methods described in previous Examples. 5×10^6 fusosomes are maintained in Opti-Mem. 0.5 μg of purified protein is mixed with 25 μL of Opti-MEM medium, followed by the addition of 25 μL of Opti-MEM containing 2 μL of lipid transfection reagent 3000. The mixture of protein, Opti-MEM, and lipid transfection reagent is maintained at room temperature for 15 minutes, then is added to the fusosomes. The entire solution is mixed by gently swirling the plate and incubating at 37 C for 6 hours. Fusosomes are then washed with PBS, resuspended in PBS, and kept on ice.

[000621] See, also, Liang et al., Rapid and highly efficiency mammalian cell engineering via Cas9 protein transfection, *Journal of Biotechnology* 208: 44-53, 2015.

Example 27: Fusosomes with lipid bilayer structure

[000622] This example describes the composition of fusosomes. In some embodiments, a fusosome composition will comprise a lipid bilayer structure, with a lumen in the center.

[000623] Without wishing to be bound by theory, the lipid bilayer structure of a fusosome promotes fusion with a target cell, and allows fusosomes to load different therapeutics.

[000624] Fusosomes are freshly prepared using the methods described in the previous Examples. The positive control is the native cell line (HEK293), and the negative control is cold DPBS and membrane-disrupted HEK293 cell prep, which has been passed through 36 gauge needles for 50 times.

[000625] Samples are spin down in Eppendorf tube, and the supernatant is carefully removed. Then a pre-warmed fixative solution (2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1M NaCl, pH 7.5; keep at 37 °C for 30 min before use) is added to the sample pellet and kept at room temperature for 20 minutes. The samples are washed twice with PBS after fixation. Osmium tetroxide solution is added to the sample pellet and incubated 30 minutes. After rinsing once with PBS, 30%, 50%, 70% and 90% hexylene glycol is added and washed with swirling, 15 minutes each. Then 100% hexylene glycol is added with swirling, 3 times, 10 minutes each.

[000626] Resin is combined with hexylene glycol at 1:2 ratio, and then added to the samples and incubated at room temperature for 2 hours. After incubation, the solution is replaced with 100% resin and incubated for 4-6 hours. This step is repeated one more time with fresh 100%

resin. Then it is replaced with 100% fresh resin, the level is adjusted to ~1-2 mm in depth, and baked for 8-12 hours. The Eppendorf tube is cut and pieces of epoxy cast with the sample is baked for an additional 16-24 hours. The epoxy cast is then cut into small pieces making note of the side with the cells. Pieces are glued to blocks for sectioning, using commercial 5-minute epoxy glue. A transmission electron microscope (JOEL, USA) is used to image the samples at a voltage of 80kV.

[000627] In some embodiments, fusosomes will show a lipid bilayer structure similar to the positive control (HEK293 cells), and no obvious structure is observed in the DPBS control. In some embodiments no lumenal structures will be observed in the disrupted cell preparation.

Example 28: Detecting fusogen expression

[000628] This example quantifies fusogen expression in fusosomes.

[000629] Transposase vectors (System Biosciences, Inc.) that include the open reading frame of the Puromycin resistance gene together with an open reading frame of a cloned fragment (e.g. Glycoprotein from Vesicular stomatitis virus [VSV-G], Oxford Genetics # OG592) are electroporated into 293Ts using an electroporator (Amaxa) and a 293T cell line specific nuclear transfection kit (Lonza).

[000630] Following selection with 1 μg/μL puromycin for 3-5 days in DMEM containing 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin, fusosomes are prepared from the stably expressing cell line or from control cells by any one of the methods described in previous Examples.

[000631] The fusosomes are then washed with 1xPBS, ice-cold lysis buffer (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0 and Protease Inhibitor Cocktail III (Abcam, ab201117)), sonicated 3 times, 10-15 seconds each time and centrifuged at 16,000 x g for 20min. A western blot is conducted on the recovered supernatant fraction with a probe specific to VSV-G to determine the non-membrane specific concentration of VSV-G from the fusosomes prepared from stably transfected cells or control cells and compared to the standard of VSV-G protein.

[000632] In some embodiments, fusosomes from stably transfected cells will have more

VSV-G than fusosomes generated from cells that were not stably transfected.

Example 29: Quantification of fusogens

[000633] This example describes quantification of the absolute number of fusogens per fusosome.

[000634] A fusosome composition is produced by any one of the methods described in the previous Examples, except the fusosome is engineered as described in a previous Example to express a fusogen (VSV-G) tagged with GFP. In addition, a negative control fusosome is engineered with no fusogen (VSV-G) or GFP present.

[000635] The fusosomes with the GFP-tagged fusogen and the negative control(s) are then assayed for the absolute number of fusogens as follows. Commercially acquired recombinant GFP is serially diluted to generate a calibration curve of protein concentration. The GFP fluorescence of the calibration curve and a sample of fusosomes of known quantity is then measured in a fluorimeter using a GFP light cube (469/35 excitation filter and a 525/39 emission filter) to calculate the average molar concentration of GFP molecules in the fusosome preparation. The molar concentration is then converted to the number of GFP molecules and divided by the number of fusosomes per sample to achieve an average number of GFP-tagged fusogen molecules per fusosome and thus provides a relative estimate of the number of fusogens per fusosome.

[000636] In some embodiments, GFP fluorescence will be higher in the fusosomes with GFP tag as compared to the negative controls, where no fusogen or GFP is present. In some embodiments, GFP fluorescence is relative to the number of fusogen molecules present.

[000637] Alternatively, individual fusosomes are isolated using a single cell prep system (Fluidigm) per manufacturer's instructions, and qRT-PCR is performed using a commercially available probeset (Taqman) and master mix designed to quantify fusogen or GFP cDNA levels based upon the C_t value. A RNA standard of the same sequence as the cloned fragment of the fusogen gene or the GFP gene is generated by synthesis (Amsbio) and then added to single cell prep system qRT-PCR experimental reaction in serial dilutions to establish a standard curve of C_t vs concentration of fusogen or GFP RNA.

[000638] The C_t value from fusosomes is compared to the standard curve to determine the

amount of fusogen or GFP RNA per fusosome.

[000639] In some embodiments, fusogen and GFP RNA will be higher in the fusosomes with engineered to express the fusogens as compared to the negative controls, where no fusogen or GFP is present.

[000640] Fusogens may further be quantified in the lipid bilayer by analyzing the lipid bilayer structure as previously described and quantifying fusogens in the lipid bilayer by LC-MS as described in other Examples herein.

Example 30: Measuring the average diameter of fusosomes

[000641] This Example describes measurement of the average diameter of fusosomes.

[000642] Fusosomes are prepared by any one of the methods described in previous Examples. The fusosomes measured to determine the average diameter using commercially available systems (iZON Science). The system is used with software according to manufacturer's instructions and a nanopore designed to analyze particles within the 40 nm to 10 μm diameter range. Fusosomes and parental cells are resuspended in phosphate-buffered saline (PBS) to a final concentration range of 0.01-0.1 μg protein/mL. Other instrument settings are adjusted as indicated in the following table:

Table 16: Fusosome measurement parameters and settings

Measurement Parameter	Setting
Pressure	6
Nanopore type	NP300
Calibration sample	CPC400_6P
Gold standard analysis	no
Capture assistant	none

[000643] All fusosomes are analyzed within 2 hours of isolation. In some embodiments, fusosomes will have a diameter within about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than parental source cells.

Example 31: Measuring the average diameter distribution of fusosomes

[000644] This Example describes measurement of the diameter distribution of fusosomes.

[000645] Fusosomes are generated by any one of the methods described in previous Examples, and are tested to determine the average diameter of particles using a commercially available system, such as described in a previous Example. In some embodiments, diameter thresholds for 10%, 50%, and 90% of the fusosomes centered around the median are compared to parental cells to assess fusosome diameter distribution.

[000646] In some embodiments, fusosomes will have less than about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, or less of the parental cell's variability in diameter distribution within 10%, 50%, or 90% of the sample.

Example 32: Average volume of fusosomes

[000647] This example describes measurement of the average volume of fusosomes. Without wishing to be bound by theory, varying the size (e.g., diameter, volume, surface area, etc.) of fusosomes can make them versatile for distinct cargo loading, therapeutic design or application.

[000648] Fusosomes are prepared as described in previous Examples. The positive control is HEK293 cells or polystyrene beads with a known size. The negative control is HEK293 cells that are passed through a 36 gauge needle approximately 50 times.

[000649] Analysis with a transmission electron microscope, as described in a previous Example, is used to determine the size of the fusosomes. The diameter of the fusosome is measured and volume is then calculated.

[000650] In some embodiments, fusosomes will have an average size of approximately 50nm or greater in diameter.

Example 33: Average density of fusosomes

[000651] Fusosome density is measured via a continuous sucrose gradient centrifugation assay as described in Théry et al., Curr Protoc Cell Biol. 2006 Apr; Chapter 3:Unit 3.22. Fusosomes are obtained as described in previous Examples.

[000652] First, a sucrose gradient is prepared. A 2 M and a 0.25 sucrose solution are generated by mixing 4 mL HEPES/sucrose stock solution and 1 mL HEPES stock solution or 0.5 mL HEPES/sucrose stock solution and 4.5 mL HEPES stock solution, respectively. These two

fractions are loaded into the gradient maker with all shutters closed, the 2 M sucrose solution in the proximal compartment with a magnetic stir bar, and the 0.25 M sucrose solution in the distal compartment. The gradient maker is placed on a magnetic stir plate, the shutter between proximal and distal compartments is opened and the magnetic stir plate is turned on. HEPES stock solution is made as follows: 2.4 g N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 20mMfinal), 300 H2O, adjust pH to 7.4 with 10 N NaOH and finally adjust volume to 500 mL with H2O. HEPES/sucrose stock solution is made as follows: 2.4 g hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 20 mM final), 428 g protease-free sucrose (ICN; 2.5 M final), 150 mL H2O, adjust pH to 7.4 with 10 N NaOH and finally adjust volume to 500 mL with H2O.

[000653] The fusosomes are resuspended in 2 mL of HEPES/sucrose stock solution and are poured on the bottom of an SW 41 centrifuge tube. The outer tubing is placed in the SW 41 tube, just above the 2 mL of fusosomes. The outer shutter is opened, and a continuous 2 M (bottom) to 0.25 M (top) sucrose gradient is slowly poured on top of the fusosomes. The SW 41 tube is lowered as the gradient is poured, so that the tubing is always slightly above the top of the liquid.

[000654] All tubes with gradients are balanced with each other, or with other tubes having the same weight of sucrose solutions. The gradients are centrifuged overnight (≥14 hr) at 210,000 x g, 4°C, in the SW 41 swinging-bucket rotor with the brake set on low.

[000655] With a micropipettor, eleven 1-mL fractions, from top to bottom, are collected and placed in a 3-mL tube for the TLA-100.3 rotor. The samples are set aside and, in separate wells of a 96-well plate, 50 µl of each fraction is used to measure the refractive index. The plate is covered with adhesive foil to prevent evaporation and stored for no more than 1 hour at room temperature. A refractometer is used to measure the refractive index (hence the sucrose concentration, and the density) of 10 to 20 µl of each fraction from the material saved in the 96-well plate.

[000656] A table for converting the refractive index into g/mL is available in the ultracentrifugation catalog downloadable from the Beckman website.

[000657] Each fraction is then prepared for protein content analysis. Two milliliters of 20 mM HEPES, pH 7.4, is added to each 1-mL gradient fraction, and mixed by pipetting up and down two to three times. One side of each tube is marked with a permanent marker, and the tubes are placed marked side up in a TLA-100.3 rotor.

[000658] The 3 mL-tubes with diluted fractions are centrifuged for 1 hr at 110,000 x g, 4°C. The TLA-100.3 rotor holds six tubes, so two centrifugations for each gradient is performed with the other tubes kept at 4°C until they can be centrifuged.

[000659] The supernatant is aspirated from each of the 3-mL tubes, leaving a drop on top of the pellet. The pellet most probably is not visible, but its location can be inferred from the mark on the tube. The invisible pellet is resuspended and transferred to microcentrifuge tubes. Half of each resuspended fraction is used for protein contentment analysis by bicinchoninic acid assay, described in another Example. This provides a distribution across the various gradient fractions of the fusosome preparation. This distribution is used to determine the average density of the fusosomes. The second half volume fraction is stored at -80°C and used for other purposes (e.g. functional analysis, or further purification by immunoisolation) once protein analysis has revealed the fusosome distribution across fractions.

[000660] In some embodiments, using this assay or an equivalent, the average density of the preparation comprising a plurality of fusosomes will be 1.25 g/mL +/- 0.05 standard deviation. In some embodiments, the average density of the preparation will be in the range of 1-1.1, 1.05-1.15, 1.1-1.2, 1.15-1.25, 1.2-1.3, or 1.25-1.35 g/mL. In some embodiments, average density of the preparation will be less than 1 or more than 1.35.

Example 34: Measuring organelle content in fusosomes

[000661] This Example describes detection of organelles in fusosomes.

[000662] Fusosomes were prepared as described herein. For detection of endoplasmic reticulum (ER) and mitochondria, fusosomes or C2C12 cells were stained with 1 μM ER stain (E34251, Thermo Fisher, Waltham, MA) and 1 μM mitochondria stain (M22426, Thermo Fisher Waltham, MA). For detection of lysosomes, fusosomes or cells were stained with 50 nM lysosome stain (L7526, Thermo Fisher, Waltham, MA).

[000663] Stained fusosomes were run on a flow cytometer (Thermo Fisher, Waltham, MA) and fluorescence intensity was measured for each dye according to the table below. Validation for the presence of organelles was made by comparing fluorescence intensity of stained fusosomes to unstained fusosomes (negative control) and stained cells (positive control).

[000664] Fusosomes stained positive for endoplasmic reticulum (Figure 1), mitochondria (Figure 2), and lysosomes (Figure 3) 5 hours post-enucleation.

Table 17: Fusosome stains

Stain	Attune Laser/Filter	Laser Wavelength	Emission Filter (nm)
Hoechst 33342	VL1	405	450/40
ER-Tracker Green	BL1	488	530/30
MitoTracker Deep Red FM	RL1	638	670/14
LysoTracker Green	BL1	488	530/30

Example 35: Measuring nuclear content in fusosomes

[000665] This Example describes measuring nuclear content in a fusosome. To validate that fusosomes do not contain nuclei, fusosomes are stained with 1 $\mu g \cdot mL^{-1}$ Hoechst 33342 and 1 μM CalceinAM (C3100MP, Thermo Fisher, Waltham, MA) and the stained fusosomes are run on an Attune NXT Flow Cytometer (Thermo Fisher, Waltham, MA) to determine the fluorescence intensity of each dye according to the table below. In some embodiments, validation for the presence of cytosol (CalceinAM) and the absence of a nucleus (Hoechst 33342) will be made by comparing the mean fluorescence intensity of stained fusosomes to unstained fusosomes and stained cells.

Table 18: Flow cytometer settings

Stain	Attune Laser/Filter	Laser Wavelength	Emission Filter (nm)
Hoechst 33342	VL1	405	450/40
Calcein AM	BL1	488	530/30

Example 36: Measuring nuclear envelope content

[000666] This Example describes a measurement of the nuclear envelope content in enucleated fusosomes. The nuclear envelope isolates DNA from the cytoplasm of the cell.

[000667] In some embodiments, a purified fusosome composition comprises a mammalian

cell, such as HEK-293Ts (293 [HEK-293] (ATCC® CRL-1573™), that has been enucleated as described herein. This Example describes the quantification of different nuclear membrane proteins as a proxy to measure the amount of intact nuclear membrane that remains after fusosome generation.

[000668] In this Example, $10x10^6$ HEK-293Ts and the equivalent amount of fusosomes prepared from $10x10^6$ HEK-293Ts are fixed for 15 min using 3.7% PFA, washed with 1xPBS buffer, pH 7.4 and permeabilized simultaneously, and then blocked for 15 min using 1xPBS buffer containing 1% Bovine Serum Albumin and 0.5% Triton® X-100, pH 7.4. After permeabilization, fusosomes and cells are incubated for 12 hours at 4°C with different primary antibodies, e.g. (anti-RanGAP1 antibody [EPR3295] (Abcam - ab92360), anti-NUP98 antibody [EPR6678] - nuclear pore marker (Abcam - ab124980), anti-nuclear pore complex proteins antibody [Mab414] - (Abcam- ab24609), anti-importin 7 antibody (Abcam - ab213670), at manufacturer suggested concentrations diluted in 1xPBS buffer containing 1% bovine serum albumin and 0.5% Triton® X-100, pH 7.4. Fusosomes and cells are then washed with 1xPBS buffer, pH 7.4, and incubated for 2hr at 21°C with an appropriate fluorescent secondary antibody that detects the previous specified primary antibody at manufacturer suggested concentrations diluted in 1xPBS buffer containing 1% bovine serum albumin and 0.5% detergent, pH 7.4. Fusosomes and cells are then washed with 1xPBS buffer, re-suspended in 300µL of 1xPBS buffer, pH 7.4 containing 1 µg/mL Hoechst 33342, filtered through a 20 µm FACS tube and analyzed by flow cytometry.

[000669] Negative controls are generated using the same staining procedure but with no primary antibody added. Flow cytometry is performed on a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 488nm argon laser excitation, and a 530+/-30nm emission spectrum is collected. FACS acquisition software is used for acquisition and analysis. The light scatter channels are set on linear gains, and the fluorescence channels on a logarithmic scale, with a minimum of 10,000 cells analyzed in each condition. The relative intact nuclear membrane content is calculated based on the median intensity of fluorescence in each sample. All events are captured in the forward and side scatter channels.

[000670] The normalized fluorescence intensity value for the fusosomes is determined by subtracting from the median fluorescence intensity value of the fusosome the median fluorescence

intensity value of the respective negative control sample. Then the normalized fluorescence for the fusosomes samples is normalized to the respective nucleated cell samples in order to generate quantitative measurements of intact nuclear membrane content.

[000671] In some embodiments, enucleated fusosomes will comprise less than 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% fluorescence intensity or nuclear envelope content compared to the nucleated parental cells.

Example 37: Measuring chromatin levels

[000672] This Example describes measurement of chromatin in enucleated fusosomes.

[000673] DNA can be condensed into chromatin to allow it to fit inside the nucleus. In some embodiments, a purified fusosome composition as produced by any one of the methods described herein will comprise low levels of chromatin.

[000674] Enucleated fusosomes prepared by any of the methods previously described and positive control cells (e.g., parental cells) are assayed for chromatin content using an ELISA with antibodies that are specific to histone protein H3 or histone protein H4. Histones are the chief protein component of chromatin, with H3 and H4 the predominant histone proteins.

[000675] Histones are extracted from the fusosome preparation and cell preparation using a commercial kit (e.g. Abcam Histone Extraction Kit (ab113476)) or other methods known in the art. These aliquots are stored at -80°C until use. A serial dilution of standard is prepared by diluting purified histone protein (either H3 or H4) from 1 to 50 ng/μL in a solution of the assay buffer. The assay buffer may be derived from a kit supplied by a manufacturer (e.g. Abcam Histone H4 Total Quantification Kit (ab156909) or Abcam Histone H3 total Quantification Kit (ab115091)). The assay buffer is added to each well of a 48- or 96-well plate, which is coated with an anti-histone H3 or anti-H4 antibody and sample or standard control is added to the well to bring the total volume of each well to 50 μL. The plate is then covered and incubated at 37 degrees for 90 to 120 minutes.

[000676] After incubation, any histone bound to the anti-histone antibody attached to the plate is prepared for detection. The supernatant is aspirated and the plate is washed with 150 μL of wash buffer. The capture buffer, which includes an anti-histone H3 or anti-H4 capture antibody, is then added to the plate in a volume of 50 μL and at a concentration of 1 μg/mL. The plate is

then incubated at room temperature on an orbital shaker for 60 minutes.

[000677] Next, the plate is aspirated and washed 6 times using wash buffer. Signal reporter molecule activatable by the capture antibody is then added to each well. The plate is covered and incubated at room temperature for 30 minutes. The plate is then aspirated and washed 4 times using wash buffer. The reaction is stopped by adding stop solution. The absorbance of each well in the plate is read at 450 nm, and the concentration of histones in each sample is calculated according to the standard curve of absorbance at 450 nm vs. concentration of histone in standard samples.

[000678] In some embodiments, fusosome samples will comprise less than 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% the histone concentration of the nucleated parental cells.

Example 38: Measuring DNA content in fusosomes

[000679] This example describes quantification of the amount of DNA in a fusosome relative to nucleated counterparts. In some embodiments, fusosomes will have less DNA than nucleated counterparts. Nucleic acid levels are determined by measuring total DNA or the level of a specific house-keeping gene. In some embodiments, fusosomes having reduced DNA content or substantially lacking DNA will be unable to replicate, differentiate, or transcribe genes, ensuring that their dose and function is not altered when administered to a subject.

[000680] Fusosomes are prepared by any one of the methods described in previous Examples. Preparations of the same mass as measured by protein of fusosomes and source cells are used to isolate total DNA (e.g. using a kit such as Qiagen DNeasy catalog #69504), followed by determination of DNA concentration using standard spectroscopic methods to assess light absorbance by DNA (e.g. with Thermo Scientific NanoDrop).

[000681] In some embodiments, concentration of DNA in enucleated fusosomes will be less than about 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or less than in parental cells.

[000682] Alternatively, the concentration of a specific house-keeping gene, such as GAPDH, can be compared between nucleated cells and fusosomes with semi-quantitative real-time PCR (RT-PCR). Total DNA is isolated from parental cells and fusosome and DNA concentration is

measured as described herein. RT-PCR is carried out with a PCR kit (Applied Biosystems, catalog #4309155) using the following reaction template:

SYBR Green Master Mix:	10 µL
0.45 µM Forward Primer:	1 µL
0.45 µM Reverse Primer:	1 µL
DNA Template:	10 ng
PCR-Grade Water: Variable	

[000683] Forward and reverse primers are acquired from Integrated DNA Technologies. The table below details the primer pairs and their associated sequences:

Table 19: Primer sequences

Target	**Forward Primer Sequence (5'→3')**	**Reverse Primer Sequence (5'→3')**
Human nDNA (GAPDH)	GGAGTCCACTGGCGTCTTCAC (SEQ ID NO: 39)	GAGGCATTGCTGATGATCTTGAGG (SEQ ID NO: 40)

[000684] A real-time PCR system (Applied Biosystems) is used to perform the amplification and detection with the following protocol:

Denaturation, 94°C	2 min
40 Cycles of the following sequence:	
Denaturation, 94°C	15 sec
Annealing, Extension, 60°C	1 min

[000685] A standard curve of the C_t vs. DNA concentration is prepared with serial dilutions of GAPDH DNA and used to normalize the Ct nuclear value from fusosome PCR results to a specific amount (ng) of DNA.

[000686] In some embodiments, concentration of GAPDH DNA in enucleated fusosomes will be less than about 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or less than in parental cells.

Example 39: Measuring miRNA content in fusosomes

[000687] This example describes quantification of microRNAs (miRNAs) in fusosomes. In some embodiments, a fusosome comprises miRNAs.

[000688] MiRNAs are regulatory elements that, among other activities, control the rate by which messenger RNAs (mRNAs) are translated into proteins. In some embodiments, fusosomes carrying miRNA may be used to deliver the miRNA to target sites.

[000689] Fusosomes are prepared by any one of the methods described in previous Examples. RNA from fusosomes or parental cells is prepared as described previously. At least one miRNA gene is selected from the Sanger Center miRNA Registry at www.sanger.ac.uk/Software/Rfam/mirna/index.shtml. miRNA is prepared as described in Chen *et al, Nucleic Acids Research*, 33(20), 2005. All TaqMan miRNA assays are available through Thermo Fisher (A25576, Waltham, MA).

[000690] qPCR is carried out according to manufacturer's specifications on miRNA cDNA, and C_T values are generated and analyzed using a real-time PCR system as described herein.

[000691] In some embodiments, miRNA content of fusosomes will be at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than that of their parental cells.

Example 40: Quantifying expression of an endogenous RNA or synthetic RNA in fusosomes

[000692] This example describes quantification of levels of endogenous RNA with altered expression, or a synthetic RNA that is expressed in a fusosome.

[000693] The fusosome or parental cell is engineered to alter the expression of an endogenous or synthetic RNA that mediates a cellular function to the fusosomes.

[000694] Transposase vectors (System Biosciences, Inc.) includes the open reading frame of the Puromycin resistance gene together with an open reading frame of a cloned fragment of a protein agent. The vectors are electroporated into 293Ts using an electroporator (Amaxa) and a 293T cell line specific nuclear transfection kit (Lonza).

[000695] Following selection with puromycin for 3-5 days in DMEM containing 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin, fusosomes are prepared from the stably expressing cell line by any one of the methods described in previous Examples.

[000696] Individual fusosomes are isolated and protein agent or RNA per fusosome is quantified as described in a previous Example.

[000697] In some embodiments, fusosomes will have at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, or more of the RNA per fusosome.

Example 41: Measuring lipid composition in fusosomes

[000698] This Example describes quantification of the lipid composition of fusosomes. In some embodiments, lipid composition of fusosomes is similar to the cells that they are derived from. Lipid composition affects important biophysical parameters of fusosomes and cells, such as size, electrostatic interactions, and colloidal behavior.

[000699] The lipid measurements are based on mass spectrometry. Fusosomes are prepared by any one of the methods described in previous Examples.

[000700] Mass spectrometry-based lipid analysis is performed at a lipid analysis service (Dresden, Germany) as described (Sampaio, et al., Proc Natl Acad Sci, 2011, Feb 1;108(5):1903–7). Lipids are extracted using a two-step chloroform/methanol procedure (Ejsing, et al., Proc Natl Acad Sci, 2009, Mar 17;106(7):2136–41). Samples are spiked with an internal lipid standard mixture of: cardiolipin 16:1/15:0/15:0/15:0 (CL), ceramide 18:1;2/17:0 (Cer), diacylglycerol 17:0/17:0 (DAG), hexosylceramide 18:1;2/12:0 (HexCer), lysophosphatidate 17:0 (LPA), lyso-phosphatidylcholine 12:0 (LPC), lyso-phosphatidylethanolamine 17:1 (LPE), lyso-phosphatidylglycerol 17:1 (LPG), lyso-phosphatidylinositol 17:1 (LPI), lyso-phosphatidylserine 17:1 (LPS), phosphatidate 17:0/17:0 (PA), phosphatidylcholine 17:0/17:0 (PC), phosphatidylethanolamine 17:0/17:0 (PE), phosphatidylglycerol 17:0/17:0 (PG), phosphatidylinositol 16:0/16:0 (PI), phosphatidylserine 17:0/17:0 (PS), cholesterol ester 20:0 (CE), sphingomyelin 18:1;2/12:0;0 (SM) and triacylglycerol 17:0/17:0/17:0 (TAG).

[000701] After extraction, the organic phase is transferred to an infusion plate and dried in a speed vacuum concentrator. The first step dry extract is resuspended in 7.5 mM ammonium acetate

in chloroform/methanol/propanol (1:2:4, V:V:V) and the second step dry extract is resuspended in 33% ethanol solution of methylamine in chloroform/methanol (0.003:5:1; V:V:V). All liquid handling steps are performed using a robotic platform for organic solvent with an anti-droplet control feature (Hamilton Robotics) for pipetting.

