COMPOSITIONS AND METHODS FOR INTERNALIZING ENZYMES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Nos. 62/673,098, filed May 17, 2018; 62/574,719 filed October 19, 2017; and 62/516,656 filed June 7, 2017, each of which is hereby incorporated in their entireties by reference.

FIELD OF THE INVENTION

[0002] This application is generally directed to compositions and methods for treating lysosomal storage diseases. This application is directed specifically to targeted protein complexes that contain replacement enzymes and their use in treating lysosomal storage diseases.

BACKGROUND

[0003] Lysosomal storage diseases are a class of rare diseases that affect the degradation of myriad substrates in the lysosome. Those substrates include sphingolipids, mucopolysaccharides, glycoproteins, glycogen, and oligosaccharides, which can accumulate in the cells of those with disease leading to cell death. Organs affected by lysosomal storage diseases include the central nervous system (CNS), the peripheral nervous system (PNS), lungs, liver, bone, skeletal and cardiac muscle, and the reticuloendothelial system.

[0004] Options for the treatment of lysosomal storage diseases include enzyme replacement therapy (ERT), substrate reduction therapy, pharmacological chaperone-mediated therapy, hematopoietic stem cell transplant therapy, and gene therapy. An example of substrate reduction therapy includes the use of Miglustat or Eliglustat to treat Gaucher Type 1. These drugs act by blocking synthase activity, which reduces subsequent substrate production. Hematopoietic stem cell therapy (HSCT), for example, is used to ameliorate and slow-down the negative central nervous system phenotype in patients with some forms of MPS. See R.M. Boustany, "Lysosomal storage

diseases--the horizon expands," 9(10) Nat. Rev. Neurol. 583-98, Oct. 2013. Table 1 lists some lysosomal storage diseases and their associated enzymes or other proteins.

Table 1: Lysosomal Storage Diseases

Class	Disease	Involved Enzyme/Protein
Sphingolipidoses	Fabry disease	α-Galactosidase A
	Farber lipogranulomatosis	Ceramidase
	Gaucher disease type I	β-Glucosidase
	Gaucher disease types II and III	Saposin-C activator
	Niemann-Pick diseases types A and B	Sphingomyelinase
	GM1-gangliosidosis	β -Galactosidase
	GM2-gangliosidosis (Sandhoff)	β -Hexosaminidase A and B
	GM2-gangliosidosis (Tay-Sachs)	β -Hexosaminidase A
	GM2-gangliosidosis (GM2-activator deficiency)	GM2-activator protein
	GM3-gangliosidosis	GM3 synthase
	Metachromatic leukodystrophy	Arylsulfatase A
	Sphingolipid-activator deficiency	Sphingolipid activator
Mucopoly-saccharidoses	MPS I (Scheie, Hurler-Scheie, and Hurler disease)	α-Iduronidase
	MPS II (Hunter)	Iduronidase-2-sulphatase
	MPS IIIA (Sanfilippo A)	Heparan *N*-sulphatase
	MPS IIIB (Sanfilippo B)	*N*-acetyl-α-glucosaminidase
	MPS IIIC (Sanfilippo C)	Acetyl-CoA; α-glucosamide *N*-acetyltransferase
	MPS IIID (Sanfilippo D)	*N*-acetylglucosamine-6-sulphatase
	MPS IVA (Morquio syndrome A)	*N*-acetylgalactosamine-6-sulphate sulphatase
	MPS IVB (Morquio syndrome B)	β -Galactosidase
	MPS VI (Maroteaux-Lamy)	*N*-acetylgalactosamine-4-sulphatase (arylsulphatase B)
	MPS VII (Sly disease)	β -Glucuronidase
	MPS IX	Hylauronidase
Glycogen storage disease	Pompe (glycogen storage disease type II)	α-Glucosidase 2
Lipid metabolism	Lysosomal acid lipase deficiency (LAL-D; Wolman disease)	Lysosomal acid lipase

[0005] Two of the most common LSDs are Pompe disease and Fabry disease. Pompe disease, which has an estimated incidence of 1 in 10,000, is caused by defective lysosomal enzyme alpha-glucosidase (GAA), which results in the deficient processing of lysosomal glycogen. Accumulation

of lysosomal glycogen occurs predominantly in skeletal, cardiac, and hepatic tissues. Infantile onset Pompe causes cardiomegaly, hypotonia, hepatomegaly, and death due to cardiorespiratory failure, usually before 2 years of age. Adult onset Pompe occurs as late as the second to sixth decade and usually involves only skeletal muscle. Treatments currently available include Genzyme's MYOZYME®/LUMIZYME® (alglucosidase alfa), which is a recombinant human alpha-glucosidase produced in CHO cells and administered by intravenous infusion.