[000702] Samples are analyzed by direct infusion on a mass spectrometer (Thermo Scientific) equipped with an ion source (Advion Biosciences). Samples are analyzed in both positive and negative ion modes with a resolution of Rm/z=200=280000 for MS and Rm/z=200=17500 for tandem MS/MS experiments, in a single acquisition. MS/MS is triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1 Da increments (Surma, et al., Eur J lipid Sci Technol, 2015, Oct;117(10):1540–9). Both MS and MS/MS data are combined to monitor CE, DAG and TAG ions as ammonium adducts; PC, PC O-, as acetate adducts; and CL, PA, PE, PE O-, PG, PI and PS as deprotonated anions. MS only is used to monitor LPA, LPE, LPE O-, LPI and LPS as deprotonated anions; Cer, HexCer, SM, LPC and LPC O- as acetate.

[000703] Data are analyzed with in-house developed lipid identification software as described in the following references (Herzog, et al., Genome Biol, 2011, Jan 19;12(1):R8; Herzog, et al., PLoS One, 2012, Jan;7(1):e29851). Only lipid identifications with a signal-to-noise ratio >5, and a signal intensity 5-fold higher than in corresponding blank samples are considered for further data analysis.

[000704] Fusosome lipid composition is compared to parental cells' lipid composition. In some embodiments, fusosomes and parental cells will have a similar lipid composition if >50% of the identified lipids in the parental cells are present in the fusosomes, and of those identified lipids, the level in the fusosome will be >25% of the corresponding lipid level in the parental cell.

Example 42: Measuring proteomic composition in fusosomes

[000705] This Example describes quantification of the protein composition of fusosomes. In some embodiments, protein composition of fusosomes will be similar to the cells that they are derived from.

[000706] Fusosomes are prepared by any one of the methods described in previous Examples.

Fusosomes are resuspended in lysis buffer (7M Urea, 2M Thiourea, 4% (w/v) Chaps in 50 mM Tris pH 8.0) and incubated for 15 minutes at room temperature with occasional vortexing. Mixtures are then lysed by sonication for 5 minutes in an ice bath and spun down for 5 minutes at 13,000 RPM. Protein content is determined by a colorimetric assay (Pierce) and protein of each sample is transferred to a new tube and the volume is equalized with 50 mM Tris pH 8.

[000707] Proteins are reduced for 15 minutes at 65 Celsius with 10 mM DTT and alkylated with 15 mM iodoacetamide for 30 minutes at room temperature in the dark. Proteins are precipitated with gradual addition of 6 volumes of cold (-20°Celsius) acetone and incubated overnight at -80°C. Protein pellets are washed 3 times with cold (-20°Celsius) methanol. Proteins are resuspended in 50 mM Tris pH 8.3.

[000708] Next, trypsin/lysC is added to the proteins for the first 4h of digestion at 37 Celsius with agitation. Samples are diluted with 50mM Tris pH 8 and 0.1% sodium deoxycholate is added with more trypsin/lysC for digestion overnight at 37 Celsius with agitation. Digestion is stopped and sodium deoxycholate is removed by the addition of 2% v/v formic acid. Samples are vortexed and cleared by centrifugation for 1 minute at 13,000 RPM. Peptides are purified by reversed phase solid phase extraction (SPE) and dried down. Samples are reconstituted in 20 µl of 3% DMSO, 0.2% formic acid in water and analyzed by LC-MS.

[000709] To have quantitative measurements, a protein standard is also run on the instrument. Standard peptides (Pierce, equimolar, LC-MS grade, #88342) are diluted to 4, 8, 20, 40 and 100 fmol/µL and are analyzed by LC-MS/MS. The average AUC (area under the curve) of the 5 best peptides per protein (3 MS/MS transition/peptide) is calculated for each concentration to generate a standard curve.

[000710] Acquisition is performed with a high resolution mass spectrometer (ABSciex, Foster City, CA, USA) equipped with an electrospray interface with a 25 µm iD capillary and coupled with micro-ultrahigh performance liquid chromatography (µUHPLC) (Eksigent, Redwood City, CA, USA). Analysis software is used to control the instrument and for data processing and acquisition. The source voltage is set to 5.2 kV and maintained at 225°C, curtain gas is set at 27 psi, gas one at 12 psi and gas two at 10 psi. Acquisition is performed in Information Dependent Acquisition (IDA) mode for the protein database and in SWATH acquisition mode for

the samples. Separation is performed on a reversed phase column 0.3 μm i.d., 2.7 μm particles, 150mm long (Advance Materials Technology, Wilmington, DE) which is maintained at 60°C. Samples are injected by loop overfilling into a 5μL loop. For the 120 minute (samples) LC gradient, the mobile phase includes the following: solvent A (0.2% v/v formic acid and 3% DMSO v/v in water) and solvent B (0.2% v/v formic acid and 3% DMSO in EtOH) at a flow rate of 3 μL/min.

[000711] For the absolute quantification of the proteins, a standard curve (5 points, R2>0.99) is generated using the sum of the AUC of the 5 best peptides (3 MS/MS ion per peptide) per protein. To generate a database for the analysis of the samples, the DIAUmpire algorithm is run on each of the 12 samples and combined with the output MGF files into one database. This database is used with software (ABSciex) to quantify the proteins in each of the samples, using 5 transition/peptide and 5 peptide/protein maximum. A peptide is considered as adequately measured if the score computed is superior to 1.5 or had a FDR < 1%. The sum of the AUC of each of the adequately measured peptides is mapped on the standard curve, and is reported as fmol.

[000712] The resulting protein quantification data is then analyzed to determine protein levels and proportions of known classes of proteins as follows: enzymes are identified as proteins that are annotated with an Enzyme Commission (EC) number; ER associated proteins are identified as proteins that had a Gene Ontology (GO; http://www.geneontology.org) cellular compartment classification of ER and not mitochondria; exosome associated proteins are identified as proteins that have a Gene Ontology cellular compartment classification of exosomes and not mitochondria; and mitochondrial proteins are identified as proteins that are identified as mitochondrial in the MitoCarta database (Calvo et al., NAR 2015l doi:10.1093/nar/gkv1003). The molar ratios of each of these categories are determined as the sum of the molar quantities of all the proteins in each class divided by the sum of the molar quantities of all identified proteins in each sample.

[000713] Fusosome proteomic composition is compared to parental cell proteomic composition. In some embodiments, similar proteomic compositions between fusosomes and parental cells will be observed when >50% of the identified proteins are present in the fusosome, and of those identified proteins the level is >25% of the corresponding protein level in the parental

cell.

Example 43: Quantifying an endogenous or synthetic protein level per fusosome

[000714] This example describes quantification of an endogenous or synthetic protein cargo in fusosomes. In some embodiments, fusosomes comprise an endogenous or synthetic protein cargo.

[000715] The fusosome or parental cell is engineered to alter the expression of an endogenous protein or express a synthetic cargo that mediates a therapeutic or novel cellular function.

[000716] Transposase vectors (System Biosciences, Inc.) that include the open reading frame of the puromycin resistance gene together with an open reading frame of a cloned fragment of a protein agent, optionally translationally fused to the open reading frame of a green fluorescent protein (GFP). The vectors are electroporated into 293Ts using an electroporator (Amaxa) and a 293T cell line specific nuclear transfection kit (Lonza).

[000717] Following selection with puromycin for 3-5 days in DMEM containing 20% fetal bovine serum and 1x penicillin/streptomycin, fusosomes are prepared from the stably expressing cell line by any one of the methods described in previous Examples.

[000718] Altered expression levels of an endogenous protein or expression levels of a synthetic protein that are not fused to GFP are quantified by mass spectrometry as described above. In some embodiments, fusosomes will have at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, or more protein agent molecules per fusosome.

[000719] Alternatively, purified GFP is serially diluted in DMEM containing 20% fetal bovine serum and 1x Penicillin/Streptomycin to generate a standard curve of protein concentration. GFP fluorescence of the standard curve and a sample of fusosomes is measured in a fluorimeter (BioTek) using a GFP light cube (469/35 excitation filter and a 525/39 emission filter) to calculate the average molar concentration of GFP molecules in the fusosomes. The molar concentration is then converted to number of GFP molecules and divided by the number of fusosomes per sample to achieve an average number of protein agent molecules per fusosome.

[000720] In some embodiments, fusosomes will have at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 10^3, 5.0 x 10^3, 10^4, 5.0 x 10^4, 10^5, 5.0 x 10^5, 10^6, 5.0 x 10^6, or more protein agent molecules

per fusosome.

Example 44: Measuring markers of exosomal proteins in fusosomes

[000721] This assay describes quantification of the proteomics makeup of the sample preparation, and quantifies the proportion of proteins that are known to be specific markers of exosomes.

[000722] Fusosomes are pelleted and shipped frozen to the proteomics analysis center per standard biological sample handling procedures.

[000723] The fusosomes are thawed for protein extraction and analysis. First, they are resuspended in lysis buffer (7M urea, 2M thiourea, 4% (w/v) chaps in 50 mM Tris pH 8.0) and incubated for 15 minutes at room temperature with occasional vortexing. The mixtures are then lysed by sonication for 5 minutes in an ice bath and spun down for 5 minutes at 13,000 RPM. Total protein content is determined by a colorimetric assay (Pierce) and 100 µg of protein from each sample is transferred to a new tube and the volume is adjusted with 50 mM Tris pH 8.

[000724] The proteins are reduced for 15 minutes at 65° Celsius with 10 mM DTT and alkylated with 15 mM iodoacetamide for 30 minutes at room temperature in the dark. The proteins are then precipitated with gradual addition of 6 volumes of cold (-20° Celsius) acetone and incubated over night at -80° Celsius.

[000725] The proteins are pelleted, washed 3 times with cold (-20° Celsius) methanol, and resuspended in 50 mM Tris pH 8. 3.33µg of trypsin/lysC is added to the proteins for a first 4h of digestion at 37° Celsius with agitation. The samples are diluted with 50 mM Tris pH 8 and 0.1 % sodium deoxycholate is added with another 3.3 µg of trypsin/lysC for digestion overnight at 37° Celsius with agitation. Digestion is stopped and sodium deoxycholate is removed by the addition of 2% v/v formic acid. Samples are vortexed and cleared by centrifugation for 1 minute at 13,000 RPM.

[000726] The proteins are purified by reversed phase solid phase extraction (SPE) and dried down. The samples are reconstituted in 3% DMSO, 0.2% formic acid in water and analyzed by LC-MS as described previously.

[000727] The resulting protein quantification data is analyzed to determine protein levels and

proportions of know exosomal marker proteins. Specifically: tetraspanin family proteins (CD63, CD9, or CD81), ESCRT-related proteins (TSG101, CHMP4A-B, or VPS4B), Alix, TSG101, MHCI, MHCII, GP96, actinin-4, mitofilin, syntenin-1, TSG101, ADAM10, EHD4, syntenin-1, TSG101, EHD1, flotillin-1, heat-shock 70-kDa proteins (HSC70/HSP73, HSP70/HSP72). The molar ratio these exosomal marker proteins relative to all proteins measured is determined as the molar quantity of each specific exosome marker protein listed above divided by the sum of the molar quantities of all identified proteins in each sample and expressed as a percent.

[000728] Similarly, the molar ratio for all exosomal marker proteins relative to all proteins measured is determined as the sum of the molar quantity of all specific exosome marker protein listed above divided by the sum of the molar quantities of all identified proteins in each sample and expressed as a percent of the total.

[000729] In some embodiments, a sample will comprise less than 5% of any individual exosomal marker protein and less than 15% of total exosomal marker proteins.

[000730] In some embodiments, an individual exosomal marker protein will be present at less than 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, , or 10%.

[000731] In some embodiments, the sum of all exosomal marker proteins will be less than 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25%.

Example 45: Measuring GAPDH in fusosomes

[000732] This assay describes quantification of the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the fusosomes, and the relative level of GAPDH in the fusosomes compared to the parental cells.

[000733] GAPDH is measured in the parental cells and the fusosomes using a standard commercially available ELISA for GAPDH (ab176642, Abcam) per the manufacturer's directions.

[000734] Total protein levels are similarly measured via bicinchoninic acid assay as previously described in the same volume of sample used to measure GAPDH. In embodiments, using this assay, the level of GAPDH per total protein in the fusosomes will be <100ng GAPDH / µg total protein. Similarly, in embodiments, the decrease in GAPDH levels relative to total protein from the parental cells to the fusosomes will be greater than a 10% decrease.

[000735] In some embodiments, GAPDH content in the preparation in ng GAPDH / µg total protein will be less than 500, less than 250, less than 100, less than 50, less than 20, less than 10, less than 5, or less than1.

[000736] In some embodiments, a decrease in GAPDH per total protein in ng/µg from the parent cell to the preparation will be more than 1%, more than 2.5%, more than 5%, more than 10%, more than 15%, more than 20%, more than 30%, more than 40%, more than 50%, more than 60%, more than 70%, more than 80%, or more than 90%.

[000737]

Example 46: Measuring calnexin in fusosomes

[000738] This assay describes quantification of the level of calnexin (CNX) in the fusosomes, and the relative level of CNX in the fusosomes compared to the parental cells.

[000739] Calnexin is measured in the starting cells and the preparation using a standard commercially available ELISA for calnexin (MBS721668, MyBioSource) per the manufacturer's directions.

[000740] Total protein levels are similarly measured via bicinchoninic acid assay as previously described in the same volume of sample used to measure calnexin. In embodiments, using this assay, the level of calnexin per total protein in the fusosomes will be <100ng calnexin / µg total protein. Similarly, in embodiments, the increase in calnexin levels relative to total protein from the parental cell to the fusosomes will be greater than a 10% increase.

[000741] In some embodiments, calnexin content in the preparation in ng calnexin / µg total protein will be less than 500, 250, 100, 50, 20, 10, 5, or 1.

[000742] In some embodiments, a decrease in calnexin per total protein in ng/µg from the parent cell to the preparation will be more than 1%, 2.5%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%.

Example 47: Comparison of soluble to insoluble protein mass

[000743] This Example describes quantification of the soluble:insoluble ratio of protein mass in fusosomes. In some embodiments, a soluble:insoluble ratio of protein mass in fusosomes will

be similar to nucleated cells.

[000744] Fusosomes are prepared by any one of the methods described in previous Examples. The fusosome preparation is tested to determine the soluble : insoluble protein ratio using a standard bicinchoninic acid assay (BCA) (e.g. using the commercially available Pierce™ BCA Protein Assay Kit, Thermo Fischer product# 23225). Soluble protein samples are prepared by suspending the prepared fusosomes or parental cells at a concentration of $1x10^7$ cells or fusosomes/mL in PBS and centrifuging at 1600g to pellet the fusosomes or cells. The supernatant is collected as the soluble protein fraction.

[000745] The fusosomes or cells in the pellet are lysed by vigorous pipetting and vortexing in PBS with 2% Triton-X-100. The lysed fraction represents the insoluble protein fraction.

[000746] A standard curve is generated using the supplied BSA, from 0 to 20 µg of BSA per well (in triplicate). The fusosome or cell preparation is diluted such that the quantity measured is within the range of the standards. The fusosome preparation is analyzed in triplicate and the mean value is used. The soluble protein concentration is divided by the insoluble protein concentration to yield the soluble:insoluble protein ratio.

[000747] In some embodiments, a fusosome soluble:insoluble protein ratio will be within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater compared to the parental cells.

Example 48: Measuring LPS in fusosomes

[000748] This example describes quantification of levels of lipopolysaccharides (LPS) in fusosomes as compared to parental cells. In some embodiments, fusosomes will have lower levels of LPS compared to parental cells.

[000749] LPS are a component of bacterial membranes and potent inducer of innate immune responses.

[000750] The LPS measurements are based on mass spectrometry as described in the previous Examples.

[000751] In some embodiments, less than 5%, 1%, 0.5%, 0.01%, 0.005%, 0.0001%,

0.00001% or less of the lipid content of fusosomes will be LPS.

Example 49: Ratio of lipids to proteins in fusosomes

[000752] This Example describes quantification of the ratio of lipid mass to protein mass in fusosomes. In some embodiments, fusosomes will have a ratio of lipid mass to protein mass that is similar to nucleated cells.

[000753] Total lipid content is calculated as the sum of the molar content of all lipids identified in the lipidomics data set outlined in a previous Example. Total protein content of the fusosomes is measured via bicinchoninic acid assay as described herein.

[000754] Alternatively, the ratio of lipids to proteins can be described as a ratio of a particular lipid species to a specific protein. The particular lipid species is selected from the lipidomics data produced in a previous Example. The specific protein is selected from the proteomics data produced in a previous Example. Different combinations of selected lipid species and proteins are used to define specific lipid:protein ratios.

Example 50: Ratio of proteins to DNA in fusosomes

[000755] This Example describes quantification of the ratio of protein mass to DNA mass in fusosomes. In some embodiments, fusosomes will have a ratio of protein mass to DNA mass that is much greater than cells.

[000756] Total protein content of the fusosomes and cells is measured as described in in a previous Example. The DNA mass of fusosomes and cells is measured as described in a previous Example. The ratio of proteins to total nucleic acids is then determined by dividing the total protein content by the total DNA content to yield a ratio within a given range for a typical fusosome preparation.

[000757] Alternatively, the ratio of proteins to nucleic acids is determined by defining nucleic acid levels as the level of a specific house-keeping gene, such as GAPDH, using semi-quantitative real-time PCR (RT-PCR).

[000758] The ratio of proteins to GAPDH nucleic acids is then determined by dividing the total protein content by the total GAPDH DNA content to define a specific range of protein:nucleic

acid ratio for a typical fusosome preparation.

Example 51: Ratio of lipids to DNA in fusosomes

[000759] This Example describes quantification of the ratio of lipids to DNA in fusosomes compared to parental cells. In some embodiments, fusosomes will have a greater ratio of lipids to DNA compared to parental cells.

[000760] This ratio is defined as total lipid content (outlined in an Example above) or a particular lipid species. In the case of a particular lipid species, the range depends upon the particular lipid species selected. The particular lipid species is selected from the lipidomics data produced in the previously described Example. Nucleic acid content is determined as described in the previously described Example.

[000761] Different combinations of selected lipid species normalized to nucleic acid content are used to define specific lipid:nucleic acid ratios that are characteristic of a particular fusosome preparation.

Example 52: Analyzing surface markers on fusosomes

[000762] This assay describes identification of surface markers on the fusosomes.

[000763] Fusosomes are pelleted and shipped frozen to the proteomics analysis center per standard biological sample handling procedures.

[000764] To identify surface marker presence or absence on the fusosomes, they are stained with markers against phosphatidyl serine and CD40 ligand and analyzed by flow cytometry using a FACS system (Becton Dickinson). For detection of surface phosphatidylserine, the product is analyzed with an annexin V assay (556547, BD Biosciences) as described by the manufacturer.

[000765] Briefly, the fusosomes are washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1 x 10^6 fusosomes/mL. 10% of the resuspension is transferred to a 5 mL culture tube and 5 µl of FITC annexin V is added. The cells are gently vortexed and incubated for 15 min at room temperature (25°C) in the dark.

[000766] In parallel, a separate 10% of the resuspension is transferred to a different tube to act as an unstained control. 1X binding buffer is added to each tube. The samples are analyzed by

flow cytometry within 1 hr.

[000767] In some embodiments, using this assay, the mean of the population of the stained fusosomes will be determined to be above the mean of the unstained cells indicating that the fusosomes comprise phosphatidyl serine.

[000768] Similarly, for the CD40 ligand, the following monoclonal antibody is added to another 10% of the washed fusosomes: PE-CF594 mouse anti-human CD154 clone TRAP1 (563589, BD Pharmigen) as per the manufacturer's directions. Briefly, saturating amounts of the antibody are used. In parallel, a separate 10% of the fusosomes are transferred to a different tube to act as an unstained control. The tubes are centrifuged for 5 min at 400 × g, at room temperature. The supernatant is decanted and the pellet is washed twice with flow cytometry wash solution. 0.5 mL of 1% paraformaldehyde fixative is added to each tube. Each is briefly vortexed and stored at 4°C until analysis on the flow cytometer.

[000769] In some embodiments, using this assay or equivalent, the mean of the population of the stained fusosomes will be above the mean of the unstained cells indicating that the fusosomes comprise CD40 ligand.

Example 53: Analysis of viral capsid proteins in fusosomes

[000770] This assay describes analysis of the makeup of the sample preparation and assesses the proportion of proteins that are derived from viral capsid sources.

[000771] Fusosomes are pelleted and shipped frozen to a proteomics analysis center per standard biological sample handling procedures.

[000772] The fusosomes are thawed for protein extraction and analysis. First, they are resuspended in lysis buffer (7M urea, 2M thiourea, 4% (w/v) chaps in 50 mM Tris pH 8.0) and incubated for 15 minutes at room temperature with occasional vortexing. The mixtures are then lysed by sonication for 5 minutes in an ice bath and spun down for 5 minutes at 13,000 RPM. Total protein content is determined by a colorimetric assay (Pierce) and 100 µg of protein from each sample is transferred to a new tube and the volume is adjusted with 50 mM Tris pH 8.

[000773] The proteins are reduced for 15 minutes at 65° Celsius with 10 mM DTT and alkylated with 15 mM iodoacetamide for 30 minutes at room temperature in the dark. The proteins

are then precipitated with gradual addition of 6 volumes of cold (-20° Celsius) acetone and incubated over night at -80° Celsius.

[000774] The proteins are pelleted, washed 3 times with cold (-20° Celsius) methanol, and resuspended in 50 mM Tris pH 8. 3.33µg of trypsin/lysC is added to the proteins for a first 4h of digestion at 37° Celsius with agitation. The samples are diluted with 50 mM Tris pH 8 and 0.1 % sodium deoxycholate is added with another 3.3 µg of trypsin/lysC for digestion overnight at 37° Celsius with agitation. Digestion is stopped and sodium deoxycholate is removed by the addition of 2% v/v formic acid. Samples are vortexed and cleared by centrifugation for 1 minute at 13,000 RPM.

[000775] The proteins are purified by reversed phase solid phase extraction (SPE) and dried down. The samples are reconstituted in 3% DMSO, 0.2% formic acid in water and analyzed by LC-MS as described previously.

[000776] The molar ratio of the viral capsid proteins relative to all proteins measured is determined as the molar quantity of all viral capsid proteins divided by the sum of the molar quantities of all identified proteins in each sample and expressed as a percent.

[000777] In some embodiments, using this approach or an equivalent, the sample will comprise less than 10% viral capsid protein. In some embodiments, a sample will comprise less than 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, or 90% viral capsid protein.

Example 54: Measuring fusion with a target cell

[000778] This example describes quantification of fusosome fusion with a target cell compared to a non-target cell.

[000779] In some embodiments, fusosome fusion with a target cell allows the cell-specific delivery of a cargo, carried within the lumen of the fusosome, to the cytosol of the recipient cell. Fusosomes produced by the herein described methods are assayed for fusion rate with a target cell as follows.

[000780] In this example, the fusosome comprises a HEK293T cell expressing Myomaker on its plasma membrane. In addition, the fusosome expresses mTagBFP2 fluorescent protein and

Cre recombinase. The target cell is a myoblast cell, which expresses both Myomaker and Myomixer, and the non-target cell is a fibroblast cell, which expresses neither Myomaker nor Myomixer. A Myomaker-expressing fusosome is predicted to fuse with the target cell that expresses both Myomaker and Myomixer but not the non-target cell (Quinn et al., 2017, Nature Communications, 8, 15665. doi.org/10.1038/ncomms15665) (Millay et al., 2013, Nature, 499(7458), 301–305. doi.org/10.1038/nature12343). Both the target and non-target cell types are isolated from mice and stably-express "LoxP-stop-Loxp-tdTomato" cassette under a CMV promoter, which upon recombination by Cre turns on tdTomato expression, indicating fusion.

[000781] The target or non-target recipient cells are plated into a black, clear-bottom 96-well plate. Both target and non-target cells are plated for the different fusion groups. Next, 24 hours after plating the recipient cells, the fusosomes expressing Cre recombinase protein and Myomaker are applied to the target or non-target recipient cells in DMEM media. The dose of fusosomes is correlated to the number of recipient cells plated in the well. After applying the fusosomes, the cell plate is centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells.

[000782] Starting at four hours after fusosome application, the cell wells are imaged to positively identify RFP-positive cells versus GFP-positive cells in the field or well.

[000783] In this example, cell plates are imaged using an automated microscope (www.biotek.com/products/imaging-microscopy-automated-cell-imagers/lionheart-fx-automated-live-cell-imager/). The total cell population in a given well is determined by first staining the cells with Hoechst 33342 in DMEM media for 10 minutes. Hoechst 33342 stains cell nuclei by intercalating into DNA and therefore is used to identify individual cells. After staining, the Hoechst media is replaced with regular DMEM media.

[000784] The Hoechst is imaged using the 405 nm LED and DAPI filter cube. GFP is imaged using the 465 nm LED and GFP filter cube, while RFP is imaged using 523 nm LED and RFP filter cube. Images of target and non-target cell wells are acquired by first establishing the LED intensity and integration times on a positive-control well; i.e., recipient cells treated with adenovirus coding for Cre recombinase instead of fusosomes.

[000785] Acquisition settings are set so that RFP and GFP intensities are at the maximum

pixel intensity values but not saturated. The wells of interest are then imaged using the established settings. Wells are imaged every 4 hours to acquire time-course data for rates of fusion activity.

[000786] Analysis of GFP and RFP-positive wells is performed with software provided with the fluorescent microscope or other software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, rsb.info.nih.gov/ij/, 1997–2007).

[000787] The images are pre-processed using a rolling ball background subtraction algorithm with a 60 μm width. The total cell mask is set on the Hoechst-positive cells. Cells with Hoechst intensity significantly above background intensities are thresholded and areas too small or large to be Hoechst-positive cells are excluded.

[000788] Within the total cell mask, GFP and RFP-positive cells are identified by again thresholding for cells significantly above background and extending the Hoechst (nuclei) masks for the entire cell area to include the entire GFP and RFP cellular fluorescence. The number of RFP-positive cells identified in control wells containing target or non-target recipient cells is used to subtract from the number of RFP-positive cells in the wells containing fusosome (to subtract for non-specific Loxp recombination). The number of RFP-positive cells (fused recipient cells) is then divided by the sum of the GFP-positive cells (recipient cells that have not fused) and RFP-positive cells at each time point to quantify the rate of fusosome fusion within the recipient cell population. The rate is normalized to the given dose of fusosome applied to the recipient cells. For rates of targeted fusion (fusosome fusion to targeted cells), the rate of fusion to the non-target cell is subtracted from the rate of fusion to the target cell in order to quantify rates of targeted fusion.

In some embodiments, average rate of fusion for the fusosomes with the target cells will be in the range of 0.01-4.0 RFP/GFP cells per hour for target cell fusion or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than non-target recipient cells with fusosomes. In some embodiments, groups with no fusosome applied will show a background rate of <0.01 RFP/GFP cells per hour.

Example 55: In vitro fusion to deliver a membrane protein

[000789] This example describes fusosome fusion with a cell *in vitro*. In some embodiments, fusosome fusion with a cell in vitro results in delivery of an active membrane protein to the recipient cell.

[000790] In this example, the fusosomes are generated from a HEK293T cell expressing the Sendai virus HVJ-E protein (Tanaka et al., 2015, Gene Therapy, 22(October 2014), 1–8. doi.org/10.1038/gt.2014.12). In some embodiments, fusosomes are generated to express the membrane protein, GLUT4, which is found primarily in muscle and fat tissues and is responsible for the insulin-regulated transport of glucose into cells. Fusosomes with and without GLUT4 are prepared from HEK293T cells as described by any of the methods described in a previous Example.

[000791] Muscles cells, such as, C2C12 cells, are then treated with fusosomes expressing GLUT4, fusosomes that do not express GLUT4, PBS (negative control), or insulin (positive control). The activity of GLUT4 on C2C12 cells is measured by the uptake of the fluorescent 2-deoxyglucose analog, 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). The fluorescence of C2C12 cells is assessed via microscopy using methods described in previous Examples.

[000792] In some embodiments, C2C12 cells that are treated with fusosomes that express GLUT4 and insulin are expected to demonstrate increased fluorescence compared to C2C12 cells treated with PBS or fusosomes not expressing GLUT4. See, also, Yang et al., *Advanced Materials* 29, 1605604, 2017.

Example 56: In vivo delivery of membrane protein

[000793] This example describes fusosome fusion with a cell *in vivo*. In some embodiments, fusosome fusion with a cell in vivo results in delivery of an active membrane protein to the recipient cell.

[000794] In this example, the fusosomes are generated from a HEK293T cell expressing the Sendai virus HVJ-E protein as in the previous Example. In some embodiments, fusosomes are generated to express the membrane protein, GLUT4. Fusosomes with and without GLUT4 are prepared from HEK293T cells as described by any of the methods described in a previous Example.

[000795] BALB/c-nu mice are administered fusosomes expressing GLUT4, fusosomes that do not express GLUT4, or PBS (negative control). Mice are injected intramuscularly in the tibialis

anterior muscle with fusosomes or PBS. Immediately prior to fusosome administration, mice are fasted for 12 hours and injected with [18F] 2-fluoro-2deoxy-d-glucose (18F-FDG), which is an analog of glucose that enables positron emission tomography (PET imaging). Mice are injected with 18F-FDG via the tail vein under anesthesia (2% isoflurane). PET imaging is performed using a nanoscale imaging system (1T, Mediso, Hungary). Imaging is conducted 4 hours after administration of fusosomes. Immediately after imaging, mice are sacrificed and the tibialis anterior muscle is weighed. PET images are reconstructed using a 3D imaging system in full detector mode, with all corrections on, high regularization, and eight iterations. Three-dimensional volume of interest (VOI) analysis of the reconstructed images is performed using the imaging software package (Mediso, Hungary) and applying standard uptake value (SUV) analysis. VOI fixed with a diameter of 2 mm sphere, is drawn for the tibialis anterior muscle site. The SUV of each VOI sites is calculated using the following formula: SUV = (radioactivity in volume of interest, measured as Bq/cc × body weight)/ injected radioactivity.

[000796] In some embodiments, mice that are administered fusosomes expressing GLUT4 are expected to demonstrate an increased radioactive signal in VOI as compared to mice administered PBS or fusosomes that do not express GLUT4. See, also, Yang et al., *Advanced Materials* 29, 1605604, 2017.

Example 57: Measuring extravasation from blood vessels

[000797] This Example describes quantification of fusosome extravasation across an endothelial monolayer as tested with an in vitro microfluidic system (J.S Joen et al. 2013, journals.plos.org/plosone/article?id=10.1371/journal.pone.0056910).

[000798] Cells extravasate from the vasculature into surrounding tissue. Without wishing to be bound by theory, extravasation is one way for fusosomes to reach extravascular tissues.

[000799] The system includes three independently addressable media channels, separated by chambers into which an ECM-mimicking gel can be injected. In brief, the microfluidics system has molded PDMS (poly-dimethyl siloxane; Silgard 184; Dow Chemical, MI) through which access ports are bored and bonded to a cover glass to form microfluidic channels. Channel cross-sectional dimensions are 1 mm (width) by 120 μm (height). To enhance matrix adhesion, the PDMS channels are coated with a PDL (poly-D-lysine hydrobromide; 1 mg/mL; Sigma-Aldrich,

St. Louis, MO) solution.

[000800] Next, collagen type I (BD Biosciences, San Jose, CA, USA) solution (2.0 mg/mL) with phosphate-buffered saline (PBS; Gibco) and NaOH is injected into the gel regions of the device via four separate filling ports and incubated for 30 min to form a hydrogel. When the gel is polymerized, endothelial cell medium (acquired from suppliers such as Lonza or Sigma) is immediately pipetted into the channels to prevent dehydration of the gel. Upon aspirating the medium, diluted hydrogel (BD science) solution (3.0 mg/mL) is introduced into the cell channel and the excess hydrogel solution is washed away using cold medium.

[000801] Endothelial cells are introduced into the middle channel and allowed to settle to form an endothelium. Two days after endothelial cell seeding, fusosomes or macrophage cells (positive control) are introduced into the same channel where endothelial cells had formed a complete monolayer. The fusosomes are introduced so they adhere to and transmigrate across the monolayer into the gel region. Cultures are kept in a humidified incubator at 37°C and 5% CO_2. A GFP-expressing version of the fusosome is used to enable live-cell imaging via fluorescent microscopy. On the following day, cells are fixed and stained for nuclei using DAPI staining in the chamber, and multiple regions of interest are imaged using confocal microscope to determine how many fusosomes passed through the endothelial monolayer.

[000802] In some embodiments, DAPI staining will indicate that fusosomes and positive control cells are able to pass through the endothelial barrier after seeding.

Example 58: Measuring chemotactic cell mobility

[000803] This Example describes quantification of fusosome chemotaxis. Cells can move towards or away from a chemical gradient via chemotaxis. In some embodiments, chemotaxis will allow fusosomes to home to a site of injury, or track a pathogen. A purified fusosome composition as produced by any one of the methods described in previous Examples is assayed for its chemotactic abilities as follows.

[000804] A sufficient number of fusosomes or macrophage cells (positive control) are loaded in a micro-slide well according to the manufacturer's provided protocol in DMEM media (ibidi.com/img/cms/products/labware/channel_slides/S_8032X_Chemotaxis/IN_8032X_Chemot

axis.pdf). Fusosomes are left at 37°C and 5% CO_2 for 1h to attach. Following cell attachment, DMEM (negative control) or DMEM containing MCP1 chemoattractant is loaded into adjacent reservoirs of the central channel and the fusosomes are imaged continuously for 2 hours using a Zeiss inverted widefield microscope. Images are analyzed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2007). Migration co-ordination data for each observed fusosome or cell is acquired with the manual tracking plugin (Fabrice Cordelières, Institut Curie, Orsay, France). Chemotaxis plots and migration velocities is determined with the Chemotaxis and Migration Tool (ibidi).

[000805] In some embodiments, an average accumulated distance and migration velocity of fusosomes will be within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the response of the positive control cells to chemokine. The response of cells to a chemokine is described, e.g., in Howard E. Gendelman et al., *Journal of Neuroimmune Pharmacology*, 4(1): 47-59, 2009.

Example 59: Measuring homing potential

[000806] This Example describes homing of fusosomes to a site of injury. Cells can migrate from a distal site and/or accumulate at a specific site, e.g., home to a site. Typically, the site is a site of injury. In some embodiments, fusosomes will home to, e.g., migrate to or accumulate at, a site of injury.

[000807] Eight week old C57BL/6J mice (Jackson Laboratories) are dosed with notexin (NTX) (Accurate Chemical & Scientific Corp), a myotoxin, in sterile saline by intramuscular (IM) injection using a 30G needle into the right tibialis anterior (TA) muscle at a concentration of 2 µg/mL. The skin over the tibialis anterior (TA) muscle is prepared by depilating the area using a chemical hair remover for 45 seconds, followed by 3 rinses with water. This concentration is chosen to ensure maximum degeneration of the myofibers, as well as minimal damage to their satellite cells, the motor axons and the blood vessels.

[000808] On day 1 after NTX injection, mice receive an IV injection of fusosomes or cells that express firefly luciferase. Fusosomes are produced from cells that stably express firefly luciferase by any one of the methods described in previous Examples. A bioluminescent imaging system (Perkin Elmer) is used to obtain whole animal images of bioluminescence at 0, 1, 3, 7, 21,

and 28 post injection.

[000809] Five minutes before imaging, mice receive an intraperitoneal injection of bioluminescent substrate (Perkin Elmer) at a dose of 150mg/kg in order to visualize luciferase. The imaging system is calibrated to compensate for all device settings. The bioluminescent signal is measured using Radiance Photons, with Total Flux used as a measured value. The region of interest (ROI) is generated by surrounding the signal of the ROI in order to give a value in photons/second. An ROI is assessed on both the TA muscle treated with NTX and on the contralateral TA muscle, and the ratio of photons/second between NTX-treated and NTX-untreated TA muscles is calculated as a measure of homing to the NTX-treated muscle.

[000810] In some embodiments, a ratio of photons/second between NTX-treated and NTX-untreated TA muscles in fusosomes and cells will be greater than 1 indicating site specific accumulation of luciferase-expressing fusosomes at the injury.

[000811] See, for example, Plant et al., *Muscle Nerve* 34(5)L 577-85, 2006.

Example 60: Measuring phagocytic activity

[000812] This Example demonstrates phagocytic activity of fusosomes. In some embodiments, fusosomes have phagocytic activity, e.g., are capable of phagocytosis. Cells engage in phagocytosis, engulfing particles, enabling the sequestration and destruction of foreign invaders, like bacteria or dead cells.

[000813] A purified fusosome composition as produced by any one of the methods described in previous Examples comprising a fusosome from a mammalian macrophage having partial or complete nuclear inactivation was capable of phagocytosis assayed via pathogen bioparticles. This estimation was made by using a fluorescent phagocytosis assay according to the following protocol.

[000814] Macrophages (positive control) and fusosomes were plated immediately after harvest in separate confocal glass bottom dishes. The macrophages and fusosomes were incubated in DMEM+10%FBS+1%P/S for 1h to attach. Fluorescein-labeled *E. coli* K12 and non-fluorescein-labeled *Escherichia coli* K-12 (negative control) were added to the macrophages/fusosomes as indicated in the manufacturer's protocol, and were incubated for 2h,

tools.thermofisher.com/content/sfs/manuals/mp06694.pdf. After 2h, free fluorescent particles were quenched by adding Trypan blue. Intracellular fluorescence emitted by engulfed particles was imaged by confocal microscopy at 488 excitation. The number of phagocytotic positive fusosome were quantified using image J software.

[000815] The average number of phagocytotic fusosomes was at least 30% 2h after bioparticle introduction, and was greater than 30% in the positive control macrophages.

Example 61: Measuring ability to cross a cell membrane or the blood brain barrier

[000816] This Example describes quantification of fusosomes crossing the blood brain barrier. In some embodiments, fusosomes will cross, e.g., enter and exit, the blood brain barrier, e.g., for delivery to the central nervous system.

[000817] Eight week old C57BL/6J mice (Jackson Laboratories) are intravenously injected with fusosomes or leukocytes (positive control) that express firefly luciferase. Fusosomes are produced from cells that stably express firefly luciferase or cells that do not express luciferase (negative control) by any one of the methods described in previous Examples. A bioluminescent imaging system (Perkin Elmer) is used to obtain whole-animal images of bioluminescence at one, two, three, four, five, six, eight, twelve, and twenty-four hours after fusosome or cell injection.

[000818] Five minutes before imaging, mice receive an intraperitoneal injection of bioluminescent substrate (Perkin Elmer) at a dose of 150mg/kg in order to visualize luciferase. The imaging system is calibrated to compensate for all device settings. The bioluminescent signal is measured, with total flux used as a measured value. The region of interest (ROI) is generated by surrounding the signal of the ROI in order to give a value in photons/second. The ROI selected is the head of the mouse around the area that includes the brain.

[000819] In some embodiments, the photons/second in the ROI will be greater in the animals injected with cells or fusosomes that express luciferase than the negative control fusosomes that do not express luciferase indicating accumulation of luciferase-expressing fusosomes in or around the brain.

Example 62: Measuring potential for protein secretion

[000820] This Example describes quantification of secretion by fusosomes. In some

embodiments, fusosomes will be capable of secretion, e.g., protein secretion. Cells can dispose or discharge of material via secretion. In some embodiments, fusosomes will chemically interact and communicate in their environment via secretion.

[000821] The capacity of fusosomes to secrete a protein at a given rate is determined using the Gaussia luciferase flash assay from ThermoFisher Scientific (catalog #16158). Mouse embryonic fibroblast cells (positive control) or fusosomes as produced by any one of the methods described in previous Examples are incubated in growth media and samples of the media are collected every 15 minutes by first pelleting the fusosomes at 1600g for 5min and then collecting the supernatant. The collected samples are pipetted into a clear-bottom 96-well plate. A working solution of assay buffer is then prepared according to the manufacturer's instructions.

[000822] Briefly, colenterazine, a luciferin or light-emitting molecule, is mixed with flash assay buffer and the mixture is pipetted into each well of the 96 well plate containing samples. Negative control wells that lack cells or fusosomes include growth media or assay buffer to determine background Gaussia luciferase signal. In addition, a standard curve of purified Gaussia luciferase (Athena Enzyme Systems, catalog #0308) is prepared in order to convert the luminescence signal to molecules of Gaussia luciferase secretion per hour.

[000823] The plate is assayed for luminescence, using 500 msec integration. Background Gaussia luciferase signal is subtracted from all samples and then a linear best-fit curve is calculated for the Gaussia luciferase standard curve. If sample readings do not fit within the standard curve, they are diluted appropriately and re-assayed. Using this assay, the capacity for fusosomes to secrete Gaussia luciferase at a rate (molecules/hour) within a given range is determined.

[000824] In some embodiments, fusosomes will be capable of secreting proteins at a rate that is 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the positive control cells.

Example 63: Measuring signal transduction potential

[000825] This Example describes quantification of signal transduction in fusosomes. In some embodiments, fusosomes are capable of signal transduction. Cells can send and receive molecular signals from the extracellular environment through signaling cascades, such as

phosphorylation, in a process known as signal transduction. A purified fusosome composition as produced by any one of the methods described in previous Examples comprising a fusosome from a mammalian cell having partial or complete nuclear inactivation is capable of signal transduction induced by insulin. Signal transduction induced by insulin is assessed by measuring AKT phosphorylation levels, a key pathway in the insulin receptor signaling cascade, and glucose uptake in response to insulin.

[000826] To measure AKT phosphorylation, cells, e.g., Mouse Embryonic Fibroblasts (MEFs) (positive control), and fusosomes are plated in 48-well plates and left for 2 hours in a humidified incubator at 37°C and 5% CO_2. Following cell adherence, insulin (e.g. at 10 nM), or a negative control solution without insulin, is add to the well containing cells or fusosomes for 30 min. After 30 minutes, protein lysate is made from the fusosomes or cells, and phospho-AKT levels are measured by western blotting in insulin stimulated and control unstimulated samples.

[000827] Glucose uptake in response to insulin or negative control solution is measured as it is explained in the glucose uptake section by using labeled glucose (2-NBDG). (S. Galic et al., *Molecular Cell Biology* 25(2): 819-829, 2005).

[000828] In some embodiments, fusosomes will enhance AKT phosphorylation and glucose uptake in response to insulin over the negative controls by at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater.

Example 64: Measuring ability to transport glucose across cell membrane

[000829] This Example describes quantification of the levels of a 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) a fluorescent glucose analog that can be used to monitor glucose uptake in live cells, and thus measure active transport across the lipid bilayer. In some embodiments, this assay or an equivalent can be used to measure the level of glucose uptake and active transport across the lipid bilayer of the fusosome.

[000830] A fusosome composition is produced by any one of the methods described in previous Examples. A sufficient number of fusosomes are then incubated in DMEM with no glucose, 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin for 2hr at 37°C and 5% CO_2. After a 2hr glucose starvation period, the medium is changed such that it includes DMEM with no

glucose, 20% Fetal Bovine Serum, 1x Penicillin/Streptomycin and 20 μM 2-NBDG (ThermoFisher) and incubated for an additional 2hr at 37°C and 5% CO_2.

[000831] Negative control fusosomes are treated the same, except an equal amount of DMSO is added in place of 2-NBDG.

[000832] The fusosomes are then washed thrice with 1xPBS and re-suspended in an appropriate buffer, and transferred to a 96 well imaging plate. 2-NBDG fluorescence is then measured in a fluorimeter using a GFP light cube (469/35 excitation filter and a 525/39 emission filter) to quantify the amount of 2-NBDG that has been transported across the fusosome membrane and accumulated in the fusosome in the 1hr loading period.

[000833] In some embodiments, 2-NBDG fluorescence will be higher in the fusosome with 2-NBDG treatment as compared to the negative (DMSO) control. Fluorescence measure with a 525/39 emission filter will correlate with to the number of 2-NBDG molecules present.

Example 65: Lumen of fusosomes are miscible with aqueous solutions

[000834] This example assesses the miscibility of a fusosome lumen with aqueous solutions, such as water.

[000835] The fusosomes are prepared as described in previous Examples. The controls are dialysis membranes with either hypotonic solution, hyperosmotic solution or normal osmotic solutions.

[000836] Fusosomes, positive control (normal osmotic solution) and negative control (hypotonic solution) are incubated with hypotonic solution (150 mOsmol). The cell size is measured under a microscope after exposing each sample to the aqueous solution. In some embodiments, fusosome and positive control sizes in the hypotonic solution increase in comparison to a negative control.

[000837] Fusosomes, positive control (normal osmotic solution) and negative control (hyperosmotic solution) are incubated with a hyperosmotic solution (400 mOsmol). The cell size is measured under a microscope after exposing each sample to the aqueous solution. In some embodiments, fusosome and positive control sizes in a hyperosmotic solution will decrease in comparison to the negative control.

[000838] Fusosomes, positive control (hypotonic or hyperosmotic solution) and negative control (normal osmotic) are incubated with a normal osmotic solution (290 mOsmol). The cell size is measured under a microscope after exposing each sample to the aqueous solution. In some embodiments, fusosome and positive control sizes in a normal osmotic solution will remain substantially the same in comparison to the negative control.

Example 66: Measuring esterase activity in the cytosol

[000839] This Example describes quantification of esterase activity, as a surrogate for metabolic activity, in fusosomes. The cytosolic esterase activity in fusosomes is determined by quantitative assessment of calcein-AM staining (Bratosin et al., Cytometry 66(1): 78-84, 2005).

[000840] The membrane-permeable dye, calcein-AM (Molecular Probes, Eugene OR USA), is prepared as a stock solution of 10 mM in dimethylsulfoxide and as a working solution of 100 mM in PBS buffer, pH 7.4. Fusosomes as produced by any one of the methods described in previous Examples or positive control parental Mouse Embryonic Fibroblast cells are suspended in PBS buffer and incubated for 30 minutes with calcein-AM working solution (final concentration in calcein-AM: 5 mM) at 37°C in the dark and then diluted in PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention.

[000841] Fusosomes and control parental Mouse Embryonic Fibroblast cells are experimental permeabilized as a negative control for zero esterase activity with saponin as described in (Jacob et al., *Cytometry* 12(6): 550-558, 1991). Fusosomes and cells are incubated for 15 min in 1% saponin solution in PBS buffer, pH 7.4, containing 0.05% sodium azide. Due to the reversible nature of plasma membrane permeabilization, saponin is included in all buffers used for further staining and washing steps. After saponin permeabilization, fusosomes and cells are suspended in PBS buffer containing 0.1% saponin and 0.05% sodium azide and incubated (37°C in the dark for 45 min) with calcein-AM to a final concentration of 5 mM, washed three times with the same PBS buffer containing 0.1% saponin and 0.05% sodium azide, and analyzed by flow cytometry. Flow cytometric analyses are performed on a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 488nm argon laser excitation and emission is collected at 530+/-30nm. FACS software is used for acquisition and analysis. The light scatter channels are set on linear gains, and the fluorescence channels are set on a logarithmic scale, with a minimum of 10,000 cells analyzed

in each condition. Relative esterase activities are calculated based on the intensity of calcein-AM in each sample. All events are captured in the forward and side scatter channels (alternatively, a gate can be applied to select only the fusosome population). The fluorescence intensity (FI) value for the fusosomes is determined by subtracting the FI value of the respective negative control saponin-treated sample. The normalized esterase activity for the fusosomes samples are normalized to the respective positive control cell samples in order to generate quantitative measurements for cytosolic esterase activities.

[000842] In some embodiments, a fusosome preparation will have within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater esterase activity compared to the positive control cell.

[000843] See also, Bratosin D, Mitrofan L, Palii C, Estaquier J, Montreuil J. Novel fluorescence assay using calcein-AM for the determination of human erythrocyte viability and aging. Cytometry A. 2005 Jul;66(1):78-84; and Jacob BC, Favre M, Bensa JC. Membrane cell permeabilisation with saponin and multiparametric analysis by flow cytometry. Cytometry 1991;12:550–558.

Example 67: Measuring acetylcholinesterase activity in fusosomes

[000844] Acetylcholinesterase activity is measured using a kit (MAK119, SIGMA) that follows a procedure described previously (Ellman, et al., Biochem. Pharmacol. 7, 88, 1961) and following the manufacturer's recommendations.

[000845] Briefly, fusosomes are suspended in 1.25 mM acetylthiocholine in PBS, pH 8, mixed with 0.1 mM 5,5-dithio-bis(2-nitrobenzoic acid) in PBS, pH 7. The incubation is performed at room temperature but the fusosomes and the substrate solution are pre-warmed at 37 °C for 10 min before starting the optical density readings.

[000846] Changes in absorption are monitored at 450 nm for 10 min with a plate reader spectrophotometer (ELX808, BIO-TEK instruments, Winooski, VT, USA). Separately, a sample is used for determining the protein content of the fusosomes via bicinchoninic acid assay for normalization. Using this assay, the fusosomes are determined to have <100 AChE activity units/μg of protein.

[000847] In some embodiments, AChE activity units/μg of protein values will be less than 0.001, 0.01, 0.1, 1, 10, 100, or 1000.

Example 68: Measuring metabolic activity level

[000848] This Example describes quantification of the measurement of citrate synthase activity in fusosomes.

[000849] Citrate synthase is an enzyme within the tricarboxylic acid (TCA) cycle that catalyzes the reaction between oxaloacetate (OAA) and acetyl-CoA to generate citrate. Upon hydrolysis of acetyl-CoA, there is a release of CoA with a thiol group (CoA-SH). The thiol group reacts with a chemical reagent, 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB), to form 5-thio-2-nitrobenzoic acid (TNB), which is a yellow product that can be measured spectrophotometrically at 412 nm (Green 2008). Commercially-available kits, such as the Abcam Human Citrate Synthase Activity Assay Kit (Product #ab119692) provide all the necessary reagents to perform this measurement.

[000850] The assay is performed as per the manufacturer's recommendations. Fusosome sample lysates are prepared by collecting the fusosomes as produced by any one of the methods described in previous Examples and solubilizing them in Extraction Buffer (Abcam) for 20 minutes on ice. Supernatants are collected after centrifugation and protein content is assessed by bicinchoninic acid assay (BCA, ThermoFisher Scientific) and the preparation remains on ice until the following quantification protocol is initiated.

[000851] Briefly, fusosome lysate samples are diluted in 1X Incubation buffer (Abcam) in the provided microplate wells, with one set of wells receiving only 1X Incubation buffer. The plate is sealed and incubated for 4 hours at room temperature with shaking at 300rpm. The buffer is then aspirated from the wells and 1X Wash buffer is added. This washing step is repeated once more. Then, 1X Activity solution is added to each well, and the plate is analyzed on a microplate reader by measuring absorbance at 412nm every 20 seconds for 30 minutes, with shaking between readings.

[000852] Background values (wells with only 1X Incubation buffer) are subtracted from all wells, and the citrate synthase activity is expressed as the change in absorbance per minute per μg

of fusosome lysate sample loaded (ΔmOD@412nm/min/μg protein). Only the linear portion from 100-400 seconds of the kinetic measurement is used to calculate the activity.

[000853] In some embodiments, a fusosome preparation will have within 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater synthase activity compared to the control cell.

[000854] See, for example, Green HJ et al. Metabolic, enzymatic, and transporter response in human muscle during three consecutive days of exercise and recovery. Am J Physiol Regul Integr Comp Physiol 295: R1238-R1250, 2008.

Example 69: Measuring respiration levels

[000855] This Example describes quantification of the measurement of respiration level in fusosomes. Respiration level in cells can be a measure of oxygen consumption, which powers metabolism. Fusosome respiration is measured for oxygen consumption rates by a Seahorse extracellular flux analyzer (Agilent) (Zhang 2012).

[000856] Fusosomes as produced by any one of the methods described in previous Examples or cells are seeded in a 96-well Seahorse microplate (Agilent). The microplate is centrifuged briefly to pellet the fusosomes and cells at the bottom of the wells. Oxygen consumption assays are initiated by removing growth medium, replacing with a low-buffered DMEM minimal medium containing 25mM glucose and 2mM glutamine (Agilent) and incubating the microplate at 37°C for 60 minutes to allow for temperature and pH equilibrium.

[000857] The microplate is then assayed in an extracellular flux analyzer (Agilent) that measures changes in extracellular oxygen and pH in the media immediately surrounding adherent fusosomes and cells. After obtaining steady state oxygen consumption (basal respiration rate) and extracellular acidification rates, oligomycin (5μM), which inhibits ATP synthase, and proton ionophore FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; 2μM), which uncouples mitochondria, are added to each well in the microplate to obtain values for maximal oxygen consumption rates.

[000858] Finally, 5 μM antimycin A (inhibitor of mitochondria complex III) is added to confirm that respiration changes are due mainly to mitochondrial respiration. The minimum rate

of oxygen consumption after antimycin A addition is subtracted from all oxygen consumption measurements to remove the non-mitochondrial respiration component. Cell samples that do not appropriately respond to oligomycin (at least a 25% decrease in oxygen consumption rate from basal) or FCCP (at least a 50% increase in oxygen consumption rate after oligomycin) are excluded from the analysis. Fusosomes respiration level is then measured as pmol O2/min/1e4 fusosomes.

[000859] This respiration level is then normalized to the respective cell respiration level. In some embodiments, fusosomes will have at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater respiration level compared to the respective cell samples.

[000860] See, for example, Zhang J, Nuebel E, Wisidagama DRR, et al. Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. Nature protocols. 2012;7(6):10.1038/nprot.2012.048. doi:10.1038/nprot.2012.048.

Example 70: Measuring phosphatidylserine levels of fusosomes

[000861] This Example describes quantification of the level of annexin-V binding to the surface of fusosomes.

[000862] Dying cells can display phosphatidylserine on the cell surface which is a marker of apoptosis in the programmed cell death pathway. Annexin-V binds to phosphatidylserine, and thus, annexin-V binding is a proxy for viability in cells.

[000863] Fusosomes were produced as described herein. For detection of apoptosis signals, fusosomes or positive control cells were stained with 5% annexin V fluor 594 (A13203, Thermo Fisher, Waltham, MA). Each group (detailed in the table below) included an experimental arm that was treated with an apoptosis-inducer, menadione. Menadione was added at 100 μM menadione for 4 h. All samples were run on a flow cytometer (Thermo Fisher, Waltham, MA) and fluorescence intensity was measured with the YL1 laser at a wavelength of 561 nm and an emission filter of 585/16 nm. The presence of extracellular phophatidyl serine was quantified by comparing fluorescence intensity of annexin V in all groups.

[000864] The negative control unstained fusosomes were not positive for annexin V staining.

[000865] In some embodiments, fusosomes were capable of upregulating phosphatidylserine

display on the cell surface in response to menadione, indicating that non-menadione stimulated fusosomes are not undergoing apoptosis. In some embodiments, positive control cells that were stimulated with menadione demonstrated higher-levels of annexin V staining than fusosomes not stimulated with menadione.

Table 20: Annexin V staining parameter

Experimental Arm	Mean Fluorescence Intensity of Annexin V Signal (and standard deviation)
Unstained Fusosomes (negative control)	941 (937)
Stained Fusosomes	11257 (15826)
Stained Fusosomes + Menadione	18733 (17146)
Stained Macrophages + Menadione (positive control)	14301 (18142)

Example 71: Measuring juxtacrine-signaling levels

[000866] This Example describes quantification of juxtacrine-signaling in fusosomes.

[000867] Cells can form cell-contact dependent signaling via juxtacrine signaling. In some embodiments, presence of juxtacrine signaling in fusosomes will demonstrate that fusosomes can stimulate, repress, and generally communicate with cells in their immediate vicinity.

[000868] Fusosomes produced by any one of the methods described in previous Examples from mammalian bone marrow stromal cells (BMSCs) having partial or complete nuclear inactivation trigger IL-6 secretion via juxtacrine signaling in macrophages. Primary macrophages and BMSCs are co-cultured. Bone marrow-derived macrophages are seeded first into 6-well plates, and incubated for 24h, then primary mouse BMSC-derived fusosomes or BMSC cells (positive control parental cells) are placed on the macrophages in a DMEM medium with 10% FBS. The supernatant is collected at different time points (2, 4, 6, 24 hours) and analyzed for IL-6 secretion by ELISA assay. (Chang J. et al., 2015).

[000869] In some embodiments, a level of juxtacrine signaling induced by BMSC fusosomes is measured by an increase in macrophage-secreted IL-6 levels in the media. In some embodiments, a level of juxtacrine signaling will be at least 1%, 2%, 3%, 4%, 5%, 10%, 20%,

30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the levels induced by the positive control bone marrow stromal cells (BMSCs).

Example 72: Measuring paracrine-signaling levels

[000870] This Example describes quantification of paracrine signaling in fusosomes.

[000871] Cells can communicate with other cells in the local microenvironment via paracrine signaling. In some embodiments, fusosomes will be capable of paracrine signaling, e.g., to communicate with cells in their local environment. In asome n embodiments, ability of fusosomes to trigger Ca^{2+} signaling in endothelial cells via paracrine-derived secretion with the following protocol will measure Ca^{2+} signaling via the calcium indicator, fluo-4 AM.

[000872] To prepare the experimental plate, murine pulmonary microvascular endothelial cells (MPMVECs) are plated on a 0.2% gelatin coated 25mm glass bottom confocal dish (80% confluence). MPMVECs are incubated at room temperature for 30 min in ECM containing 2% BSA and 0.003% pluronic acid with 5 μM fluo-4 AM (Invitrogen) final concentration to allow loading of fluo-4 AM. After loading, MPMVECs are washed with experimental imaging solution (ECM containing 0.25% BSA) containing sulfinpyrazone to minimize dye loss. After loading fluo-4, 500μl of pre-warmed experimental imaging solution is added to the plate, and the plate is imaged by a Zeiss confocal imaging system.

[000873] In a separate tube, freshly isolated murine macrophages are either treated with 1μg/mL LPS in culture media (DMEM+10% FBS) or not treated with LPS (negative control). After stimulation, fusosomes are generated from macrophages by any one of the methods described in previous Examples.

[000874] Fusosomes or parental macrophages (positive control) are then labeled with cell tracker red, CMTPX (Invitrogen), in ECM containing 2%BSA and 0.003% pluronic acid. Fusosomes and macrophages are then washed and resuspended in experimental imaging solution. Labeled fusosomes and macrophages are added onto the fluo-4 AM loaded MPMVECs in the confocal plate.

[000875] Green and red fluorescence signal is recorded every 3s for 10-20 min using Zeiss confocal imaging system with argon ion laser source with excitation at 488 and 561 nm for fluo-4

AM and cell tracker red fluorescence respectively. Fluo-4 fluorescence intensity changes are analyzed using imaging software (Mallilankaraman, K. et al., *J Vis Exp.* (58): 3511, 2011). The level of Fluo-4 intensity measured in negative control fusosome and cell groups is subtracted from LPS-stimulated fusosome and cell groups.

[000876] In some embodiments, fusosomes, e.g., activated fusosomes, will induce an increase in Fluo-4 fluorescence intensity that is at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the positive control cell groups.

Example 73: Measuring ability to polymerize actin for mobility

[000877] This Example describes quantification of cytoskeletal components, such as actin, in fusosomes. In some embodiments, fusosomes comprise cytoskeletal components such as actin, and are capable of actin polymerization.

[000878] Cells use actin, which is a cytoskeletal component, for motility and other cytoplasmic processes. The cytoskeleton is essential to creating motility driven forces and coordinating the process of movement

[000879] C2C12 cells were enucleated as described herein. Fusosomes obtained from the 12.5% and 15% Ficoll layers were pooled and labeled 'Light', while fusosomes from the 16 – 17% layers were pooled and labeled 'Medium'. Fusosomes or cells (parental C2C12 cells, positive control) were resuspended in DMEM + Glutamax + 10% Fetal Bovine Serum (FBS), plated in 24-well ultra-low attachment plates (#3473, Corning Inc, Corning, NY) and incubated at 37 °C + 5% CO_2. Samples were taken periodically (5.25 hr, 8.75 hr, 26.5 hr) and stained with 165 μM rhodamine phalloidin (negative control was not stained) and measured on a flow cytometer (#A24858, Thermo Fisher, Waltham, MA) with a FC laser YL1 (561 nm with 585/16 filter) to measure F-actin cytoskeleton content. The fluorescence intensity of rhodamine phalloidin in fusosomes was measured along with unstained fusosomes and stained parental C2C12 cells.

[000880] Fusosome fluorescence intensity was greater (Figure 4) than the negative control at all timepoints, and fusosomes were capable of polymerizing actin at a similar rate to the parental C2C12 cells.

[000881] Additional cytoskeletal components, such as those listed in the table below, are

measured via a commercially available ELISA systems (Cell Signaling Technology and MyBioSource), according to manufacturer's instructions.

Table 21: Cytoskeletal components

Cytoskeletal protein measured	Commercial Kit Type	Kit ID
Actin	Path Scan Total B-Actin Sandwich ELISA Kit	Cell Signaling, 7880
Arp2/3	Human Actin Related protein 2/3 complex subunit(APRC2) ELISA KIT	MyBioSource, MBS7224740
Formin	**Formin Binding Protein 1 (FNBP1), ELISA Kit**	MyBioSource, MBS9308864
Coronin	Human Coronin 1A ELISA Kit	MyBioSource, MBS073640
Dystrophin	Human dystrophin ELISA Kit	MyBioSource MBS722223
Keratin	Human Keratin 5 ELISA Kit	MyBioSource, MBS081200
Myosin	Human Myosin IG (MYO1G) ELISA Kit	MyBioSource, MBS9312965
Tubulin	Human Tubulin Beta 3 ELISA Kit	MyBioSource, MBS097321

[000882] Then 100 µL of appropriately-diluted lysate is added to the appropriate well from the microwell strips. The microwells are sealed with tape and incubated for 2 hrs at 37°C. After incubation, the sealing tape is removed and the contents are discarded. Each microwell is washed four times with 200 µL of 1X Wash Buffer. After each individual wash, plates are struck onto an absorbent cloth so that the residual wash solution is removed from each well. However, wells are not completely dry at any time during the experiment.

[000883] Next, 100 µL of the reconstituted Detection Antibody (green) is added each

individual well, except for negative control wells. Then wells are sealed and incubated for 1 hour at 37°C. The washing procedure is repeated after incubation is complete. 100 µL of reconstituted HRP-Linked secondary antibody (red) is added to each of the wells. The wells are sealed with tape and incubated for 30 minutes at 37°C. The sealing tape is then removed and the washing procedure is repeated. 100 µL of TMB Substrate is then added to each well. The wells are sealed with tape, then incubated for 10 minutes at 37°C. Once this final incubation is complete, 100 µL of STOP solution is added to each of the wells and the plate is shaken gently for several seconds.

[000884] Spectrophotometric analysis of the assay is conducted within 30 minutes of adding the STOP solution. The underside of the wells is wiped with lint-free tissue and then absorbance is read at 450nm. In some embodiments, fusosome samples that have been stained with the detection antibody will absorb more light at 450 nm that negative control fusosome samples, and absorb less light than cell samples that have been stained with the detection antibody.

Example 74: Measuring average membrane potential

[000885] This Example describes quantification of the mitochondrial membrane potential of fusosomes. In some embodiments, fusosomes comprising a mitochondrial membrane will maintain mitochondrial membrane potential.

[000886] Mitochondrial metabolic activity can be measured by mitochondrial membrane potential. The membrane potential of the fusosome preparation is quantified using a commercially available dye, TMRE, for assessing mitochondrial membrane potential (TMRE: tetramethyl rhodamine, ethyl ester, perchlorate, Abcam, Cat# T669).

[000887] Fusosomes are generated by any one of the methods described in previous Examples. Fusosomes or parental cells are diluted in growth medium (phenol-red free DMEM with 10% fetal bovine serum) in 6 aliquots (untreated and FCCP-treated triplicates). One aliquot of the samples is incubated with FCCP, an uncoupler that eliminates mitochondrial membrane potential and prevents TMRE staining. For FCCP-treated samples, 2µM FCCP is added to the samples and incubated for 5 minutes prior to analysis. Fusosomes and parental cells are then stained with 30nM TMRE. For each sample, an unstained (no TMRE) sample is also prepared in parallel. Samples are incubated at 37°C for 30 minutes. The samples are then analyzed on a flow cytometer with 488nm argon laser, and excitation and emission is collected at 530+/-30nm.

[000888] Membrane potential values (in millivolts, mV) are calculated based on the intensity of TMRE. All events are captured in the forward and side scatter channels (alternatively, a gate can be applied to exclude small debris). The fluorescence intensity (FI) value for both the untreated and FCCP-treated samples are normalized by subtracting the geometric mean of the fluorescence intensity of the unstained sample from the geometric mean of the untreated and FCCP-treated sample. The membrane potential state for each preparation is calculated using the normalized fluorescent intensity values with a modified Nernst equation (see below) that can be used to determine mitochondrial membrane potential of the fusosomes or cells based on TMRE fluorescence (as TMRE accumulates in mitochondria in a Nernstian fashion).

[000889] Fusosome or cell membrane potential is calculated with the following formula: (mV) = -61.5 * log(FIuntreated-normalized/FIFCCP-treated-normalized). In some embodiments, using this assay or an equivalent on fusosome preparations from C2C12 mouse myoblast cells, the membrane potential state of the fusosome preparation will be within about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the parental cells. In some embodiments, the range of membrane potential is about -20 to -150mV.

Example 75: Measuring persistence half-life in a subject

[000890] This Example describes the measurement of fusosome half-life.

[000891] Fusosomes are derived from cells that express gaussia-luciferase produced by any one of the methods described in previous Examples, and pure, 1:2, 1:5, and 1:10 dilutions in buffered solution are made. A buffered solution lacking fusosomes is used as a negative control.

[000892] Each dose is administered to three eight week old male C57BL/6J mice (Jackson Laboratories) intravenously. Blood is collected from the retro-orbital vein at 1, 2, 3, 4, 5, 6, 12, 24, 48, and 72 hours after intravenous administration of the fusosomes. The animals are sacrificed at the end of the experiment by CO_2 inhalation.

[000893] Blood is centrifuged for 20 min at room temperature. The serum samples are immediately frozen at −80°C until bioanalysis. Then, each blood sample is used to carry out a Gaussia-luciferase activity assay after mixing the samples with Gaussia-luciferase substrate (Nanolight, Pinetop, AZ). Briefly, colenterazine, a luciferin or light-emitting molecule, is mixed

with flash assay buffer and the mixture is pipetted into wells containing blood samples in a 96 well plate. Negative control wells that lack blood contain assay buffer to determine background Gaussia luciferase signal.

[000894] In addition, a standard curve of positive-control purified Gaussia luciferase (Athena Enzyme Systems, catalog #0308) is prepared in order to convert the luminescence signal to molecules of Gaussia luciferase secretion per hour. The plate is assayed for luminescence, using 500 msec integration. Background Gaussia luciferase signal is subtracted from all samples and then a linear best-fit curve is calculated for the Gaussia luciferase standard curve. If sample readings do not fit within the standard curve, they are diluted appropriately and re-assayed. The luciferase signal from samples taken at 1, 2, 3, 4, 5, 6, 12, 24, 48, and 72 hours is interpolated to the standard curve. The elimination rate constant k_e (h^{-1}) is calculated using the following equation of a one-compartment model: $C(t) = C_0 \times e^{-kext}$, in which $C(t)$ (ng/mL) is the concentration of fusosomes at time t (h) and C_0 the concentration of fusosomes at time = 0 (ng/mL). The elimination half-life $t_{1/2,e}$ (h) is calculated as $\ln(2)/k_e$.

[000895] In some embodiments, fusosomes will have a half-life of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the negative control cells.

Example 76: Measuring retention of fusosomes in circulation

[000896] This example describes quantification of fusosome delivery into the circulation and retention at organs. In some embodiments, fusosomes are delivered into the circulation, and are not captured and retained in organ sites.

[000897] In some embodiments, fusosomes delivered into the peripheral circulation evade capture and retention by the reticulo-endothelial system (RES) in order to reach target sites with high efficiency. The RES comprises a system of cells, primarily macrophages, which reside in solid organs such as the spleen, lymph nodes and the liver. These cells are usually tasked with the removal of "old" cells, such as red blood cells.

[000898] Fusosomes are derived from cells expressing CRE recombinase (agent), or cells not expressing CRE (negative control). These fusosomes are prepared for in vivo injection as in

Example 62.

[000899] The recipient mice harbor a loxp-luciferase genomic DNA locus that is modified by CRE protein made from mRNA delivered by the fusosomes to unblock the expression of luciferase (JAX#005125). Luciferase can be detected by bioluminescent imaging in a living animal. The positive control for this example are offspring of recipient mice mated to a mouse strain that expresses the same protein exclusively in macrophage and monocyte cells from its own genome (Cx3cr1-CRE JAX#025524). Offspring from this mating harbor one of each allele (loxp-luciferase, Cx3cr1-CRE).

[000900] Fusosomes are injected into the peripheral circulation via tail vein injection (IV, Example #48) into mice that harbor a genetic locus that when acted on by the CRE protein results in the expression of luciferase. The non-specific capture mechanism of the RES is phagocytic in nature releasing a proportion of the CRE protein from the fusosome into the macrophage resulting in genomic recombination. IVIS measurements (as described in Example 62) identify where non-fusogen controls accumulate and fuse. Accumulation in the spleen, lymph nodes and liver will be indicative of non-specific RES-mediated capture of the fusosome. IVIS is carried out at 24, 48 and 96 hours post-fusosome injection.

[000901] Mice are euthanized and spleen, liver and major lymphatic chain in the gut are harvested.

[000902] Genomic DNA is isolated from these organs and subjected to quantitative polymerase chain reaction against the recombined genomic DNA remnant. An alternative genomic locus (not targeted by CRE) is also quantified to provide a measure of the number of cells in the sample.

[000903] In embodiments, low bioluminescent signals will be observed for both the agent and negative control throughout the animal and specifically at the liver and splenic sites. In embodiments, the positive control will show increased signal in the liver (over negative control and agent) and high signals in the spleen and a distribution consistent with lymph nodes.

[000904] In some embodiments, genomic PCR quantification of these tissues will indicate a high proportion of the recombination signals over the alternative locus in the positive control in

all tissues examined, while for or agent and negative controls, the level of recombination will be negligible in all tissues.

[000905] In some embodiments, the result of this Example will indicate that the non-fusogen controls are not retained by the RES and will be able to achieve broad distribution and exhibit high bioavailability.

Example 77: Fusosome longevity with immunosuppression

[000906] This Example describes quantification of the immunogenicity of a fusosome composition when it is co-administered with an immunosuppressive drug.

[000907] Therapies that stimulate an immune response can sometimes reduce the therapeutic efficacy or cause toxicity to the recipient. In some embodiments, fusosomes will be substantially non-immunogenic.

[000908] A purified composition of fusosomes as produced by any one of the methods described in previous Examples is co-administered with an immunosuppressive drug, and immunogenic properties are assayed by the longevity of the fusosome in vivo. A sufficient number of fusosomes, labeled with luciferase, are injected locally into the gastrocnemius muscle of a normal mouse with tacrolimus (TAC, 4mg/kg/day; Sigma Aldrich), or vehicle (negative control), or without any additional agent (positive control). The mice are then subjected to in vivo imaging at 1, 2, 3, 4, 5, 6, 12, 24, 48, and 72 hours post injection.

[000909] Briefly, mice are anesthetized with isoflurane and D-luciferin is administered intraperitoneally at a dose of 375 mg per kilogram of body weight. At the time of imaging, animals are placed in a light-tight chamber, and photons emitted from luciferase expressing fusosomes transplanted into the animals are collected with integration times of 5 sec to 5 min, depending on the intensity of the bioluminescence emission. The same mouse is scanned repetitively at the various timepoints set forth above. BLI signal is quantified in units of photons per second (total flux) and presented as log [photons per second]. The data is analyzed by comparing the intensity and fusosome injection with and without TAC.

[000910] In embodiments, the assay will show an increase in fusosome longevity in the TAC co-administered group relative to the fusosome alone and vehicle groups at the final timepoint. In

addition to the increase in fusosome longevity, in some embodiments, an increase in BLI signal from the fusosome plus TAC arm versus the fusosome plus vehicle or fusosomes alone at each of the time points will be observed.

Example 78: Measuring pre-existing IgG and IgM antibodies reactive against fusosomes

[000911] This Example describes quantification of pre-existing anti-fusosome antibody titers measured using flow cytometry.

[000912] A measure of immunogenicity for fusosomes is antibody responses. Antibodies that recognize fusosomes can bind in manner that can limit fusosome activity or longevity. In some embodiments, some recipients of a fusosome described herein will have pre-existing antibodies which bind to and recognize fusosomes.

[000913] In this Example, anti-fusosome antibody titers are tested using fusosomes produced using a xenogeneic source cell by any one of the methods described in a previous Example. In this Example, a fusosome naïve mouse is assessed for the presence of anti-fusosome antibodies. Notably, the methods described herein may be equally applicable to humans, rats, monkeys with optimization to the protocol.

[000914] The negative control is mouse serum which has been depleted of IgM and IgG, and the positive control is serum derived from a mouse that has received multiple injections of fusosomes generated from a xenogeneic source cell.

[000915] To assess the presence of pre-existing antibodies which bind to fusosomes, sera from fusosome-naïve mice is first decomplemented by heating to 56°C for 30 min and subsequently diluted by 33% in PBS containing 3% FCS and 0.1% NaN3. Equal amounts of sera and fusosomes ($1x10^2$ - $1x10^8$ fusosomes per mL) suspensions are incubated for 30 min at 4°C and washed with PBS through a calf-serum cushion.

[000916] IgM xenoreactive antibodies are stained by incubation of the cells with PE-conjugated goat antibodies specific for the Fc portion of mouse IgM (BD Bioscience) at 4°C for 45 min. Notably, anti-mouse IgG1 or IgG2 secondary antibodies may also be used. Cells from all groups are washed twice with PBS containing 2% FCS and then analyzed on a FACS system (BD

Biosciences). Fluorescence data are collected by use of logarithmic amplification and expressed as mean fluorescent intensity. In some embodiments, the negative control serum will show negligible fluorescence comparable to the no serum or secondary alone controls. In an embodiment, the positive control will show more fluorescence than the negative control, and more than the no serum or secondary alone controls. In an embodiment, in cases where immunogenicity occurs, serum from fusosome-naïve mice will show more fluorescence than the negative control. In an embodiment, in cases where immunogenicity does not occur, serum from fusosome-naïve mice will show similar fluorescence compared to the negative control.

Example 79: Measuring IgG and IgM antibody responses after multiple administrations of fusosomes

[000917] This Example describes quantification of the humoral response of a modified fusosome following multiple administrations of the modified fusosome. In some embodiments, a modified fusosome, e.g., modified by a method described herein, will have a reduced (e.g., reduced compared to administration of an unmodified fusosome) humoral response following multiple (e.g., more than one, e.g., 2 or more), administrations of the modified fusosome.

[000918] A measure of immunogenicity for fusosomes is the antibody responses. In some embodiments, repeated injections of a fusosome can lead to the development of anti-fusosome antibodies, e.g., antibodies that recognize fusosomes. In some embodiments, antibodies that recognize fusosomes can bind in a manner that can limit fusosome activity or longevity.

[000919] In this Example, anti-fusosome antibody titers are examined after one or more administrations of fusosomes. Fusosomes are produced by any one of the previous Examples. Fusosomes are generated from: unmodified mesenchymal stem cells (hereafter MSCs), mesenchymal stem cells modified with a lentiviral-mediated expression of HLA-G (hereafter MSC-HLA-G), and mesenchymal stem cells modified with a lentiviral-mediated expression of an empty vector (hereafter MSC-empty vector). Serum is drawn from the different cohorts: mice injected systemically and/or locally with 1, 2, 3, 5, 10 injections of vehicle (Fusosome naïve group), MSC fusosomes, MSC-HLA-G fusosomes, or MSC-empty vectors fusosomes.

[000920] To assess the presence and abundance of anti-fusosomes antibodies, sera from the mice is first decomplemented by heating to 56°C for 30 min and subsequently diluted by 33% in

PBS with 3% FCS and 0.1% NaN3. Equal amounts of sera and fusosomes ($1x10^2$ - $1x10^8$ fusosomes per mL) are incubated for 30 min at 4°C and washed with PBS through a calf-serum cushion.

[000921] Fusosome reactive IgM antibodies are stained by incubation of the cells with PE-conjugated goat antibodies specific for the Fc portion of mouse IgM (BD Bioscience) at 4°C for 45 min. Notably, anti-mouse IgG1 or IgG2 secondary antibodies may also be used. Cells from all groups are washed twice with PBS containing 2% FCS and then analyzed on a FACS system (BD Biosciences). Fluorescence data are collected by use of logarithmic amplification and expressed as mean fluorescent intensity.

[000922] In some embodiments, MSC-HLA-G fusosomes will have decreased anti-fusosome IgM (or IgG1/2) antibody titers (as measured by fluorescence intensity on FACS) after injections, as compared to MSC fusosomes or MSC-empty vector fusosomes.

Example 80: Modification of fusosome source cells to express tolerogenic protein to reduce immunogenicity

[000923] This Example describes quantification of immunogenicity in fusosomes derived from a modified cell source. In some embodiments, fusosomes derived from a modified cell source have reduced immunogenicity in comparison to the fusosomes derived from an unmodified cell source.

[000924] Therapies that stimulate an immune response can sometimes reduce the therapeutic efficacy or cause toxicity to the recipient. In some embodiments, substantially non-immunogenic fusosomes are administered to a subject. In some embodiments, immunogenicity of the cell source can be assayed as a proxy for fusosome immunogenicity.

[000925] iPS cells modified using lentiviral mediated expression of HLA-G or expressing an empty vector (Negative control) are assayed for immunogenic properties as follows. A sufficient number of iPS cells, as a potential fusosome cell source, are injected into C57/B6 mice, subcutaneously in the hind flank and are given an appropriate amount of time to allow for teratomas to form.

[000926] Once teratomas are formed, tissues are harvested. Tissues prepared for fluorescent

staining are frozen in OCT, and those prepared for immunohistochemistry and H&E staining are fixed in 10% buffered formalin and embedded in paraffin. The tissue sections are stained with antibodies, polyclonal rabbit anti-human CD3 anti-body (DAKO), mouse anti–human CD4 mAb (RPA-T4, BD PharMingen), mouse anti-human CD8 mAb (RPA-T8, BD PharMingen), in accordance with general immunohistochemistry protocols. These are detected by using an appropriate detection reagent, namely an anti-mouse secondary HRP (Thermofisher), or anti-rabbit secondary HRP (Thermofisher).

[000927] Detection is achieved using peroxidase-based visualization systems (Agilent). The data is analyzed by taking the average number of infiltrating CD4+ T-cells, CD8+ T-cells, CD3+ NK-cells present in 25, 50 or 100 tissue sections examined in a 20x field using a light microscope. In embodiments, iPSCs which are not modified or iPSCs expressing an empty vector will have a higher number of infiltrating CD4+ T-cells, CD8+ T-cells, CD3+ NK-cells present in the fields examined as compared to iPSCs that express HLA-G.

[000928] In some embodiments, a fusosome's immunogenic properties will be substantially equivalent to that of the source cell. In some embodiments, fusosomes derived from iPS cells modified with HLA-G will have reduced immune cell infiltration versus their unmodified counterparts.

Example 81: Modification of fusosome source cells to knockdown immunogenic protein to reduce immunogenicity

[000929] This Example describes quantification of the generation of a fusosome composition derived from a cell source, which has been modified to reduce expression of a molecule which is immunogenic. In some embodiments, a fusosome can be derived from a cell source, which has been modified to reduce expression of a molecule which is immunogenic.

[000930] Therapies that stimulate an immune response can reduce the therapeutic efficacy or cause toxicity to the recipient. Thus, immunogenicity is an important property for a safe and effective therapeutic fusosomes. Expression of certain immune activating agents can create an immune response. MHC class I represents one example of an immune activating agent.

[000931] In this Example, fusosomes are generated by any one of the methods described in

previous Examples. Fusosomes are generated from: unmodified mesenchymal stem cells (hereafter MSCs, positive control), mesenchymal stem cells modified with a lentiviral-mediated expression of an shRNA targeting MHC class I (hereafter MSC-shMHC class I), and mesenchymal stem cells modified with a lentiviral-mediated expression of a non-targeted scrambled shRNA (hereafter MSC-scrambled, negative control).

[000932] Fusosomes are assayed for expression of MHC class I using flow cytometry. An appropriate number of fusosomes are washed and resuspended in PBS, held on ice for 30 minutes with 1: 10-1: 4000 dilution of fluorescently conjugated monoclonal antibodies against MHC class I (Harlan Sera-Lab, Belton, UK). Fusosomes are washed three times in PBS and resuspended in PBS. Nonspecific fluorescence is determined, using equal aliquots of fusosomes preparation incubated with and appropriate fluorescently conjugated isotype control antibody at equivalent dilutions. Fusosomes are assayed in a flow cytometer (FACSort, Becton-Dickinson) and the data is analyzed with flow analysis software (Becton-Dickinson).

[000933] The mean fluorescence data of the fusosomes derived from MSCs, MSCs-shMHC class I, MSC-scrambled, is compared. In some embodiments, fusosomes derived from MSCs-shMHC class I will have lower expression of MHC class I compared to MSCs and MSC-scrambled.

Example 82: Modification of fusosome source cells to evade macrophage phagocytosis

[000934] This Example describes quantification of the evasion of phagocytosis by modified fusosomes. In some embodiments, modified fusosomes will evade phagocytosis by macrophages.

[000935] Cells engage in phagocytosis, engulfing particles, enabling the sequestration and destruction of foreign invaders, like bacteria or dead cells. In some embodiments, phagocytosis of fusosomes by macrophages would reduce their activity.

[000936] Fusosomes are generated by any one of the methods described in previous Examples. Fusosomes are created from: CSFE-labelled mammalian cells which lack CD47 (hereafter NMC, positive control), CSFE-labelled cells that are engineered to express CD47 using lentiviral mediated expression of a CD47 cDNA (hereafter NMC-CD47), and CSFE-labelled cells engineered using lentiviral mediated expression of an empty vector control (hereafter NMC-empty

vector, negative control).

[000937] Reduction of macrophage mediate immune clearance is determined with a phagocytosis assay according to the following protocol. Macrophages are plated immediately after harvest in confocal glass bottom dishes. Macrophages are incubated in DMEM+10%FBS+1%P/S for 1h to attach. An appropriate number of fusosomes derived from NMC, NMC-CD47, NMC-empty vector are added to the macrophages as indicated in the protocol, and are incubated for 2h, tools.thermofisher.com/content/sfs/manuals/mp06694.pdf.

[000938] After 2h, the dish is gently washed and intracellular fluorescence is examined. Intracellular fluorescence emitted by engulfed particles is imaged by confocal microscopy at 488 excitation. The number of phagocytotic positive macrophage is quantified using imaging software. The data is expressed as the phagocytic index = (total number of engulfed cells/total number of counted macrophages) × (number of macrophages containing engulfed cells/total number of counted macrophages) × 100.

[000939] In some embodiments, a phagocytic index will be reduced when macrophages are incubated with fusosomes derived from NMC-CD47, versus those derived from NMC, or NMC-empty vector.

Example 83: Modification of fusosome source cells for decreased cytotoxicity mediated by PBMC cell lysis

[000940] This Example described the generation of fusosomes derived from cells modified to have decreased cytotoxicity due to cell lysis by PBMCs.

[000941] In some embodiments, cytotoxicity mediated cell lysis of source cells or fusosomes by PBMCs is a measure of immunogenicity for fusosomes, as lysis will reduce, e.g., inhibit or stop, the activity of a fusosome.

[000942] In this Example, fusosomes are generated by any one of the methods described in a previous Example. Fusosomes are created from: unmodified mesenchymal stem cells (hereafter MSCs, positive control), mesenchymal stem cells modified with a lentiviral-mediated expression of HLA-G (hereafter MSC-HLA-G), and mesenchymal stem cells modified with a lentiviral-mediated expression of an empty vector (hereafter MSC-empty vector, negative control).

[000943] PMBC mediated lysis of a fusosome is determined by europium release assays as described in Bouma, et al. Hum. Immunol. 35(2):85-92; 1992 & van Besouw et al. Transplantation 70(1):136-143; 2000. PBMCs (hereafter effector cells) are isolated from an appropriate donor, and stimulated with allogeneic gamma irradiated PMBCs and 200IU/mL IL-2 (proleukin, Chiron BV Amsterdam, The Netherlands) in a round bottom 96 well plate for 7 days at 37°C. The fusosomes are labeled with europium-diethylenetriaminepentaacetate (DTPA) (sigma, St. Louis, MO, USA).

[000944] At day 7 cytotoxicity-mediated lysis assays is performed by incubating ^{63}Eu-labelled fusosomes with effector cells in a 96-well plate for 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 24, 48 hours after plating at effector/target ratios ranging from 1000:1-1:1 and 1:1.25-1:1000. After incubation, the plates are centrifuged and a sample of the supernatant is transferred to 96-well plates with low background fluorescence (fluoroimmunoplates, Nunc, Roskilde, Denmark).

[000945] Subsequently, enhancement solution (PerkinElmer, Groningen, The Netherlands) is added to each well. The released europium is measured in a time-resolved fluorometer (Victor 1420 multilabel counter, LKB-Wallac, Finland). Fluorescence is expressed in counts per second (CPS). Maximum percent release of europium by a target fusosome is determined by incubating an appropriate number (1x 10^2 -1x 10^8) of fusosomes with 1% triton (sigma-aldrich) for an appropriate amount of time. Spontaneous release of europium by target fusosomes is measured by incubation of labeled target fusosomes without effector cells. Percentage leakage is then calculated as: (spontaneous release/maximum release) x100%. Finally, the percentage of cytotoxicity mediated lysis is calculated as %lysis= [(measured lysis-spontaneous lysis- spontaneous release)/(maximum release-spontaneous release)]x100%. The data is analyzed by looking at the percentage of lysis as a function of different effector target ratios.

[000946] In some embodiments, fusosomes generated from MSC-HLA-G cells will have a decreased percentage of lysis by target cells, at specific timepoints as compared to MSCs or MSC-scrambled generated fusosomes.

Example 84: Modification of fusosome source cells for decreased NK lysis activity

[000947] This Example describes the generation of a fusosome composition derived from a cell source, which has been modified to decrease cytotoxicity mediated cell lysis by NK cells. In some embodiments cytotoxicity mediated cell lysis of source cells or fusosomes by NK cells is a

measure of immunogenicity for fusosomes.

[000948] In this Example, fusosomes are generated by any one of the methods described in a previous Example. Fusosomes are created from: unmodified mesenchymal stem cells (hereafter MSCs, positive control), mesenchymal stem cells modified with a lentiviral-mediated expression of HLA-G (hereafter MSC-HLA-G), and mesenchymal stem cells modified with a lentiviral-mediated expression of an empty vector (hereafter MSC-empty vector, negative control).

[000949] NK cell mediated lysis of a fusosome is determined by europium release assays as described in Bouma, et al. Hum. Immunol. 35(2):85-92; 1992 & van Besouw et al. Transplantation 70(1):136-143; 2000. NK cells (hereafter effector cells) are isolated from an appropriate donor according to the methods in Crop et al. Cell transplantation (20):1547-1559; 2011, and stimulated with allogeneic gamma irradiated PMBCs and 200IU/mL IL-2 (proleukin, Chiron BV Amsterdam, The Netherlands) in a round bottom 96 well plate for 7 days at 37°C. The fusosomes are labeled with europium-diethylenetriaminepentaacetate (DTPA) (sigma, St. Louis, MO, USA).

[000950] At day 7 cytotoxicity-mediated lysis assays is performed by incubating ^{63}Eu-labelled fusosomes with effector cells in a 96-well plate for 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 24, 48 hours after plating at effector/target ratios ranging from 1000:1-1:1 and 1:1.25-1:1000. After incubation, the plates are centrifuged and a sample of the supernatant is transferred to 96-well plates with low background fluorescence (fluoroimmunoplates, Nunc, Roskilde, Denmark).

[000951] Subsequently, enhancement solution (PerkinElmer, Groningen, The Netherlands) is added to each well. The released europium is measured in a time-resolved fluorometer (Victor 1420 multilabel counter, LKB-Wallac, Finland). Fluorescence is expressed in counts per second (CPS). Maximum percent release of europium by a target fusosome is determined by incubating an appropriate number (1x 10^2 -1x 10^8) of fusosomes with 1% triton (Sigma-Aldrich) for an appropriate amount of time. Spontaneous release of europium by target fusosomes is measured by incubation of labeled target fusosomes without effector cells. Percentage leakage is then calculated as: (spontaneous release/maximum release) x100%. Finally, the percentage of cytotoxicity mediated lysis is calculated as %lysis= [(measured lysis-spontaneous lysis- spontaneous release)/(maximum release-spontaneous release)]x100%. The data is analyzed by looking at the percentage of lysis as a function of different effector target ratios.

[000952] In some embodiments, fusosomes generated from MSC-HLA-G cells will have a decreased percentage of lysis at appropriate timepoints as compared to MSCs or MSC-scrambled generated fusosomes.

Example 85: Modification of fusosome source cells for decreased CD8 Killer T cell lysis

[000953] This Example describes the generation of a fusosome composition derived from a cell source, which has been modified to decrease cytotoxicity mediated cell lysis by CD8+ T-cells. In some embodiments, cytotoxicity mediated cell lysis of source cells or fusosomes by CD8+ T-cells is a measure of immunogenicity for fusosomes.

[000954] In this Example, fusosomes are generated by any one of the methods described in a previous Example. Fusosomes are created from: unmodified mesenchymal stem cells (hereafter MSCs, positive control), mesenchymal stem cells modified with a lentiviral-mediated expression of HLA-G (hereafter MSC-HLA-G), and mesenchymal stem cells modified with a lentiviral-mediated expression of an empty vector (hereafter MSC-empty vector, negative control).

[000955] CD8+ T cell mediated lysis of a fusosome is determined by europium release assays as described in Bouma, et al. Hum. Immunol. 35(2):85-92; 1992 & van Besouw et al. Transplantation 70(1):136-143; 2000. CD8+ T-cells (hereafter effector cells) are isolated from an appropriate donor according to the methods in Crop et al. Cell transplantation (20):1547-1559; 2011, and stimulated with allogeneic gamma irradiated PMBCs and 200IU/mL IL-2 (proleukin, Chiron BV Amsterdam, The Netherlands) in a round bottom 96 well plate for 7 days at 37°C. The fusosomes are labeled with europium-diethylenetriaminepentaacetate (DTPA) (sigma, St. Louis, MO, USA).

[000956] At day 7 cytotoxicity-mediated lysis assays is performed by incubating ^{63}Eu-labelled fusosomes with effector cells in a 96-well plate for 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, 24, 48 hours after plating at effector/target ratios ranging from 1000:1-1:1 and 1:1.25-1:1000. After incubation, the plates are centrifuged and 20 µL of the supernatant is transferred to 96-well plates with low background fluorescence (fluoroimmunoplates, Nunc, Roskilde, Denmark).

[000957] Subsequently, enhancement solution (PerkinElmer, Groningen, The Netherlands)

is added to each well. The released europium is measured in a time-resolved fluorometer (Victor 1420 multilabel counter, LKB-Wallac, Finland). Fluorescence is expressed in counts per second (CPS). Maximum percent release of europium by a target fusosome is determined by incubating an appropriate number (1x 10^2 -1x 10^8) of fusosomes with 1% triton (sigma-aldrich) for an appropriate amount of time. Spontaneous release of europium by target fusosomes is measured by incubation of labeled target fusosomes without effector cells. Percentage leakage is then calculated as: (spontaneous release/maximum release) x100%. Finally, the percentage of cytotoxicity mediated lysis is calculated as %lysis= [(measured lysis-spontaneous lysis- spontaneous release)/(maximum release-spontaneous release)]x100%. The data is analyzed by looking at the percentage of lysis as a function of different effector target ratios.

[000958] In some embodiments, fusosomes generated from MSC-HLA-G cells will have a decreased percentage of lysis at appropriate timepoints as compared to MSCs or MSC-scrambled generated fusosomes.

Example 86: Modification of fusosome source cells for decreased T-cell activation

This Example describes the generation of modified fusosomes that will have reduced T cell activation and proliferation as assessed by a mixed lymphocyte reaction (MLR).

[000959] T-cell proliferation and activation are measures of immunogenicity for fusosomes. Stimulation of T cell proliferation in an MLR reaction by a fusosome composition, could suggest a stimulation of T cell proliferation in vivo.

[000960] In some embodiments, fusosomes generated from modified source cells have reduced T cell activation and proliferation as assessed by a mixed lymphocyte reaction (MLR). In some embodiments, fusosomes generated from modified source cells do not generate an immune response in vivo, thus maintaining the efficacy of the fusosome composition.

[000961] In this Example, fusosomes are generated by any one of the methods described in a previous Example. Fusosomes are generated from: unmodified mesenchymal stem cells (hereafter MSCs, positive control), mesenchymal stem cells modified with a lentiviral-mediated expression of IL-10 (hereafter MSC-IL-10), and mesenchymal stem cells modified with a lentiviral-mediated expression of an empty vector (hereafter MSC-empty vector, negative control).

[000962] BALB/c and C57BL/6 splenocytes are used as either stimulator or responder cells. Notably, the source of these cells can be exchanged with commonly used human-derived stimulator/responder cells. Additionally, any mammalian purified allogeneic CD4+ T cell population, CD8+ T-cell population, or CD4-/CD8- may be used as responder population.

[000963] Mouse Splenocytes are isolated by mechanical dissociation using fully frosted slides followed by red blood cell lysis with lysing buffer (Sigma-Aldrich, St-Louis, MO). Prior to the experiment, stimulator cells are irradiated with 20 Gy of γray to prevent them from reacting against responder cells. A co-culture is then made by adding equal numbers of stimulator and responder cells (or alternative concentrations while maintaining a 1:1 ratio) to a round bottom 96-well plate in complete DMEM-10 media. An appropriate number of fusosomes (at several concentrations from a range of $1x10^1$-$1x10^8$) are added to the co-culture at different time intervals, t = 0, 6, 12, 24, 36, 48h.

[000964] Proliferation is assessed by adding 1μCi of [^{3}H]- thymidine (Amersham, Buckinghamshire, UK) to allow for incorporation. [^{3}H]- thymidine is added to the MLR at t= 2, 6, 12, 24, 36, 48, 72h, and the cells are harvested onto glass fiber filters using a 96 well cell harvester (Inoteck, Bertold, Japan) after 2, 6, 12, 18, 24, 36 and 48h of extended culture. All of the T-cell proliferation experiments are done in triplicate. [^{3}H]- thymidine incorporation is measured using a microbeta lLuminescence counter (Perkin Elmer, Wellesley, MA). The results can be represented as counts per minute (cpm).

[000965] In some embodiments, MSC-IL10 fusosomes will show a decrease in T-cell proliferation as compared to the MSC-Empty vector or the MSC unmodified fusosome controls.

Example 87: Measuring targeting potential in a subject

[000966] This Example assesses the ability of a fusosome to target a specific body site. In some embodiments, a fusosome can target a specific body site. Targeting is a way to restrict activity of a therapeutic to one or more relevant therapeutic sites.

[000967] Eight week old C57BL/6J mice (Jackson Laboratories) are intravenously injected with fusosomes or cells that express firefly luciferase. Fusosomes are produced from cells that stably express firefly luciferase or cells that do not express luciferase (negative control) by any

one of the methods described in previous Examples. Groups of mice are euthanized at one, two, three, four, five, six, eight, twelve, and twenty-four hours after fusosome or cell injection.

[000968] Five minutes before euthanization, mice receive an IP injection of bioluminescent substrate (Perkin Elmer) at a dose of 150mg/kg in order to visualize luciferase. The bioluminescent imaging system is calibrated to compensate for all device settings. Mice are then euthanized and liver, lungs, heart, spleen, pancreas, GI, and kidney are collected. The imaging system (Perkin Elmer) is used to obtain images of bioluminescence of these ex vivo organs. The bioluminescent signal is measured using Radiance Photons, with Total Flux used as a measured value. The region of interest (ROI) is generated by surrounding the ex vivo organ in order to give a value in photons/second. The ratio of photons/second between target organs (e.g. liver) and non-target organs (e.g. the sum of photons/second from lungs, heart, spleen, pancreas, GI, and kidney) is calculated as a measure of targeting to the liver.

[000969] In some embodiments, in both fusosomes and cells, the ratio of photons/second between liver and the other organs will be greater than 1, which would indicate that fusosomes target the liver. In some embodiments, negative control animals will display much lower photons/second in all organs.

Example 88: Measuring delivery of an exogenous agent in a subject

[000970] This Example describes quantification of delivery of fusosomes comprising an exogenous agent in a subject. Fusosomes are prepared from cells expressing Gaussia-luciferase or from cells not expressing luciferase (negative control) by any one of the methods described in previous Examples.

[000971] Positive control cells or fusosomes are intravenously injected into mice. Fusosomes or cells are delivered within 5–8 seconds using a 26-gauge insulin syringe-needle. In vivo bioluminescent imaging is performed on mice 1, 2, or 3 days after injection using an in vivo imaging system (Xenogen Corporation, Alameda, CA).

[000972] Immediately before use, coelenterazine, a luciferin or light-emitting molecule, (5 mg/mL) is prepared in acidified methanol and injected immediately into the tail vein of the mice. Mice are under continuous anesthesia on a heated stage using the XGI-8 Gas Anesthesia System.

[000973] Bioluminescence imaging is obtained by acquiring photon counts over 5 min immediately after intravenous tail-vein injection of coelenterazine (4 μg/g body weight). Acquired data are analyzed using software (Xenogen) with the overlay on light-view image. Regions of interest (ROI) are created using an automatic signal intensity contour tool and normalized with background subtraction of the same animal. A sequential data acquisition using three filters at the wavelengths of 580, 600 and 620 nm with exposure time 3–10 min is conducted to localize bioluminescent light sources inside a mouse.

[000974] Furthermore, at each timepoint, urine samples are collected by abdominal palpation.

[000975] Blood samples (50 μL) are obtained from the tail vein of each mouse into heparinized or EDTA tubes. For plasma isolation, blood samples are centrifuged for 25 min at 1.3×g at 4°C.

[000976] Then, 5 μL of blood, plasma or urine sample are used to carry out a Gaussia-luciferase activity assay after mixing the samples with 50 μM Gaussia-luciferase substrate (Nanolight, Pinetop, AZ).

[000977] In some embodiments, a negative control sample will be negative for luciferase, and a positive control sample will be from animals administered cells. In some embodiments, samples from animals administered fusosomes expressing Gaussia-luciferase will be positive for luciferase in each sample.

[000978] See, for example, El-Amouri SS et al., *Molecular biotechnology* 53(1): 63-73, 2013.

Example 89: Active transport across a lipid bilayer of a fusosome

[000979] This example describes quantification of the level of 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose), a fluorescent glucose analog that can be used to monitor glucose uptake in live cells and thus active transport across the lipid bilayer. In some embodiments, this assay or an equivalent can be used to measure the level of glucose uptake and active transport across the lipid bilayer of the fusosome.

[000980] A fusosome composition as produced by any one of the methods described in

previous Examples. A sufficient number of fusosomes are then incubated in DMEM containing no glucose, 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin for 2hr at 37°C and 5% CO_2. After the 2hr glucose starvation period, the medium is changed such that it includes DMEM with no glucose, 20% Fetal Bovine Serum, 1x Penicillin/Streptomycin, and 20 μM 2-NBDG (ThermoFisher) and incubated for 2hr at 37°C and 5% CO_2. Negative control fusosomes are treated the same, except an equal amount of DMSO, the vehicle for 2-NBDG is added in place of 2-NBDG.

[000981] The fusosomes are then washed thrice with 1xPBS and re-suspended in an appropriate buffer, and transferred to a 96 well imaging plate. 2-NBDG fluorescence is then measured in a fluorimeter using a GFP light cube (469/35 excitation filter and a 525/39 emission filter) to quantify the amount of 2-NBDG that has transported across the fusosome membrane and accumulated in the fusosome in the 1hr loading period.

[000982] In some embodiments, 2-NBDG fluorescence will be higher in the fusosomes with 2-NBDG treatment as compared to the negative (DMSO) control. Fluorescence measure with a 525/39 emission filter will be relatively to the number of 2-NBDG molecules present.

Example 90: Delivery of fusosomes via non-endocytic pathway

[000983] This example describes quantification of fusosome delivery of Cre to a recipient cell via a non-endocytic pathway.

[000984] In some embodiments, fusosomes will deliver agents via a fusosome-mediated, non-endocytic pathway. Without wishing to be bound by theory, delivery of an agent, e.g., Cre, which is carried within the lumen of the fusosomes, directly to the cytosol of the recipient cells without any requirement for endocytosis-mediated uptake of the fusosomes, will occur through a fusosome-mediated, non-endocytic pathway delivery.

[000985] In this example, the fusosome comprises a HEK293T cell expressing the Sendai virus H and F protein on its plasma membrane (Tanaka et al., 2015, Gene Therapy, 22(October 2014), 1–8. https://doi.org/10.1038/gt.2014.123). In addition, the fusosome expresses mTagBFP2 fluorescent protein and Cre recombinase. The target cell is a RPMI8226 cell which stably-expresses "LoxP-GFP-stop-LoxP-RFP" cassette under a CMV promoter, which upon

recombination by Cre switches from GFP to RFP expression, indicating fusion and Cre, as a marker, delivery.

[000986] Fusosomes produced by the herein described methods are assayed for delivery of Cre via a non-endocytic pathway as follows. The recipient cells are plated into a black, clear-bottom 96-well plate. Next, 24 hours after plating the recipient cells, the fusosomes expressing Cre recombinase protein and possessing the particular fusogen protein are applied to the recipient cells in DMEM media. To determine the level of Cre delivery via a non-endocytic pathway, a parallel group of recipient cells receiving fusosomes is treated with an inhibitor of endosomal acidification, chloroquine (30 μg/mL). The dose of fusosomes is correlated to the number of recipient cells plated in the well. After applying the fusosomes, the cell plate is centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells. The cells are then incubated for 16 hours and agent delivery, Cre, is assessed via imaging.

[000987] The cells are imaged to positively identify RFP-positive cells versus GFP-positive cells in the field or well. In this example cell plates are imaged using an automated fluorescence microscope. The total cell population in a given well is determined by first staining the cells with Hoechst 33342 in DMEM media for 10 minutes. Hoechst 33342 stains cell nuclei by intercalating into DNA and therefore is used to identify individual cells. After staining, the Hoechst media is replaced with regular DMEM media.

[000988] The Hoechst is imaged using the 405 nm LED and DAPI filter cube. GFP is imaged using the 465 nm LED and GFP filter cube, while RFP is imaged using 523 nm LED and RFP filter cube. Images of the different cell groups are acquired by first establishing the LED intensity and integration times on a positive-control well; i.e., recipient cells treated with adenovirus coding for Cre recombinase instead of fusosomes.

[000989] Acquisition settings are set so that RFP and GFP intensities are at the maximum pixel intensity values but not saturated. The wells of interest are then imaged using the established settings.

[000990] Analysis of GFP and RFP-positive wells is performed with software provided with the fluorescence microscope or other software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, 1997–2007). The images are pre-processed using a rolling

ball background subtraction algorithm with a 60 μm width. The total cell mask is set on the Hoechst-positive cells. Cells with Hoechst intensity significantly above background intensities are used to set a threshold, and areas too small or large to be Hoechst-positive cells are excluded.

[000991] Within the total cell mask, GFP and RFP-positive cells are identified by again setting a threshold for cells significantly above background and extending the Hoechst (nuclei) masks for the entire cell area to include the entire GFP and RFP cellular fluorescence.

[000992] The number of RFP-positive cells identified in control wells containing recipient cells is used to subtract from the number of RFP-positive cells in the wells containing fusosomes (to subtract for non-specific Loxp recombination). The number of RFP-positive cells (recipient cells that received Cre) is then divided by the sum of GFP-positive cells (recipient cells that have not received Cre) and RFP-positive cells to quantify the fraction of fusosome Cre delivered to the recipient cell population. The level is normalized to the given dose of fusosomes applied to the recipient cells. To calculate the value of fusosome Cre delivered via a non-endocytic pathway, the level of fusosome Cre delivery in the presence of chloroquine (FusL+CQ) is determined as well as the level of fusosome Cre delivery in the absence of chloroquine (FusL-CQ). To determine the normalized value of fusosome Cre delivered via a non-endocytic pathway, the following equation is used: [(FusL-CQ)-(FusL+CQ)]/(FusL-CQ).

[000993] In some embodiments, an average level of fusosome Cre delivered via a non-endocytic pathway for a given fusosome will be in the range of 0.1-0.95, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than chloroquine treated recipient cells.

Example 91: Delivery of fusosomes via endocytic pathway

[000994] This example describes fusosome delivery of Cre to a recipient cell via an endocytic pathway.

[000995] In some embodiments, fusosomes will deliver agents via a fusosome-mediated, endocytic pathway. Without wishing to be bound by theory, delivery of an agent, e.g., a cargo, carried in the lumen of the fusosomes, to the recipient cells with the route of uptake being endocytosis-dependent will occur through a fusosome-mediated, endocytic pathway delivery.

[000996] In this example the fusosome comprises microvesicles that were produced by extruding a HEK293T cell expressing a fusogen protein on its plasma membrane through a 2 μm filter (Lin et al., 2016, Biomedical Microdevices, 18(3). doi.org/10.1007/s10544-016-0066-y)(Riedel, Kondor-Koch, & Garoff, 1984, The EMBO Journal, 3(7), 1477–83. Retrieved from www.ncbi.nlm.nih.gov/pubmed/6086326). In addition, the fusosome expresses mTagBFP2 fluorescent protein and Cre recombinase. The target cell is a PC3 cell which stably-expresses "LoxP-GFP-stop-LoxP-RFP" cassette under a CMV promoter, which upon recombination by Cre switches from GFP to RFP expression, indicating fusion and Cre, as a marker, delivery.

[000997] Fusosomes produced by the herein described methods are assayed for delivery of Cre via an endocytic pathway as follows. The recipient cells are plated into a cell culture multi-well plate compatible with the imaging system to be used (in this example cells are plated in a black, clear-bottom 96-well plate). Next, 24 hours after plating the recipient cells, the fusosomes expressing Cre recombinase protein and possessing the particular fusogen protein are applied to the recipient cells in DMEM media. To determine the level of Cre delivery via an endocytic pathway, a parallel group of recipient cells receiving fusosomes is treated with an inhibitor of endosomal acidification, chloroquine (30 μg/mL). The dose of fusosomes is correlated to the number of recipient cells plated in the well. After applying the fusosomes, the cell plate is centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells. The cells are then incubated for 16 hours and agent delivery, Cre, is assessed via imaging.

[000998] The cells are imaged to positively identify RFP-positive cells versus GFP-positive cells in the field or well. In this example cell plates are imaged using an automated fluorescent microscope. The total cell population in a given well is determined by first staining the cells with Hoechst 33342 in DMEM media for 10 minutes. Hoechst 33342 stains cell nuclei by intercalating into DNA and therefore is used to identify individual cells. After staining the Hoechst media is replaced with regular DMEM media.

[000999] The Hoechst is imaged using the 405 nm LED and DAPI filter cube. GFP is imaged using the 465 nm LED and GFP filter cube, while RFP is imaged using 523 nm LED and RFP filter cube. Images of the different cell groups are acquired by first establishing the LED intensity and integration times on a positive-control well; i.e., recipient cells treated with adenovirus coding

for Cre recombinase instead of fusosomes.

[0001000] Acquisition settings are set so that RFP and GFP intensities are at the maximum pixel intensity values but not saturated. The wells of interest are then imaged using the established settings.

[0001001] Analysis of GFP and RFP-positive wells is performed with software provided with the fluorescent microscope or other software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, 1997–2007). The images are pre-processed using a rolling ball background subtraction algorithm with a 60 μm width. The total cell mask is set on the Hoechst-positive cells. Cells with Hoechst intensity significantly above background intensities are thresholded and areas too small or large to be Hoechst-positive cells are excluded.

[0001002] Within the total cell mask, GFP and RFP-positive cells are identified by again thresholding for cells significantly above background and extending the Hoechst (nuclei) masks for the entire cell area to include the entire GFP and RFP cellular fluorescence.

[0001003] The number of RFP-positive cells identified in control wells containing recipient cells is used to subtract from the number of RFP-positive cells in the wells containing fusosomes (to subtract for non-specific Loxp recombination). The number of RFP-positive cells (recipient cells that received Cre) is then divided by the sum of the GFP-positive cells (recipient cells that have not received Cre) and RFP-positive cells to quantify the fraction of fusosome Cre delivered to the recipient cell population. The level is normalized to the given dose of fusosomes applied to the recipient cells. To calculate the value of fusosome Cre delivered via an endocytic pathway, the level of fusosome Cre delivery in the presence of chloroquine (FusL+CQ) is determined as well as the level of fusosome Cre delivery in the absence of chloroquine (FusL-CQ). To determine the normalized value of fusosome Cre delivered via an endocytic pathway, the following equation is used: (FusL+CQ)/(FusL-CQ).

[0001004] In some embodiments, an average level of fusosome Cre delivered via an endocytic pathway for a given fusosome will be in the range of 0.01-0.6, or at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than chloroquine treated recipient cells.

Example 92: Delivery of fusosomes via a dynamin mediated pathway, a macropinocytosis pathway, or an actin mediated pathway

[0001005] This example describes fusosome delivery of Cre to a recipient cell via a dynamin mediated pathway. A fusosome comprising a microvesicle may be produced as described in the preceding example. Fusosomes are assayed for delivery of Cre via a dynamin-mediated pathway according to the preceding example, except that a group of recipient cells receiving fusosomes is treated with an inhibitor of dynamin, Dynasore (120 μM). To calculate the value of fusosome Cre delivered via a dynamin-mediated pathway, the level of fusosome Cre delivery in the presence of Dynasore (FusL+DS) is determined as well as the level of fusosome Cre delivery in the absence of Dynasore (FusL-DS). The normalized value of fusosome Cre delivered may be calculated as described in the preceding example.

[0001006] This example also describes delivery of Cre to a recipient cell via macropinocytosis. A fusosome comprising a microvesicle may be produced as described in the preceding example. Fusosomes are assayed for delivery of Cre via macropinocytosis according to the preceding example, except that a group of recipient cells receiving fusosomes is treated with an inhibitor of macropinocytosis, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (25 μM). To calculate the value of fusosome Cre delivered via macropinocytosis, the level of fusosome Cre delivery in the presence of EIPA (FusL+EPIA) is determined as well as the level of fusosome Cre delivery in the absence of EPIA (FusL-EIPA). The normalized value of fusosome Cre delivered may be calculated as described in the preceding example.

[0001007] This example also describes fusosome delivery of Cre to a recipient cell via an actin mediated pathway. A fusosome comprising a microvesicle may be produced as described in the preceding example. Fusosomes are assayed for delivery of Cre via macropinocytosis according to the preceding example, except that a group of recipient cells receiving fusosomes is treated with an inhibitor of actin polymerization, Latrunculin B (6 μM). To calculate the value of fusosome Cre delivered via an actin-mediated pathway, the level of fusosome Cre delivery in the presence of Latrunculin B (FusL+ LatB) is determined as well as the level of fusosome Cre delivery in the absence of Latrunculin B (FusL- LatB). The normalized value of fusosome Cre delivered may be calculated as described in the preceding example.

Example 93: Delivery of organelles

[0001008] This example describes fusosome fusion with a cell *in vitro*. In some embodiments, fusosome fusion with a cell *in vitro* can result in delivery of fusosomal mitochondrial cargo to the recipient cell.

[0001009] A fusosome produced by the methods described by the herein described methods was assayed for its ability to deliver its mitochondria to the recipient cell as follows.

[0001010] In this particular example, the fusosome was a HEK293T cell expressing a fusogen protein on its membrane, as well as mitochondrial-targeted DsRED (mito-DsRED) protein to label mitochondria. The recipient cells were plated into a cell culture multi-well plate compatible with the imaging system to be used (in this example cells were plated in a glass-bottom imaging dish). The recipient cells stably-expressed cytosolic GFP.

[0001011] Next, 24 hours after plating the recipient cells, the fusosome expressing mito-DsRED and possessing the particular fusogen protein was applied to the recipient cells in DMEM media. The dose of fusosomes was correlated to the number of recipient cells plated in the well. After applying the fusosomes the cell plate was centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells. The cells were then incubated for 4 hours and VSVG-mediated fusion was induced by one minute exposure to pH 6.0 phosphate-buffered saline (or control cells are exposed to pH 7.4 phosphate-buffered saline). Following induction of fusion, cells were incubated an additional 16 hours and mitochondria delivery was assessed via imaging.

[0001012] In this example, cells were imaged on a Zeiss LSM 710 confocal microscope with a 63x oil immersion objective while maintained at 37°C and 5% CO_2. GFP was subjected to 488nm laser excitation and emission was recorded through a band pass 495-530nm filter. DsRED was subjected to 543 nm laser excitation and emission was recorded through a band pass 560 to 610nm filter. The cells were scanned to positively identify cells positive for cytosolic GFP fluorescence and mito-DsRED fluorescence.

[0001013] The presence of both cytosolic GFP and mito-DsRED mitochondria were found in the same cell indicating the cell has undergone VSVG-mediated fusion, and thus mitochondria

have been delivered from the fusosome to the recipient cell.

Example 94: In vitro delivery of DNA

[0001014] This example describes the delivery of DNA using fusosomes to cells *in vitro*. This example quantifies the ability of fusosomes to deliver DNA using a plasmid encoding an exogenous gene, GFP, a surrogate therapeutic cargo.

[0001015] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre. Following production of the fusosome, it is additionally nucleofected with a plasmid having a sequence that codes for GFP (System Biosciences, Inc.).

[0001016] See, for example, Chen X, et al., Genes Dis. 2015 Mar;2(1):96-105.DOI:10.1016/j.gendis.2014.12.001.

[0001017] As a negative control, fusosomes are nucleofected with a plasmid having a sequence that codes for beta-actin.

[0001018] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with a recipient NIH/3T3 fibroblast cell line that has a loxP-STOP-loxP-tdTomato reporter for a period of 48h in in DMEM containing 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin. Following the 48 hr incubation, the tdTomato positive cells are then isolated via FACS, using a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission is collected at 590+/-20nm. Total DNA is then isolated using a DNA extraction solution (Epicentre) and PCR is performed using primers specific to GFP (see Table 222) that amplify a 600bp fragment. A 600bp fragment present on a gel following gel electrophoresis would then substantiate the present of DNA delivery to the recipient cell.

Table 22. GFP Primers sequences that amplify a 500bp fragment

Primer	SEQ ID NO:	Sequence
GFP-F	41	ATGAGTAAAGGAGAAGAACTTTTCAC
GFP-R	42	GTCCTTTTACCAGACAACCATTAC

[0001019] In some embodiments, delivery of nucleic acid cargo with fusosomes *in vitro* is higher in fusosomes with GFP plasmid as compared to the negative control. Negligible GFP fluorescence is detected in the negative control.

Example 95: In vivo delivery of DNA

[0001020] This example describes the delivery of DNA to cells *in vivo* via fusosomes. Delivery of DNA to cells *in vivo* results in the expression of proteins within the recipient cell.

[0001021] Fusosome DNA delivery in vivo will demonstrates the delivery of DNA and protein expression in recipient cells within an organism (mouse).

[0001022] Fusosomes that express a liver directed fusogen are prepared as described herein. Following production of the fusosome, it is additionally nucleofected with a plasmid having a sequence that codes for Cre recombinase.

[0001023] Fusosomes are prepared for in vivo delivery. Fusosome suspensions are subjected to centrifugation. Pellets of the fusosomes are resuspended in sterile phosphate buffered saline for injection.

[0001024] Fusosomes are verified to contain DNA using a nucleic acid detection method, e.g., PCR.

[0001025] The recipient mice harbor a loxp-luciferase genomic DNA locus that is modified by CRE protein made from DNA delivered by the fusosomes to unblock the expression of luciferase (JAX#005125). The positive control for this example are offspring of recipient mice mated to a mouse strain that expresses the same protein exclusively in the liver from its own genome (albumin-CRE JAX#003574). Offspring from this mating harbor one of each allele (loxp-luciferase, albumin-CRE). Negative controls are carried out by injection of recipient mice with fusosomes not expressing fusogens or fusosomes with fusogens but not containing Cre DNA.

[0001026] The fusosomes are delivered into mice by intravenous (IV) tailvein administration. Mice are placed in a commercially available mouse restrainer (Harvard Apparatus). Prior to restraint, animals are warmed by placing their cage on a circulating water bath. Once inside the restrainer, the animals are allowed to acclimate. An IV catheter consisting

of a 30G needle tip, a 3" length of PE-10 tubing, and a 28G needle is prepared and flushed with heparinized saline. The tail is cleaned with a 70% alcohol prep pad. Then, the catheter needle is held with forceps and slowly introduced into the lateral tail vein until blood becomes visible in the tubing. The fusosome solution (~500K-5M fusosomes) is aspirated into a 1 cc tuberculin syringe and connected to an infusion pump. The fusosome solution is delivered at a rate of 20 μL per minute for 30 seconds to 5 minutes, depending on the dose. Upon completion of infusion, the catheter is removed, and pressure is applied to the injection site until cessation of any bleeding. Mice are returned to their cages and allowed to recover.

[0001027] After fusion, the DNA will be transcribed and translated into CRE protein which will then translocates to the nucleus to carry out recombination resulting in the constitutive expression of luciferase. Intraperitoneal administration of D-luciferin (Perkin Elmer, 150 mg/kg) enables the detection of luciferase expression via the production of bioluminescence. The animal is placed into an in vivo bioluminescent imaging chamber (Perkin Elmer) which houses a cone anesthetizer (isoflurane) to prevent animal motion. Photon collection is carried out between 8-20 minutes post-injection to observe the maximum in bioluminescence due to D-luciferin pharmacokinetic clearance. A specific region of the liver is created in the software and collection exposure time set so that count rates are above 600 (in this region) to yield interpretable radiance (photons/sec/cm^2/steradians) measurements. The maximum value of bioluminescent radiance is recorded as the image of bioluminescence distribution. The liver tissue is monitored specifically for radiance measurements above background (untreated animals) and those of negative controls. Measurements are carried out at 24 hours post-injection to observe luciferase activity. Mice are then euthanized and livers are harvested.

[0001028] Freshly harvested tissue is subjected to fixation and embedding via immersion in 4% paraformaldehyde/0.1M sodium phosphate buffer pH7.4 at 4°C for 1-3hrs. Tissue is then immersed in sterile 15% sucrose/1xPBS (3 hrs. to overnight) at 4°C. Tissue is then embedded in O.C.T. (Baxter No. M7148-4). Tissue is oriented in the block appropriately for sectioning (cross-section). Tissue is then frozen in liquid nitrogen using the following method: place the bottom third of the block into the liquid nitrogen, allow to freeze until all but the center of the O.C.T. is frozen, and allow freezing to conclude on dry ice. Blocks are sectioned by cryostat into 5-7 micron

sections placed on slides and refrozen for staining.

[0001029] In situ hybridization is carried out (using standard methods) on tissue sections using digoxygenin labeled nucleic acid probes (for CRE DNA and luciferase mRNA detection), labeled by anti-digoxygenin fluorescent antibodies, and observed by confocal microscopy.

[0001030] In some embodiments, positive control animals (recombination via breeding without fusosome injection) will show bioluminescence intensity in liver as compared to untreated animals (no CRE and no fusosomes) and negative controls, while agent injected animals will show bioluminescence in liver as compared to negative controls (fusosomes without fusogen) and untreated animals.

[0001031] In embodiments, detection of nucleic acid in tissue sections in agent injected animals will reveal detection of CRE recombinase and luciferase mRNA compared to negative controls and untreated animals in cells in the tissue, while positive controls will show levels of both luciferase mRNA and CRE recombinase DNA throughout the tissue.

[0001032] Evidence of DNA delivery by fusosomes will be detected by in situ hybridization-based detection of the DNA and its colocalization in the recipient tissue of the animal. Activity of the protein expressed from the DNA will be detected by bioluminescent imaging. In embodiments, fusosomes will deliver DNA that will result in protein production and activity.

Example 96: In vitro delivery of mRNA

[0001033] This example describes fusosome fusion with a cell *in vitro*. In some embodiments, fusosome fusion with a cell *in vitro* results in delivery of a specified mRNA to the recipient cell.

[0001034] A fusosome produced by the herein described methods was assayed for its ability to deliver a specified mRNA to the recipient cell as follows. In this particular example, the fusosome was a cytobiologic (lacking a nucleus), which was generated from a 3T3 mouse fibroblast cell expressing Cre and GFP. The cytobiologic was then treated with HVJ-E fusogen protein to produce the fusosome.

[0001035] The recipient mouse macrophage cells were plated into a cell culture multi-well plate compatible with the imaging system to be used (in this example cells are plated in a glass-bottom imaging dish). The recipient cells stably-expressed "LoxP-stop-LoxP-tdTomato" cassette

under CMV promoter, which upon recombination by Cre induces tdTomato expression, indicating delivery of Cre protein to the recipient cell.

[0001036] Next, 24 hours after plating the recipient cells, the fusosome expressing Cre recombinase protein and possessing the particular fusogen protein was applied to the recipient cells in DMEM media. The dose of fusosomes was correlated to the number of recipient cells plated in the well. After applying the fusosomes the cell plate was centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells. The cells were then incubated for 16 hours and mRNA delivery was assessed via imaging.

[0001037] The cells were stained with 1 μg/mL Hoechst 33342 in DMEM media for 10 minutes prior to imaging. In this example cells were imaged on a Zeiss LSM 710 confocal microscope with a 63x oil immersion objective while maintained at 37°C and 5% CO_2. Hoechst was subjected to 405nm laser excitation and emission was recorded through a band pass 430-460nm filter. GFP was subjected to 488nm laser excitation and emission was recorded through a band pass 495-530nm filter. tdTomato was subjected to 543 nm laser excitation and emission was recorded through a band pass 560 to 610nm filter.

[0001038] First, the cells were scanned to positively identify single-nucleated, tdTomato-positive cells. The presence of a tdTomato-positive cell indicated a cell that has undergone fusion, and the single nucleus indicated the fusion was by a cytobiologic fusosome donor. These identified cells were first imaged and then subsequently photo-bleached using a 488nm laser to partially quench GFP fluorescence. The cells were then imaged over-time to assess recovery of GFP fluorescence, which would demonstrate translation of new GFP protein and thus presence of GFP mRNA delivered by the donor fusosome.

[0001039] Analysis of Hoechst, GFP, and tdTomato fluorescence in the cells of interest was performed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, rsb.info.nih.gov/ij/, 1997–2007). First the images were pre-processed using a rolling ball background subtraction algorithm with a 60 μm width. Within a photo-bleached cell, the GFP fluorescence was thresholded to remove background. Then the GFP mean fluorescence intensity for the photo-bleached cell was analyzed at different times before and after photo-bleaching.

[0001040] Within this particular Example, 3T3 mouse fibroblast cytobiologics expressing Cre and GFP and either possessing the applied fusogen HVJ-E (+fusogen) were applied to recipient mouse macrophage cells expressing "LoxP-stop-LoxP-tdTomato" cassette. Representative images and data are shown in FIG. 5. For this particular example the GFP fluorescence intensity recovered up to 25% of the original intensity 10 hours after photo-bleaching, indicating the delivery of actively-translated mRNA in the recipient cell.

Example 97: In vitro delivery of siRNA

[0001041] This example describes delivery of short interfering RNA (siRNA) to cell *in vitro* via fusosomes. Delivery of siRNA to cells *in vitro* results in the suppression of the expression of proteins within the recipient cell. This can be used to inhibit the activity of a protein whose expression is injurious to the cell, thus permitting the cell to behave normally.

[0001042] A fusosome produced by the herein described methods is assayed for its ability to deliver a specified siRNA to the recipient cell as follows. Fusosomes are prepared as described herein. Following production of the fusosome, it is additionally electroporated with an siRNA having a sequence that specifically inhibits GFP. The sequence of the double stranded siRNA targeted against GFP is 5' GACGUAAACGGCCACAAGUUC 3' (SEQ ID NO: 43) and its complement 3' CGCUGCAUUUGCCGGUGUUCA 5' (SEQ ID NO: 44) (note that there are overhangs 2 basepairs long at 3' ends of the siRNA sequence). As a negative control fusosomes are electroporated with an siRNA having a sequence that specifically inhibits luciferase. The sequence of the double stranded siRNA targeted against luciferase is 5' CUUACGCUGAGUACUUCGATT 3' (SEQ ID NO: 45) and its complement 3' TTGAAUGCGACUCAUGAAGCU 5' (SEQ ID NO: 46) (note that there are overhangs 2 basepairs long at 3' ends of the siRNA sequence).

[0001043] The fusosomes are then applied to the recipient cells that constitutively express GFP. The recipient cells are plated into a black, clear-bottom 96-well plate. Next, 24 hours after plating the recipient cells, the fusosomes expressing are applied to the recipient cells in DMEM media. The dose of fusosomes is correlated to the number of recipient cells plated in the well. After applying the fusosomes, the cell plate is centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells. The cells are then incubated for 16 hours and agent

delivery, siRNA, is assessed via imaging.

[0001044] The cells are imaged to positively identify GFP-positive cells in the field or well. In this example cell plates are imaged using an automated fluorescence microscope (www.biotek.com/products/imaging-microscopy-automated-cell-imagers/lionheart-fx-automated-live-cell-imager/). The total cell population in a given well is determined by first staining the cells with Hoechst 33342 in DMEM media for 10 minutes. Hoechst 33342 stains cell nuclei by intercalating into DNA and therefore is used to identify individual cells. After staining, the Hoechst media is replaced with regular DMEM media.

[0001045] The Hoechst is imaged using the 405 nm LED and DAPI filter cube. GFP is imaged using the 465 nm LED and GFP filter cube. Images of the different cell groups are acquired by first establishing the LED intensity and integration times on an untreated well; i.e., recipient cells that were not treated with any fusosomes.

[0001046] Acquisition settings are set so that GFP intensities are at the maximum pixel intensity values but not saturated. The wells of interest are then imaged using the established settings.

[0001047] Analysis of GFP positive wells is performed with software provided with the fluorescence microscope or other software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2007). The images are pre-processed using a rolling ball background subtraction algorithm with a 60 μm width. The total cell mask is set on the Hoechst-positive cells. Cells with Hoechst intensity significantly above background intensities are thresholded and areas too small or large to be Hoechst-positive cells are excluded.

[0001048] Within the total cell mask, GFP - positive cells are identified by again thresholding for cells significantly above background and extending the Hoechst (nuclei) masks for the entire cell area to include the entire GFP cellular fluorescence. The percentage of GFP-positive cells out of total cells is calculated.

[0001049] In embodiments, the percentage of GFP positive cells in wells treated with fusosomes containing an siRNA against GFP will be at least 1%, 2%, 3%, 4%, 5%, 10%, 20%,

30%, 40%, 50%, 60%, 70%, 80%, 90% less than the percentage of GFP positive cells in well treated with fusosomes containing an siRNA against luciferase.

Example 98: In vivo delivery of mRNA

[0001050] This example describes the delivery of messenger RNA (mRNA) to cells *in vivo* via fusosomes. In some embodiments, delivery of mRNA to cells *in vivo* results in the expression of proteins within the recipient cell. In some embodiments, this method of delivery can be used to supplement a protein not present due to a genetic mutation, permitting the cell to behave normally, or re-direct the activity of a cell to carry out a function, e.g., a therapeutic function.

[0001051] In some embodiments, fusosome mRNA delivery *in vivo* demonstrates the delivery of messenger RNA and protein expression in recipient cells within an organism (e.g., a mouse).

[0001052] In some embodiments, fusosomes that express a liver directed fusogen, and produce mRNA expressing Cre are prepared for *in vivo* delivery.

[0001053] Fusosomes are prepared as described herein. Fusosome suspensions are subjected to centrifugation. Pellets of the fusosomes are resuspended in sterile phosphate buffered saline for injection.

[0001054] Fusosomes are verified to express mRNA using a nucleic acid detection method, e.g., PCR.

[0001055] The recipient mice harbor a loxp-luciferase genomic DNA locus that is modified by CRE protein made from mRNA delivered by the fusosomes to unblock the expression of luciferase (JAX#005125). The positive controls for this example are offspring of recipient mice mated to a mouse strain that expresses the same protein exclusively in the liver from its own genome (albumin-CRE JAX#003574). Offspring from this mating harbor one of each allele (loxp-luciferase, albumin-CRE). Negative controls are carried out by injection of recipient mice with fusosomes not expressing fusogens or fusosomes with fusogens but not expressing Cre mRNA.

[0001056] The fusosomes are delivered into mice by intravenous (IV) tail vein administration. Mice are placed in a commercially available mouse restrainer (Harvard Apparatus). Prior to restraint, animals are warmed by placing their cage on a circulating water bath. Once inside the restrainer, the animals are allowed to acclimate. An IV catheter consisting

of a 30G needle tip, a 3" length of PE-10 tubing, and a 28G needle is prepared and flushed with heparinized saline. The tail is cleaned with a 70% alcohol prep pad. Then, the catheter needle is held with forceps and slowly introduced into the lateral tail vein until blood becomes visible in the tubing. The fusosome solution (~500K-5M fusosomes) is aspirated into a 1 cc tuberculin syringe and connected to an infusion pump. The fusosome solution is delivered at a rate of 20 μL per minute for 30 seconds to 5 minutes, depending on the dose. Upon completion of infusion, the catheter is removed, and pressure is applied to the injection site until cessation of any bleeding. Mice are returned to their cages and allowed to recover.

[0001057] After fusion, the mRNA is translated in the recipient cytoplasm into CRE protein which then translocates to the nucleus to carry out recombination resulting in the constitutive expression of luciferase. Intraperitoneal administration of D-luciferin (Perkin Elmer, 150 mg/kg) enables the detection of luciferase expression via the production of bioluminescence. The animal is placed into an *in vivo* bioluminescent imaging chamber (Perkin Elmer) which houses a cone anesthetizer (isoflurane) to prevent animal motion. Photon collection is carried out between 8-20 minutes post-injection to observe the maximum in bioluminescence due to D-luciferin pharmacokinetic clearance. A specific region of the liver is created in the software and collection exposure time set so that count rates are above 600 (in this region) to yield interpretable radiance (photons/sec/cm^2/steradians) measurements. The maximum value of bioluminescent radiance is recorded as the image of bioluminescence distribution. The liver tissue is monitored specifically for radiance measurements above background (untreated animals) and those of negative controls. Measurements are carried out at 24 hours post-injection to observe luciferase activity. Mice are then euthanized and livers are harvested.

[0001058] Freshly harvested tissue is subjected to fixation and embedding via immersion in 4% paraformaldehyde/0.1M sodium phosphate buffer pH7.4 at 4°C for 1-3hrs. Tissue is then immersed in sterile 15% sucrose/1xPBS (3 hrs. to overnight) at 4°C. Tissue is then embedded in O.C.T. (Baxter No. M7148-4). Tissue is oriented in the block appropriately for sectioning (cross-section). Tissue is then frozen in liquid nitrogen using the following method: place the bottom third of the block into the liquid nitrogen, allow to freeze until all but the center of the O.C.T. is frozen, and allow freezing to conclude on dry ice. Blocks are sectioned by cryostat into 5-7 micron

sections placed on slides and refrozen for staining.

[0001059] *In situ* hybridization is carried out (using standard methods) on tissue sections using digoxygenin labeled RNA probes (for CRE mRNA and luciferase mRNA detection), labeled by anti-digoxygenin fluorescent antibodies, and observed by confocal microscopy.

[0001060] In some embodiments, positive control animals (e.g., recombination via breeding without fusosome injection), will show bioluminescence intensity in liver as compared to untreated animals (e.g., no CRE or fusosomes), and negative controls. In some embodiments, fusosome injected animals will show bioluminescence in liver as compared to negative controls (e.g., fusosomes without fusogen), and untreated animals.

[0001061] In some embodiments, detection of mRNA in tissue sections in animals administered fusosomes will reveal detection of CRE recombinase and luciferase mRNA compared to negative controls, and untreated animals in cells in the tissue. In some embodiments, positive controls will show levels of both luciferase mRNA and CRE recombinase mRNA throughout the tissue.

[0001062] In some embodiments, evidence of mRNA delivery by fusosomes will be detected by *in situ* hybridization-based detection of the mRNA, and its colocalization in the recipient tissue of the animal. In some embodiments, activity of the protein expressed from the mRNA delivered by the fusosome is detected by bioluminescent imaging. In some embodiments, fusosomes deliver mRNA that will result in protein production and activity.

Example 99: In vitro delivery of protein

[0001063] This example demonstrates fusosome fusion with a cell *in vitro*. In this example, fusosome fusion with a cell in vitro results in delivery of Cre protein to the recipient cell.

[0001064] In this example, the fusosomes were generated from a 3T3 mouse fibroblast cell possessing the Sendai virus HVJ-E protein (Tanaka et al., 2015, Gene Therapy, 22(October 2014), 1–8. doi.org/10.1038/gt.2014.12). In addition, the fusosomes expressed Cre recombinase. The target cell was a primary HEK293T cell which stably-expressed "LoxP -GFP-stop-LoxP-RFP" cassette under a CMV promoter, which upon recombination by Cre switches from GFP to RFP expression, indicating fusion and Cre, as a marker, delivery.

[0001065] Fusosomes produced by the herein described methods were assayed for the ability to deliver Cre protein to recipient cells as follows. The recipient cells were plated into a cell culture multi-well plate compatible with the imaging system to be used (in this example cells were plated in a black, clear-bottom 96-well plate). Next, 24 hours after plating the recipient cells, the fusosome expressing Cre recombinase protein and possessing the particular fusogen protein were applied to the recipient cells in DMEM media. The dose of fusosomes was correlated to the number of recipient cells plated in the well. After applying the fusosomes the cell plate was centrifuged at 400g for 5 minutes to help initiate contact between the fusosomes and the recipient cells. The cells were then incubated for 16 hours and protein delivery was assessed via imaging.

[0001066] The cells were imaged to positively identify RFP-positive cells versus GFP-positive cells in the field or well. In this example cell plates were imaged using an automated microscope. The total cell population in a given well was determined by first staining the cells with 1 μg/mL Hoechst 33342 in DMEM media for 10 minutes. Hoechst 33342 stains cell nuclei by intercalating into DNA and therefore is used to identify individual cells. After staining the Hoechst media was replaced with regular DMEM media. The Hoechst was imaged using the 405 nm LED and DAPI filter cube. GFP was imaged using the 465 nm LED and GFP filter cube, while RFP was imaged using 523 nm LED and RFP filter cube. Images of the different cell groups were acquired by first establishing the LED intensity and integration times on a positive-control well; i.e., cells treated with adenovirus coding for Cre recombinase. Acquisition settings were set so that RFP and GFP intensities are at the maximum pixel intensity values but not saturated. The wells of interest were then imaged using the established settings.

[0001067] Analysis of Hoechst, GFP, and RFP-positive wells was performed in the Gen5 software provided with the LionHeart FX or by ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2007). First the images were pre-processed using a rolling ball background subtraction algorithm with a 60 μm width. Next the total cell mask was set on the Hoechst-positive cells. Cells with Hoechst intensity significantly above background intensities were thresholded and areas too small or large to be Hoechst-positive cells were excluded. Within the total cell mask GFP and RFP-positive cells were identified by again thresholding for cells significantly above background and extending the

Hoechst (nuclei) masks for the entire cell area to include the entire GFP and RFP cellular fluorescence.

[0001068] The number of RFP-positive cells identified in control wells containing only recipient cells was used to subtract from the number of RFP-positive cells in the wells containing fusosome (to subtract for non-specific Loxp recombination). The number of RFP-positive cells (recipient cells that received the agent) was then divided by the sum of the GFP-positive cells (recipient cells that have not received the agent) and RFP-positive cells to quantify the fraction of fusosome agent delivery within the recipient cell population.

[0001069] Within this particular example, 3T3 mouse fibroblast cells expressing Cre and either possessing the applied fusogen HVJ-E (+fusogen) or not (-fusogen) were applied to recipient 293T cells expressing "LoxP-GFP-stop-LoxP-RFP" cassette. Delivery of Cre protein is assessed by the induction of RFP expression in the recipient cells. The graph in Figure 6 shows the quantification of the RFP-positive cells (rightmost bar of each pair) out of the total cells stained positive for Hoechst (leftmost bar of each pair). For this particular Example the fraction of fusosome delivery to recipient cells is 0.44 for 3T3 Cre cells possessing HVJ-E fusogen.

Example 100: In vivo delivery of protein

[0001070] This example describes the delivery of therapeutic agents to the eye by fusosomes.

[0001071] Fusosomes are derived from hematopoietic stem and progenitor cells using any of the methods described in previous Examples and are loaded with a protein that is deficient in a mouse knock-out.

[0001072] Fusosomes are injected subretinally into the right eye of a mouse that is deficient for the protein and vehicle control is injected into the left eye of the mice. A subset of the mice is euthanized when they reach 2 months of age.

[0001073] Histology and H&E staining of the harvested retinal tissue is conducted to count the number of cells rescued in each retina of the mice (described in Sanges et al., The Journal of Clinical Investigation, 126(8): 3104-3116, 2016).

[0001074] The level of the injected protein is measured in retinas harvested from mice euthanized at 2 months of age via a western blot with an antibody specific to the PDE6B protein.

[0001075] In some embodiments, the left eyes of mice, which are administered fusosomes, will have an increased number of nuclei present in the outer nuclear level of the retina compared to the right eyes of mice, which are treated with vehicle. The increased protein is suggestive of complementation of the mutated PBE6B protein.

Example 101: Delivery to edit recipient DNA

[0001076] This example describes fusosomes for delivery of genome CRISPR-Cas9 editing machinery to a cell *in vitro*. In some embodiments, delivery of genome CRISPR-Cas9 editing machinery to a cell *in vitro* via a fusosome results in a loss of function of a specific protein in a recipient cell. Genome editing machinery referred to, in this example, is the *S. pyogenes* Cas9 protein complexed with a guide RNA (gRNA) specific for GFP.

[0001077] In some embodiments, fusosomes are a chassis for the delivery of therapeutic agents. In some embodiments, therapeutic agents, such as genome editing machinery that can be delivered to cells with high specificity and efficiency could be used to inactivate genes, and thus subsequent gene products (e.g. proteins) that when expressed at high levels or in the wrong cell type become pathological.

[0001078] A fusosome composition as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusosome also includes the *S. pyogenes* Cas9 protein complexed with a guide RNA (gRNA) sequence that is specific for the sequence of *A. Victoria* EGFP. This is achieved by co-nucleofecting a PiggyBac vector that has the open reading frame of the Neomycin resistance gene that is an in-frame fusion with the open reading frame of *S. pyogenes* Cas9, separated by a P2A cleavage sequence. The additional co-nucleofected PiggyBac vector also includes the gRNA sequence (GAAGTTCGAGGGCGACACCC (SEQ ID NO: 47)) driven by the U6 promoter. As a negative control a fusosome is engineered such that the fusosome includes the *S. pyogenes* Cas9 protein complexed with a scrambled gRNA (GCACTACCAGAGCTAACTCA (SEQ ID NO: 48)) sequence that is not-specific for any target in the mouse genome.

[0001079] A sufficient number of fusosomes are incubated at 37°C and 5% CO_2 together with NIH/3T3 GFP+ cells for a period of 48h in in DMEM containing 20% Fetal Bovine Serum and 1x

Penicillin/Streptomycin. Following the 48 hr incubation, genomic DNA is prepared and used as a template with primers specific for region within 500 bp of the predicted gRNA cleavage site in the GFP gene (see Table 23).

Table 23. GFP Primers sequences that amplify a 500bp fragment for TIDE analysis

Primer	SEQ ID NO:	Sequence
GFP-F	41	ATGAGTAAAGGAGAAGAACTTTTCAC
GFP-R	42	GTCCTTTTACCAGACAACCATTAC

[0001080] The PCR amplicon is then purified, sequenced by capillary sequencing and then uploaded to Tide Calculator, a web tool that rapidly assesses genome editing by CRISPR-Cas9 of a target locus determined by a guide RNA. Based on the quantitative sequence trace data from two standard capillary sequencing reactions, the software quantifies the editing efficacy. An indel (insert or deletion) at the predicted gRNA cleavage site with the GFP locus results in the loss of GFP expression in the cells and is quantified via FACS using a FACS analysis (Becton Dickinson, San Jose, CA, USA) with 488nm argon laser excitation and emission is collected at 530+/-30nm. FACS software is used for acquisition and analysis. The light scatter channels are set on linear gains, and the fluorescence channels on a logarithmic scale, with a minimum of 10,000 cells analyzed in each condition. The indel and subsequent loss of GFP function is calculated based on the intensity of GFP signal in each sample.

[0001081] In some embodiments, an indel (insert or deletion) at the predicted gRNA cleavage site with the GFP locus and loss of GFP fluorescence in the cell, in comparison to the negative control, will indicate the ability of a fusosome to edit DNA and result in a loss of protein function in vitro. In some embodiments, fusosomes with the scrambled gRNA sequence will demonstrate no indels or subsequent loss of protein function.

Example 102: Assessment of teratoma formation after administration of fusosome

[0001082] This Example describes the absence of teratoma formation with a fusosome. In some embodiments, a fusosome will not result in teratoma formation when administered to a subject.

[0001083] Fusosomes are produced by any one of the methods described in a previous Example. Fusosomes, tumor cells (positive control) or vehicle (negative control) are subcutaneously injected in PBS into the left flank of mice (12-20 weeks old). Teratoma, e.g., tumor, growth is analyzed 2-3 times a week by determination of tumor volume by caliper measurements for eight weeks after fusosome, tumor cell, or vehicle injection.

[0001084] In some embodiments, mice administered fusosomes or vehicle will not have a measurable tumor formation, e.g., teratoma, via caliper measurements. In some embodiments, positive control animals treated with tumor cells will demonstrate an appreciable tumor, e.g., teratoma, size as measured by calipers over the eight weeks of observation.

Example 103: Fusosomes deliver active protein to recipient cells of a subject in vivo

[0001085] This Example demonstrates that fusosomes can deliver a protein to a subject in vivo. This is exemplified by delivery of the nuclear editing protein Cre. Once inside of a cell, Cre translocates to the nucleus, where it recombines and excises DNA between two LoxP sites. Cre-mediated recombination can be measured microscopically when the DNA between the two LoxP sites is a stop codon and is upstream of a distal fluorescent protein, such as the red fluorescent protein tdTomato.

[0001086] Fusosomes that contain CRE and the fusogen VSV-G, purchased from Takara (Cre Recombinase Gesicles, Takara product 631449), were injected into B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice (Jackson Laboratories strain 007909). Animals were injected at the anatomical sites, injection volumes, and injection sites as described in Table 24. Mice that do not have tdTomato (FVB.129S6(B6)-*GT(ROSA)26Sor*$^{tm1(Luc)Kael}$/J, Jackson Laboratories strain 005125) and were injected with fusosomes and B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J mice that were not injected with fusosomes were used as negative controls.

Table 24: Injection parameters

Anatomical Site	Injection Volume	Injection Site(s)
Brain	10 µL	anterior posterior axis: -2 Lateral/medial axis: 1.8 ventral: 1.5 side: right
Eye	1 µL	intravitreal
Liver	25 µL	center of frontal lobe
Spleen	10 µL	approximately in the center, both lengthwise and widthwise
Kidney	20 µL	center of left kidney
Small intestine lining	10 µL	loop of small intestine nearest the peritoneal wall was isolated outside peritoneum, and injected into lining.
Heart	5 µL	near apex
White Adipose (Epididymal fat pad)	25 µL	left, top and central
Brown adipose (intrascapular)	25 µL	left lobe, as central as possible
Lung	10 µL	inferior lobe right lung
Testis	10 µL	left testis, as central as possible
Ovary	1 µL	left ovary, as central as possible

[0001087] Two days after injections, the animals were sacrificed and samples were collected. The samples were fixed for 8 hours in 2% PFA, fixed overnight in 30% sucrose, and shipped for immediate embedding in OCT and sectioning to slides. Slides were stained for nuclei with DAPI.

DAPI and tdTomato fluorescence was imaged microscopically.

[0001088] All anatomical sites listed in Table 24 demonstrated tdTomato fluorescence (Figure 9). In addition, delivery to muscle tissue was confirmed using fluorescence microscopy for tdTomato (Figure 11). Negative control mice did not have any tissues with tdTomato fluorescence. This result demonstrates that fusosomes are capable of turning on tdTomato fluorescence in the cells of a mouse at various anatomical sites, and that this does not occur if the mice are not treated with fusosomes or if the mice do not have tdTomato in their genome. Hence, fusosomes deliver active Cre recombinase to the nucleus of mouse cells *in vivo*.

[0001089] It was also shown that different routes of administration can deliver deliver fusosomes to tissue *in vivo*. Fusosomes that contain CRE and the fusogen VSV-G, purchased from Takara (Cre Recombinase Gesicles, Takara product 631449), were injected into FVB.129S6(B6)-*GT(ROSA)26Sor*$^{tm1(Luc)Kael}$/J (Jackson Laboratories strain 005125) intramuscularly (in 50 µL to the right tibialis anterior muscle), intraperitoneally (in 50 µL to the peritoneal cavity), and subcutaneously (in 50 µL under the dorsal skin).

[0001090] The legs, ventral side, and dorsal skin was prepared for intramuscular, intraperitoneal, and subcutaneous injection, respectively, by depilating the area using a chemical hair remover for 45 seconds, followed by 3 rinses with water.

[0001091] On day 3 after injection, an in vivo imaging system (Perkin Elmer) was used to obtain whole animal images of bioluminescence. Five minutes before imaging, mice received an intraperitoneal injection of bioluminescent substrate (Perkin Elmer) at a dose of 150mg/kg in order to visualize luciferase. The imaging system was calibrated to compensate for all device settings.

[0001092] Administration by all three routes resulted in luminescense (Figure 10) indicating successful delivery of active Cre recombinase to mouse cells *in vivo*.

[0001093] In conclusion, fusosomes are capable of delivering active protein to cells of a subject *in vivo*.

Example 104: Sonication-mediated loading of nucleic acid in fusosomes

[0001094] This example describes loading of nucleic acid payloads into a fusosome via sonication. Sonication methods are disclosed e.g., in Lamichhane, TN, *et al.*, Oncogene

Knockdown via Active Loading of Small RNAs into Extracellular Vesicles by Sonication. *Cell Mol Bioeng*, (2016), the entire contents of which are hereby incorporated by reference.

[0001095] Fusosomes are prepared by any one of the methods described in a previous example. Approximately 10^6 fusosomes are mixed with 5-20µg nucleic acid and incubated at room temperature for 30 minutes. The fusosome/nucleic acid mixture is then sonicated for 30 seconds at room temperature using a water bath sonicator (Brason model #1510R-DTH) operated at 40kHz. The mixture is then placed on ice for one minute followed by a second round of sonication at 40kHz for 30 seconds. The mixture is then centrifuged at 16,000g for 5 minutes at 4ºC to pellet the fusosomes containing nucleic acid. The supernatant containing unincorporated nucleic acid is removed and the pellet is resuspended in phosphate-buffered saline. After DNA loading, the fusosomes are kept on ice before use.

Example 105: Sonication-mediated loading of protein in fusosomes

[0001096] This example describes loading of protein payloads into a fusosome via sonication. Sonication methods are disclosed e.g., in Lamichhane, TN, *et al*., Oncogene Knockdown via Active Loading of Small RNAs into Extracellular Vesicles by Sonication. *Cell Mol Bioeng*, (2016), the entire contents of which are hereby incorporated by reference.

[0001097] Fusosomes are prepared by any one of the methods described in a previous example. Approximately 10^6 fusosomes are mixed with 5-20µg protein and incubated at room temperature for 30 minutes. The fusosome/protein mixture is then sonicated for 30 seconds at room temperature using a water bath sonicator (Brason model #1510R-DTH) operated at 40kHz. The mixture is then placed on ice for one minute followed by a second round of sonication at 40kHz for 30 seconds. The mixture is then centrifuged at 16,000g for 5 minutes at 4°C to pellet the fusosomes containing protein. The supernatant containing unincorporated protein is removed and the pellet is resuspended in phosphate-buffered saline. After protein loading, the fusosomes are kept on ice before use.

Example 106: Hydrophobic-carrier mediated loading of nucleic acid in fusosomes

[0001098] This example describes loading of nucleic acid payloads into a fusosome via hydrophobic carriers. Exemplary methods of hydrophobic loading are disclosed, e.g., in Didiot

et al., Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing, *Molecular Therapy* 24(10): 1836–1847, (2016), the entire contents of which are hereby incorporated by reference.

[0001099] Fusosomes are prepared by any one of the methods described in a previous example. The 3' end of a RNA molecule is conjugated to a bioactive hydrophobic conjugate (triethylene glycol–Cholesterol). Approximately 10^6 fusosomes are mixed in 1 mL with 10 µmol/l of siRNA conjugate in PBS by incubation at 37° C for 90 minutes with shaking at 500 rpm. The hydrophobic carrier mediates association of the RNA with the membrane of the fusosome. In some embodiments, some RNA molecules are incorporated into the lumen of the fusosome, and some are present on the surface of the fusosome. Unloaded fusosomes are separated from RNA-loaded fusosomes by ultracentrifugation for 1 hour at 100,000g, 4° C in a tabletop ultracentrifuge using a TLA-110 rotor. Unloaded fusosomes remain in the supernatant and RNA-loaded fusosomes form a pellet. The RNA-loaded fusosomes are resuspended in 1 mL PBS and kept on ice before use.

Example 107: Processing fusosomes

[0001100] This example described the processing of fusosomes. Fusosomes produced via any of the described methods in the previous Examples may be further processed.

[0001101] In some embodiments, fusosomes are first homogenized, e.g., by sonication. For example, the sonication protocol includes a 5 second sonication using an MSE sonicator with microprobe at an amplitude setting of 8 (Instrumentation Associates, N.Y.). In some embodiments, this short period of sonication is enough to cause the plasma membrane of the fusosomes to break up into homogenously sized fusosomes. Under these conditions, organelle membranes are not disrupted and these are removed by centrifugation (3,000 rpm, 15 min 4°C). Fusosomes are then purified by differential centrifugation as described in Example 16.

[0001102] Extrusion of fusosomes through a commercially available polycarbonate membrane (e.g., from Sterlitech, Washington) or an asymmetric ceramic membrane (e.g., Membralox), commercially available from Pall Execia, France, is an effective method for reducing fusosome sizes to a relatively well defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired fusosome size distribution is achieved. The

fusosomes may be extruded through successively smaller pore membranes (e.g., 400 nm, 100 nm and/or 50 nm pore size) to achieve a gradual reduction in size and uniform distribution.

[0001103] In some embodiments, at any step of fusosome production, though typically prior to the homogenization, sonication and/or extrusion steps, a pharmaceutical agent (such as a therapeutic), may be added to the reaction mixture such that the resultant fusosome encapsulates the pharmaceutical agent.

Example 108: Measuring total RNA in a fusosome and source cell

[0001104] This Example describes a method to quantify the amount of RNA in a fusosome relative to a source cell. In some embodiments, a fusosome will have similar RNA levels to the source cell. In this assay, RNA levels are determined by measuring total RNA.

[0001105] Fusosomes are prepared by any one of the methods described in previous Examples. Preparations of the same mass as measured by protein of fusosomes and source cells are used to isolate total RNA (e.g., using a kit such as Qiagen RNeasy catalog #74104), followed by determination of RNA concentration using standard spectroscopic methods to assess light absorbance by RNA (e.g. with Thermo Scientific NanoDrop).

[0001106] In some embodiments, the concentration of RNA in fusosomes will be 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% of that of source cells per mass of protein.

Example 109: Fusosome Fusing to T cell In Vitro

a. DNA Payload

[0001107] This example describes the delivery of DNA using fusosomes to CD3+ T cells *in vitro*. This example quantifies the ability of fusosomes to deliver DNA using a plasmid encoding an exogenous gene, a chimeric antigen receptor directed against CD19, which is a therapeutic cargo.

[0001108] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre but separated by a P2A self-cleaving peptide sequence. Following production of the fusosome, it is additionally nucleofected with a plasmid having a sequence that codes for the CAR.

[0001109] See, for example, Chen X, et al., Genes Dis. 2015 Mar;2(1):96-105.DOI:10.1016/j.gendis.2014.12.001.

[0001110] As a negative control, fusosomes are nucleofected with a plasmid that codes for GFP.

[0001111] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with recipient CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter for a period of 48h in T-cell medium comprising X-VIVO 15 medium (Lonza, Basel, CH, Switzerland) supplemented with 5% fetal bovine serum (FBS) (Gibco, LAX, CA, USA), 100 U mL– 1 penicillin, 100 µg mL – 1 streptomycin, 1.25 µg mL – 1 amphotericin B, 2 mM L-glutamine (Gibco), and 100 U mL – 1 hIL-2 (PerproTech, Rocky Hill, CT, USA). Fusosome fusion with the CD3+ T cells that have have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression. Following the 48 hr incubation, the tdTomato positive cells are then isolated via FACS, using a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission is collected at 590+/-20nm. Total DNA is then isolated using a DNA extraction solution (Epicentre). Quantitative Real-time PCR is performed using a QuantStudio3 Real-time PCR System (ThermoFisher Scientific) with TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific), 100ng of DNA template, a primer and probe set that is specific for the variable regions of the anti-CD19 CAR (designed using Taqman online primer and probe design program). cA standard curve is prepared for absolute quantitation of anti-CD19 CAR transgene DNA copies by making serial dilutions of the plasmid that encodes the CAR. A primer and probe set specific for beta-lactamase (AMP^r gene) was used to normalize for DNA quantity. The C_t value is used to compare the amount of CAR DNA in the CD3+ T cells treated with fusosomes with CAR plasmid or with negative control.

In some embodiments, delivery of DNA cargo with fusosomes *in vitro* is higher in fusosomes with CAR plasmid as compared to the negative control.

b. mRNA Payload

[0001112] This example describes the delivery of mRNA using fusosomes to CD3+ T cells *in vitro*. This example quantifies the ability of fusosomes to deliver mRNA encoding an exogenous gene, a chimeric antigen receptor directed against CD19, which is a therapeutic cargo.

[0001113] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre but separated by a P2A self-cleaving peptide sequence. Following production of the fusosome, it is additionally nucleofected with an mRNA having a sequence that codes for the CAR. See, for example, Chen X, et al., Genes Dis. 2015 Mar;2(1):96- 105.DOI:10.1016/j.gendis.2014.12.001.

[0001114] As a negative control, fusosomes are nucleofected with an mRNA that codes for GFP.

[0001115] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with recipient CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter for a period of 48h in T-cell medium comprising X-VIVO 15 medium (Lonza, Basel, CH, Switzerland) supplemented with 5% fetal bovine serum (FBS) (Gibco, LAX, CA, USA), 100 U mL– 1 penicillin, 100 µg mL – 1 streptomycin, 1.25 µg mL – 1 amphotericin B, 2 mM L-glutamine (Gibco), and 100 U mL – 1 hIL-2 (PerproTech, Rocky Hill, CT, USA). Fusosome fusion with the CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression. Following the 48 hr incubation, the tdTomato positive cells are then isolated via FACS, using a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission is collected at 590+/-20nm.

[0001116] Total RNA is isolated (e.g., using a kit such as Qiagen RNeasy catalog #74104), followed by determination of RNA concentration using standard spectroscopic methods to assess light absorbance by RNA (e.g. with Thermo Scientific NanoDrop). Reverse transcription is performed using the Superscript III First-Strand Synthesis supermix for RT-PCR (Thermo Fisher Scientific), and RNA (100 ng) is reverse transcribed into cDNA. Quantitative Real-time PCR is performed using a QuantStudio3 Real-time PCR System (ThermoFisher Scientific) with TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific), 100ng of cDNA template, a primer and probe set that is specific for the variable regions of the anti-CD19 CAR (designed using Taqman online primer and probe design program), and primer probe set designed to amplify β–actin as an endogenous loading control. The C_t value is used to compare the amount of CAR

cDNA in the qRT-PCR reaction between CD3+ T cells treated with fusosomes containing the CAR mRNA and treated with fusosomes containing negative control. The relative expression is calculated using the ΔΔCt method. A higher relative expression level of CAR is due to a higher level of CAR mRNA that is purified from the sorted CD3+ T cells.

[0001117] In some embodiments, delivery of mRNA cargo with fusosomes *in vitro* is higher in fusosomes containing CAR mRNA as compared to negative control fusosomes.

c. Protein / mRNA Payload, wherein payload is expressed by donor cell

[0001118] This example describes fusosome fusion with a cell *in vitro*. In some embodiments, fusosome fusion with a CD3+ T cell in vitro results in delivery of a chimeric antigen receptor protein to the membrane of the CD3+ T cell.

[0001119] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre and with the open reading frame of a CAR that targets CD19, separated by a P2A and T2A self-cleaving peptide sequence respectively. A negative control fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre and with the open reading frame of the blue fluorescent protein mTagBFP2, each separated by a P2A self-cleaving peptide sequence. See, for example, Chen X, et al., Genes Dis. 2015 Mar;2(1):96-105.DOI:10.1016/j.gendis.2014.12.001.

[0001120] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with recipient CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter for a period of 48h in T-cell medium comprising X-VIVO 15 medium (Lonza, Basel, CH, Switzerland) supplemented with 5% fetal bovine serum (FBS) (Gibco, LAX, CA, USA), 100 U mL– 1 penicillin, 100 μg mL – 1 streptomycin, 1.25 μg mL – 1 amphotericin B, 2 mM L-glutamine (Gibco), and 100 U mL – 1 hIL-2 (PerproTech, Rocky Hill, CT, USA. Fusosome fusion with the CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression. Following the 48 hr incubation, the tdTomato positive cells are then isolated via FACS, using a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission is collected at

590+/-20nm.

[0001121] mRNA delivery to the sorted T cells is assayed. Total RNA is isolated (e.g., using a kit such as Qiagen RNeasy catalog #74104), followed by determination of RNA concentration using standard spectroscopic methods to assess light absorbance by RNA (e.g. with Thermo Scientific NanoDrop). Reverse transcription is performed using the Superscript III First-Strand Synthesis supermix for RT-PCR (Thermo Fisher Scientific), and RNA (100 ng) is reverse transcribed into cDNA. Quantitative Real-time PCR is performed using a QuantStudio3 Real-time PCR System (ThermoFisher Scientific) with TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific), 100ng of cDNA template, a primer and probe set that is specific for the variable regions of the anti-CD19 CAR (designed using Taqman online primer and probe design program), and primer probe set designed to amplify β–actin as an endogenous loading control. The C_t value is used to compare the amount of CAR cDNA in the qRT-PCR reaction between CD3+ T cells treated with fusosomes containing the CAR mRNA and treated with fusosomes containing negative control. The relative expression is calculated using the ΔΔCt method. A higher relative expression level of CAR is due to a higher level of CAR mRNA that is purified from the sorted CD3+ T cells.

[0001122] In some embodiments, delivery of the CAR mRNA cargo with fusosomes *in vitro* is higher in fusosomes derived from cells expressing the CAR as compared to the negative control fusosomes derived from cells expressing CFP.

[0001123] CAR expression on the surface of the sorted cells is assayed. Sorted tdTomato+ CD3+ cells are incubated with CD19sIg1-4 conjugated to Alexa Fluor 488 (CD19sIg1-4:AF488), as described in De Oliveira et al., J Transl Med 11:23, 2013. CD19sIg1-4:AF488 labels cells that express CD19 CAR. 2×10^5 cells are incubated with 450 ng of CD19sIg1-4:AF488 at 4°C for 30 minutes in the dark, after being blocked by human serum from AB plasma (Sigma-Aldrich) for 10 minutes. After being washed two times with PBS, cells are analyzed on a LSR II (BD Biosciences, San Jose, CA.) machine running the FACSDiva™ software (BD Biosciences, San Jose, CA.). tdTomato+ CD3+ cells cells that were incubated with the negative control fusogen are used to set up the negative gate for CD19sIg1-4:AF488 signal. The gate is chosen such that % of positive events for CD19sIg1-4:AF488 is equal to 0.0% The percent of events that are positive for CD19sIg1-4:AF488 is measured in sorted cells that were treated with fusosomes derived from

cells expressing the CAR.

[0001124] In some embodiments, the percent of sorted cells with surface CAR expression is higher in cells treated with fusosomes derived from cells expressing the CAR as compared to the negative control fusosomes derived from cells expressing mTagBFP2.

Example 110: T-cell Specific Fusosome Fusing to T cell In Vitro

[0001125] This example describes fusosome fusion that is preferential for a CD3+ T cell *in vitro*. In some embodiments, the fusosome delivers its payload to a CD3+ T cell at a greater efficiency than an alternative cell type.

[0001126] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre but separated by a P2A self-cleaving peptide sequence.

[0001127] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with recipient CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter for a period of 48h in T-cell medium comprising X-VIVO 15 medium (Lonza, Basel, CH, Switzerland) supplemented with 5% fetal bovine serum (FBS) (Gibco, LAX, CA, USA), 100 U mL– 1 penicillin, 100 µg mL – 1 streptomycin, 1.25 µg mL – 1 amphotericin B, 2 mM L-glutamine (Gibco), and 100 U mL – 1 hIL-2 (PerproTech, Rocky Hill, CT, USA. Fusosome fusion with the CD3+ T cells that have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression.

[0001128] In a separate experiment, the same number of fusosomes are incubated at 37°C and 5% CO_2 together with a recipient NIH/3T3 fibroblast cell line that has a loxP-STOP-loxP-tdTomato reporter for a period of 48h in in DMEM containing 20% Fetal Bovine Serum and 1x Penicillin/Streptomycin. Fusosome fusion with the NIH/3T3 fibroblasts that have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression.

[0001129] Following the 48 hr incubations, cells are run on a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission collected at 590+/-

20nm. A gate is set up to measure positive tdTomato expression. The gate is chosen such that CD3+ T cells and NIH/3T3 fibroblasts that have not been contacted with fusosomes are all negative. The percent of cells that are positive for tdTomato expression is measured in CD3+ T cells and NIH/3T3 fibroblasts that have been contacted by fusosomes.

[0001130] In some embodiments, a percent of cells that are positive for tdTomato expression is higher in CD3+ cells contacted with fusosomes than in NIH/3T3 fibroblasts contacted with fusosomes, which demonstrates that fusosomes fuse preferentially with the target CD3+ cells.

Example 111: T-cell Specific Fusosome Fusing to T cell In Vivo

[0001131] This example describes fusosome fusion that is preferential for a CD3+ T cell *in vivo*. In some embodiments, a fusosome delivers its payload to a CD3+ T cell at a greater efficiency than any alternative cell type.

[0001132] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre but separated by a P2A self-cleaving peptide sequence.

[0001133] 3 x 10^{11} fusosomes or PBS are then administered slowly over 20 min through a rodent tail-vein catheter using a programmable BS-300 infusion pump (both Braintree Scientific Inc.) daily for 5 days to B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice (Jackson Laboratories strain 007909). Three days after the final treatment, peripheral blood is collected from fusosome treated mice and mice that received PBS treatment. Blood is collected into 1ml PBS containing 5 μM EDTA and mixed immediately to prevent clotting. The tubes are kept on ice and red blood cells are removed using a buffered ammonium chloride (ACK) solution. Cells are stained with a murine CD3-FITC antibody (Thermo Fisher **Catalog #:**11-0032-82), at 4°C for 30 minutes in the dark, after being blocked with bovsine serum albumin for 10 minutes. After being washed two times with PBS, cells are analyzed on a LSR II (BD Biosciences, San Jose, CA.) with 488nm laser excitation and emission collected at 530+/-30nmrunning the FACSDiva™ software (BD Biosciences, San Jose, CA.). Unstained cells from mice that received PBS treatment are used to draw a gate for negative FITC and negative tdTomato fluorescence.

[0001134] In some embodiments, a percent of cells that are positive for tdTomato fluorescence is higher in mice treated with fusosomes than in mice treated with PBS. In some embodiments, a percent of tdTomato positive cells that stain positive for FITC is greater than those that stain negative for FITC in mice treated with fusosomes. This demonstrates that fusosomes targeting CD3+ cells specifically fuse with CD3+ cells *in vivo*.

Example 112: T cells engineered *in vitro* lyse cells associated with a target antigen *in vitro*

[0001135] This example demonstrates that CD3+ T cells expressing a CAR after being contacted by a fusosome as described in any one of the methods in previous Examples are capable of lysing cells associated with a target antigen, e.g., CD19, *in vitro*.

[0001136] CD3+ T cells expressing a CAR targeting CD19 are incubated with CD19+ Eμ-ALL01 leukaemia cells (target) or CD19- B16F10 melanoma tumor cells (control). Prior to the incubation, the CD3+ T cells are activated using CD3- and CD28-specific magnetic beads at three beads/cell (Invitrogen Life Technologies, Carlsbad, CA, USA and theEμ-ALL01 leukaemia cells and B16F10 melanoma tumor cells are labeled with membrane dye PKH-26 (Sigma-Aldrich), washed with RPMI containing 10% foetal calf serum, and resuspended in the same medium at a concentration of $1x10^5$ tumor cells per mL. T cells are then added to the suspension at various ratios of T cell to tumor cell, ranging from 0 T cells : 1 tumor cells to 100 T cells : 1 tumor cells, in 96-well plates (final volume, 200 μl), and incubated for 3 h at 37 °C. Then, cells are transferred to V-bottom 96-well plates and stained with Annexin V-Brilliant Violet 421 (BioLegend). Following a wash in PBS, the cells are analyzed by flow cytometry on a LSR II (BD Biosciences, San Jose, CA.) machine running the FACSDiva™ software (BD Biosciences, San Jose, CA.). Cells from the incubation of 0 T cells : 1 tumor cells and a separate batch of T cells are first run on the flow cytometer. A gate is first drawn to distinguish T cells and tumor cells based upon PKH-26 fluorescence. The gate is drawn such that T cells are negative and tumor cells that were incubated with PKH-26 are positive. Next, a gate is drawn to measure Annexin V-Brilliant Violet 421 staining. The gate is drawn such that the cells from the incubation of 0 T cells : 1 tumor cells are all negative for Annexin V-Brilliatn Violet 421. Using these two gates, cells from each of the incubations of various ratios of T cells to tumor cells are run on the flow cytometer.

[0001137] In some embodiments, a percent of cells that are positive for PKH-26 and Annexin V-Brilliant Violet 421 increases with increasing ratios of T cells to tumor cells for the Eμ-ALL01 leukaemia cells, and the percent of cells that are positive for PKH-26 and Annexin V-Brilliant Violet 421 does not increase with increasing ratios of T cells to tumor cells for the B16F10 melanoma tumor cells. This demonstrates that T cells that express a CAR targeting CD19 after being contacted by fusomes are capable of specifically lysing cells that are CD19-positive.

[0001138] See, for example, Smith T, et al., Nature Nanotechnology. 2017. DOI: 10.1038/NNANO.2017.57

Example 113: T cells engineered *in vivo* lyse cells associated with a target antigen *in vitro*

[0001139] This example demonstrates that CD3+ T cells engineered to express a CAR after being contacted by a fusosomes *in vivo* are capable of lysing cells associated with a target antigen *in vitro*.

[0001140] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre but separated by a P2A self-cleaving peptide sequence. In addition, the fusosome is engineered to deliver a CAR targeting CD-19 to a target cell as described in previous Examples.

[0001141] 3×10^{11} fusosomes or PBS are then administered slowly over 20 min through a rodent tail-vein catheter using a programmable BS-300 infusion pump (both Braintree Scientific Inc.) daily for 5 days to B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice (Jackson Laboratories strain 007909). Three days after the final treatment, peripheral blood is collected from fusosome treated mice and mice received PBS treatment. Blood is collected into 1ml PBS containing 5 μM EDTA and mixed immediately to prevent clotting. The tubes are kept on ice and red blood cells are removed using a buffered ammonium chloride (ACK) solution. Cells are stained with a murine CD3-FITC antibody (Thermo Fisher **Catalog #:**11-0032-82), at 4°C for 30 minutes in the dark, after being blocked with bovsine serum albumin for 10 minutes. After being washed two times with PBS, cells are analyzed on a LSR II (BD Biosciences, San Jose, CA.) with 488nm laser excitation and emission collected at 530+/-30nmrunning the FACSDiva™ software (BD

Biosciences, San Jose, CA.). Sorted cells from mice treated with fusosomes or PBS are then incubated with CD19+ Eμ-ALL01 leukaemia cells. Prior to the incubation, Eμ-ALL01 leukaemia cells are labeled with membrane dye PKH-26 (Sigma-Aldrich), washed with RPMI containing 10% foetal calf serum, and resuspended in the same medium at a concentration of $1x10^5$ tumor cells per mL. T cells are then added to the suspension at various ratios of T cell to tumor cell, ranging from 0 T cells : 1 tumor cells to 100 T cells : 1 tumor cells, in 96-well plates (final volume, 200 μl), and incubated for 3 h at 37 °C. Then, cells are transferred to V-bottom 96-well plates and stained with Annexin V-Brilliant Violet 421 (BioLegend). Following a wash in PBS, the cells are analyzed by flow cytometry on a LSR II (BD Biosciences, San Jose, CA.) machine running the FACSDiva™ software (BD Biosciences, San Jose, CA.). Cells from the incubation of 0 T cells : 1 tumor cells and a separate batch of sorted T cells are first run on the flow cytometer. A first gate is first drawn to distinguish T cells and tumor cells based upon PKH-26 fluorescence. The gate is drawn such that T cells are negative and tumor cells that were incubated with PKH-26 are positive. Next, a gate is drawn to measure Annexin V-Brilliant Violet 421 staining. The gate is drawn such cells from the incubation of 0 T cells : 1 tumor cells are all negative for Annexin V-Brilliant Violet 421. Using these two gates, cells from each of the incubations of various ratios of T cells to tumor cells are run on the flow cytometer.

[0001142] In some embodiments, a percent of cells that are positive for PKH-26 and Annexin V-Brilliant Violet 421 increases with increasing ratios of T cells to Eμ-ALL01 leukaemia cells from mice treated with fusosomes, and the percent of cells that are positive for PKH-26 and Annexin V-Brilliant Violet 421 does not increase with increasing ratios of T cells to Eμ-ALL01 leukaemia cells from mice treated with PBS. This demonstrates that T cells engineered to express a CAR that targets CD19 after treatment with fusosomes are capable of lysing cells associated with CD19. See, for example, Smith T, et al., Nature Nanotechnology. 2017. DOI: 10.1038/NNANO.2017.57

Example 114: T cells engineered in vivo lyse tumor cells associated with a target antigen in vivo

[0001143] This example demonstrates that CD3+ T cells engineered to express a CAR after being contacted by a fusosomes *in vivo* are capable of treating a tumor *in vivo*.

[0001144] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre but separated by a P2A self-cleaving peptide sequence. In addition, the fusosome is engineered to deliver a CAR targeting CD-19 as described in previous Examples.

[0001145] A model of leukemia is established by systemically injecting luciferase-expressing Eμ-ALL01 leukaemia cells into 4–6-week-old female albino B6 (C57BL/6J-Tyr < c-2J>) mice (Jackson Laboratories) and allowing them to develop for 1 week. Mice are then randomly assigned to experimental cohorts. 3 x 10^{11} fusosomes or PBS are then administered slowly over 20 min through a rodent tail-vein catheter using a programmable BS-300 infusion pump (both Braintree Scientific Inc.) daily for 5 days.

[0001146] Luminescence as a proxy for the number of live leukemia cells is then measured daily. D-luciferin (Xenogen) in PBS (15 mg mL–1) is used as a substrate for F-luc expressed by the leukemia cells. Bioluminescence images are collected with a Xenogen IVIS Spectrum Imaging System (Xenogen). Living Image software version 4.3.1 (Xenogen) is used to acquire (and later quantitate) the data obtained over a range of 10-35 min after intraperitoneal injection of D-luciferin into animals anesthetized with 150 mg kg–1 of 2% isoflurane (Forane, Baxter Healthcare). Acquisition times range from 10 s to 5 min. To correct for background bioluminescence, the signals acquired from tumor-free mice (injected with D-luciferin) is subtracted from the measurement region of interest (ROI).

[0001147] In embodiments, the luciferase signal increases over the course of 21 days in mice treated with PBS and the luciferase signal decreases over the course of 21 days in mice treated with fusosomes.

[0001148] The survival of mice that received PBS or fusosomes is also tracked. In embodiments, mice that received PBS have a median survival that is less than mice treated with fusosomes.

[0001149] This demonstrates that fusosomes are capable of engineering T cells to target tumor cells *in vivo*. See, for example, Smith T, et al., Nature Nanotechnology. 2017. DOI: 10.1038/NNANO.2017.57

Example 115: Fusosomes deliver a transmembrane protein to recipient cells

[0001150] This example describes fusosome fusion with a cell *in vitro*. In some embodiments, fusosome fusion results in delivery of a transmembrane protein to a recipient cell.

[0001151] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre and with the open reading frame of the human Insulin Receptor, separated by a P2A and T2A self-cleaving peptide sequence respectively. A negative control fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre and with the open reading frame of the blue fluorescent protein mTagBFP2, each separated by a P2A self-cleaving peptide sequence. See, for example, Chen X, et al., Genes Dis. 2015 Mar;2(1):96-105.DOI:10.1016/j.gendis.2014.12.001.

[0001152] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with recipient HEK293 cells that have a loxP-STOP-loxP-tdTomato reporter for a period of 48h in complete media (DMEM + 10% FBS + Pen/Strep). Fusosome fusion with the HEK293 cells that have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression. Following the 48 hr incubation, the tdTomato positive cells are then isolated via FACS, using a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission is collected at 590+/-20nm.

[0001153] mRNA delivery to the sorted HEK293 cells is assayed. Total RNA is isolated (e.g., using a kit such as Qiagen RNeasy catalog #74104), followed by determination of RNA concentration using standard spectroscopic methods to assess light absorbance by RNA (e.g. with Thermo Scientific NanoDrop). Reverse transcription is performed using the Superscript III First-Strand Synthesis supermix for RT-PCR (Thermo Fisher Scientific), and RNA (100 ng) is reverse transcribed into cDNA. Quantitative Real-time PCR is performed using a QuantStudio3 Real-time PCR System (ThermoFisher Scientific) with TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific), 100ng of cDNA template, a primer and probe set that is specific for the variable regions of the human Insulin Receptor (designed using Taqman online primer and probe design program),

and primer probe set designed to amplify β–actin as an endogenous loading control. The C_t value is used to compare the amount of Insulin Receptor cDNA in the qRT-PCR reaction between HEK293 cells treated with fusosomes containing the Insulin Receptor mRNA and treated with fusosomes containing negative control. The relative expression is calculated using the ΔΔCt method. A higher relative expression level of Insulin Receptor is due to a higher level of Insulin Receptor mRNA that is purified from the sorted HEK293 T cells.

[0001154] In some embodiments, delivery of the Insulin Receptor mRNA cargo with fusosomes *in vitro* is higher in fusosomes derived from cells expressing the Insulin Receptor as compared to the negative control fusosomes derived from cells expressing CFP.

[0001155] Insulin Receptor expression on the surface of the sorted cells is assayed. Sorted tdTomato+ HEK293 cells are incubated with Insulin Receptor Alpha Antibody conjugated to Alexa Fluor 488 (Bioss Antibodies, catalog number bs-0260R-A488). The antibody labels cells that express the Insulin Receptor proportional to the amount of Insulin Receptor expression. 2×10^5 cells are incubated with 450 ng of the Insulin Receptor Alpha Antibody conjugated to Alexa Fluor 488 at 4°C for 30 minutes in the dark, after being blocked by human serum from AB plasma (Sigma-Aldrich) for 10 minutes. After being washed two times with PBS, cells are analyzed on a LSR II (BD Biosciences, San Jose, CA.) machine running the FACSDiva™ software (BD Biosciences, San Jose, CA.). tdTomato+ HEK293 cells that were incubated with the negative control fusogen are used to set up the negative gate for the Insulin Receptor Alpha Antibody conjugated to Alexa Fluor 488 signal. The gate is chosen such that the percent of positive events for Insulin Receptor Alpha Antibody conjugated to Alexa Fluor 488 is equal to 0.0% The percent of events that are positive for the Insulin Receptor Alpha Antibody conjugated to Alexa Fluor 488 is measured in sorted cells that were treated with fusosomes derived from cells expressing the Insulin Receptor.

[0001156] In some embodiments, the percent of sorted cells with surface Insulin Receptor expression is higher in cells treated with fusosomes derived from cells expressing the Insulin Receptor as compared to the negative control fusosomes derived from cells expressing mTagBFP2.

Example 116: Fusosomes deliver a heterologous, signal peptide-targeted transmembrane protein to recipient cells

[0001157] This example describes fusosome fusion with a cell *in vitro*. In some embodiments, fusosome fusion with a recipient cell results in the delivery of a heterologous membrane protein payload to the recipient cell's plasma membrane.

[0001158] A fusosome composition, resulting from cell-derived vesicles or cell-derived cytobiologics as produced by any one of the methods described in previous Examples, except the fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre and with the open reading frame of a membrane-targeted GFP, separated by a P2A and T2A self-cleaving peptide sequence respectively. The membrane-targeted GFP is generated by fusing the N terminus of the coding sequence of GFP to the first twenty-six amino acids of LCK, a Src family tyrosine kinase containing two palmitoylation domains and a single myristoylation domain. A negative control fusosome is engineered such that the fusogen is in-frame with the open reading frame of Cre and with the open reading frame of cytosolic GFP (the coding sequence of GFP without any additional targeting peptide sequences), each separated by a P2A self-cleaving peptide sequence. See, for example, Chen X, et al., Genes Dis. 2015 Mar;2(1):96 105.DOI:10.1016/j.gendis.2014.12.001, and Benediktsson A, et al, Journal of Neuroscience Methods 2005 141, 41-53.

[0001159] A sufficient number of fusosomes are then incubated at 37°C and 5% CO_2 together with recipient HEK293 cells that have a loxP-STOP-loxP-tdTomato reporter for a period of 48h in complete media (DMEM + 10% FBS + Pen/Strep). Fusosome fusion with the HEK293 cells that have a loxP-STOP-loxP-tdTomato reporter results in tdTomato expression due to Cre recombinase excising from DNA the stop codon that blocks tdTomato expression. Following the 48 hr incubation, the tdTomato positive cells are then isolated via FACS, using a FACS cytometer (Becton Dickinson, San Jose, CA, USA) with 561nm laser excitation and emission is collected at 590+/-20nm.

[0001160] Membrane localization of GFP in the plasma membrane of the sorted HEK293 cells is assayed via confocal microscopy. Prior to confoncal microscopy sorted HEK293 cells are stained with a reagent that labels the plasma membrane (e.g. CellMask Deep Red Plasma

Membrane Stain, Invitrogen, Catalog number C10046). Imaging experiments are performed with a Zeiss LSM 710 inverted microscope using a Plan Apochromat 63 x 1.4 numerical aperture oil objective. A 488 nm argon laser is used to excite GFP/EGFP and a 632 nm Helium-Neon laser is used to excite the plasma membrane stain. A MATLAB script is written to determine the average GFP intensity of the plasma membrane and cytosol for each cell. In the script the average intensity in the plasma membrane (defined by 6 pixels on either side of the plasma membrane as defined by the plasma membrane stain) and the cytosol (region within the plasma membrane region) are calculated. The values for plasma membrane and cytosol intensity for each cell are then used to calculate the %plasma membrane localization. The %plasma membrane localization is calculated with the following equation: plasma membrane intensity over the total (plasma membrane + cytosol) intensity x 100%. See, for example, Johnson A, et al., Scientific Reports 6: 19125 (2015).

[0001161] In some embodiments, the sorted cells treated with fusosomes containing plasma-membrane localized GFP have higher % plasma membrane localization of GFP than the sorted cells treated with fusosomes containing cytosolic GFP.

CLAIMS

What is claimed is:

1. A fusosome comprising:

(a) a lipid bilayer comprising a plurality of lipids derived from a source cell;

(b) a lumen (e.g., comprising cytosol) surrounded by the lipid bilayer;

(c) a fusogen that is exogenous or overexpressed relative to the source cell, e.g., wherein the fusogen is disposed in the lipid bilayer; and

(d) a membrane protein payload agent (e.g., which is exogenous or overexpressed relative to the source cell) that comprises or encodes one or more of:

i) a chimeric antigen receptor;

ii) an integrin membrane protein payload, e.g., chosen from Table 5;

iii) an ion channel protein chosen from Table 6;

iv) a pore forming protein, e.g., chosen from Tables 7 and 8;

v) a Toll-Like Receptor, e.g., chosen from Table 9;

vi) an interleukin receptor payload, e.g., chosen from Table 10;

vii) a cell adhesion protein chosen from Tables 11-12;

viii) a transport protein chosen from Table 15;

ix) a signal sequence that is heterologous relative to the naturally-occurring membrane protein; or

x) a signal sequence listed in Table 4;

wherein the fusosome does not comprise a nucleocapsid protein or a viral matrix protein.

2. The fusosome of claim 1, wherein the source cell is a primary cell, a cultured cell, an immortalized cell, or a cell line (e.g., myelobast cell line, e.g., C2C12).

3. The fusosome of claim 1 or 2, wherein the source cell is an endothelial cell, a fibroblast, a blood cell (e.g., a macrophage, a neutrophil, a granulocyte, a leukocyte), a stem cell (e.g., a mesenchymal stem cell, an umbilical cord stem cell, bone marrow stem cell, a hematopoietic stem cell, an induced pluripotent stem cell e.g., an induced pluripotent stem cell derived from a subject's cells), an embryonic stem cell (e.g., a stem cell from embryonic yolk sac, placenta, umbilical cord,

fetal skin, adolescent skin, blood, bone marrow, adipose tissue, erythropoietic tissue, hematopoietic tissue), a myoblast, a parenchymal cell (e.g., hepatocyte), an alveolar cell, a neuron (e.g., a retinal neuronal cell) a precursor cell (e.g., a retinal precursor cell, a myeloblast, myeloid precursor cells, a thymocyte, a meiocyte, a megakaryoblast, a promegakaryoblast, a melanoblast, a lymphoblast, a bone marrow precursor cell, a normoblast, or an angioblast), a progenitor cell (e.g., a cardiac progenitor cell, a satellite cell, a radial gial cell, a bone marrow stromal cell, a pancreatic progenitor cell, an endothelial progenitor cell, a blast cell), or an immortalized cell (e.g., HeLa, HEK293, HFF-1, MRC-5, WI-38, IMR 90, IMR 91, PER.C6, HT-1080, or BJ cell).

4. The fusosome of any of the preceding claims, wherein the source cell is allogeneic, e.g., obtained from a different organism of the same species as the target cell.

5. The fusosome of any of the preceding claims, wherein the source cell is autologous, e.g., obtained from the same organism as the target cell.

6. The fusosome of any of the preceding claims, wherein the source cell is selected from a white blood cell or a stem cell.

7. The fusosome of any of the preceding claims, wherein the source cell is selected from a neutrophil, a lymphocyte (e.g., a T cell, a B cell, a natural killer cell), a macrophage, a granulocyte, a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell, or a myeloblast.

8. The fusosome of any of the preceding claims, wherein the fusosome is from a source cell having a modified genome, e.g., having reduced immunogenicity (e.g., by genome editing to remove MHC complexes).

9. The fusosome of any of the preceding claims, wherein the fusosome has a diameter that is less than about 0.01% or 1%, of that of the source cell, e.g., as measured by an assay of Example 30.

10. The fusosome of any of the preceding claims, wherein the fusogen is a mammalian fusogen or a viral fusogen.

11. The fusosome of any of the preceding claims, wherein the fusogen is active at a pH of 6-8.

12. The fusosome of any of the preceding claims, wherein the fusosome comprises a membrane protein payload agent at a copy number of at least 1,000 copies, e.g., as measured by an assay of Example 43.

13. The fusosome of any of the preceding claims, wherein:

i) the fusosome meets a pharmaceutical or good manufacturing practices (GMP) standard;

ii) the fusosome was made according to good manufacturing practices (GMP);

iii) the fusosome has a pathogen level below a predetermined reference value, e.g., is substantially free of pathogens; or

iv) the fusosome has a contaminant level below a predetermined reference value, e.g., is substantially free of contaminants.

14. The fusosome of any of the preceding claims, wherein the membrane protein payload agent is a membrane protein, disposed in the fusosome lipid bilayer.

15. The fusosome of any of claims 1-13, wherein the membrane protein payload agent is a nucleic acid, disposed in the fusosome lumen, that encodes a membrane protein.

16. The fusosome of any of the preceding claims, wherein the membrane protein payload agent is or comprises a chimeric antigen receptor (CAR) comprising an antigen binding domain.

17. The fusosome of any of the preceding claims, wherein the target cell is in an organism.

18. The fusosome of any of claims 1-16, wherein the target cell is a primary cell isolated from an organism.

19. The fusosome of any of the preceding claims, wherein the target cell is selected from an endothelial cell, a fibroblast, a blood cell (e.g., a macrophage, a neutrophil, a granulocyte, a leukocyte), a stem cell (e.g., a mesenchymal stem cell, an umbilical cord stem cell, bone marrow stem cell, a hematopoietic stem cell, an induced pluripotent stem cell e.g., an induced pluripotent stem cell derived from a subject's cells), an embryonic stem cell (e.g., a stem cell from embryonic yolk sac, placenta, umbilical cord, fetal skin, adolescent skin, blood, bone marrow, adipose tissue, erythropoietic tissue, hematopoietic tissue), a myoblast, a parenchymal cell (e.g., hepatocyte), an alveolar cell, a neuron (e.g., a retinal neuronal cell) a precursor cell (e.g., a retinal precursor cell, a myeloblast, myeloid precursor cells, a thymocyte, a meiocyte, a megakaryoblast, a promegakaryoblast, a melanoblast, a lymphoblast, a bone marrow precursor cell, a normoblast, or an angioblast), a progenitor cell (e.g., a cardiac progenitor cell, a satellite cell, a radial gial cell, a bone marrow stromal cell, a pancreatic progenitor cell, an endothelial progenitor cell, a blast cell), or an immortalized cell (e.g., HeLa, HEK293, HFF-1, MRC-5, WI-38, IMR 90, IMR 91, PER.C6, HT-1080, or BJ cell).

20. The fusosome of any of the preceding claims, wherein the target cell is selected from a neutrophil, a lymphocyte (e.g., a T cell, a B cell, a natural killer cell), a macrophage, a granulocyte, a mesenchymal stem cell, a bone marrow stem cell, an induced pluripotent stem cell, an embryonic stem cell, or a myeloblast.

21. The fusosome of any of the preceding claims, wherein the fusosome comprises a targeting domain which localizes the fusosome to a target cell.

22. The fusosome of claim 21, wherein the targeting domain interacts with a target cell moiety on the target cell.

23. A method of manufacturing a fusosome composition, comprising:

i) providing a plurality of fusosomes according to any of claims 1-22; and

ii) formulating the plurality of fusosomes, fusosome composition, or pharmaceutical composition e.g., as a fusosome drug product suitable for administration to a subject.

24. The method of claim 23, wherein the fusosome is from a mammalian cell having a modified genome, e.g., having reduced immunogenicity (e.g., by genome editing to remove MHC complexes).

25. A method of manufacturing a fusosome drug product composition, comprising:

a) providing, e.g., producing, providing a plurality of fusosomes according to any of claims 1-22; and

b) assaying one or more fusosomes from the plurality to determine the presence or level of one or more of the following factors:

i) an immunogenic molecule, e.g., an immunogenic protein, e.g., as described herein;

ii) a pathogen, e.g., a bacterium or virus; or

iii) a contaminant;

c) (optionally) approving the plurality of fusosomes or fusosome composition for release if one or more of the factors is below a reference value;

thereby manufacturing a fusosome drug product composition.

26. A method of administering a fusosome composition to a subject, e.g., a human subject, comprising administering to the subject a fusosome composition comprising a plurality of fusosomes according to any of claims 1-22, thereby administering the fusosome composition to the subject.

27. A method of delivering a protein membrane payload to a subject, comprising administering to the subject a fusosome composition comprising a plurality of fusosomes according to any of claims 1-22, wherein the fusosome composition is administered in an amount and/or time such

that the protein membrane payload is delivered.

28. A method of treating a disease or disorder in a patient comprising administering to the subject a plurality of fusosomes according to any of claims 1-22, wherein the fusosome composition is administered in an amount and/or time such that the disease or disorder is treated.

29. The method of claim 28, wherein the disease or disorder is selected from cancer, autoimmune disorder, or infectious disease.