[0006] Fabry disease, which has including mild late onset cases an overall estimated incidence of 1 in 3,000, is caused by defective lysosomal enzyme alpha-galactosidase A (GLA), which results in the accumulation of globotriaosylceramide within the blood vessels and other tissues and organs. Symptoms associated with Fabry disease include pain from nerve damage and/or small vascular obstruction, renal insufficiency and eventual failure, cardiac complications such as high blood pressure and cardiomyopathy, dermatological symptoms such as formation of angiokeratomas, anhidrosis or hyperhidrosis, and ocular problems such as cornea verticillata, spoke-like cataract, and conjunctival and retinal vascular abnormalities. Treatments currently available include Genzyme's FABRAZYME® (agalsidase beta), which is a recombinant human alpha-galactosidase A produced in CHO cells and administered by intravenous infusion; Shire's REPLAGAL™ (agalsidase alfa), which is a recombinant human alpha-galactosidase A produced in human fibroblast cells and administered by intravenous infusion; and Amicus's GALAFOLD™ (migalastat or 1-deoxygalactonojirimycin) an orally administered small molecule chaperone that shifts the folding of abnormal alpha-galactosidase A to a functional conformation.

[0007] Current treatments for lysosomal storage diseases are less than optimal. For example, ERT generally must be administered at a high frequency and a high dose, such as biweekly and up to 40 mg/kg. Also, some replaced enzymes can be immunologically cross-reactive (CRIM), stimulating production of IgG in the subject and thus hindering delivery of the enzyme to the lysosome via the mannose-6-phosphate (M6P) receptor. The IgGs might shield the M6P residues of the replacement enzyme, and the antigen-IgG-antibody complex may be taken up into cellular lysosomes via the Fc receptor, thereby shunting the replacement enzyme preferentially to macrophages.

[0008] Delivery of replacement enzymes to the appropriate affected tissues is also inefficient (see Table 2 and Desnick & Schuchman, "Enzyme replacement therapy for lysosomal diseases: lessons from 20 years of experience and remaining challenges," 13 Annu. Rev. Genomics Hum. Genet.

307-35, 2012). For example, patients undergoing long-term enzyme replacement therapy for Infantile Pompe can still suffer from hypernasal speech, residual muscle weakness, ptosis, ostepenia, hearing loss, risk for aspiration, dysphagia, cardiac arrhythmia, and difficulty swallowing. Doses of replacement enzyme oftentimes must be increased over time to 40 mg/kg weekly or biweekly.

Table 2: Inefficient tissue targeting of ERT

Disease	Subtype(s)	Easy to reach tissue	Hard to reach tissue
Gaucher disease	Type 1	Spleen, liver, bone marrow	Bone
	Types 2 and 3	Spleen, liver, bone marrow	Bone, brain
Fabry disease	Classic and late onset	Vascular endothelium	Kidney, heart
Mucopolysaccharidoses	All	Spleen, liver, bone marrow	Bone, brain, cartilage
α-Mannosidosis	---	Spleen, liver, bone marrow	Bone, brain
Niemann-Pick disease	Type B	Spleen, liver, bone marrow	Alveolar macrophages
Pompe disease	Infantile	---	Heart, smooth and skeletal muscle
	Later onset	---	Smooth muscle and respiratory skeletal muscle

[0009] Endogenous mannose-6 phosphate receptor (MPR) mediates the transport of most recombinant enzymes to the lysosome. Two complementary forms of MPR exist: cation-independent (CI-MPR), and cation dependent (CD-MPR). Knock-outs of either form have missorted lysosomal enzymes. Lysosomal hydrolases are synthesized in the endoplasmic reticulum and move to the cis-Golgi network, where they are covalently modified by the addition of mannose-6-phosphate (M6P) groups. The formation of this marker depends on the sequential effect of two lysosomal enzymes: UDP-*N*-acetylglucosamine-l-phosphotransferase (G1cNac-phosphotransferase) and *N*-acetylglucosamine-l-phosphodiester-α-*N*-acetyl-glucosaminidase (uncovering enzyme). GlcNac-phosphotransferase catalyzes the transfer of a G1cNAc-1- phosphate residue from UDP-G1cNAc to C6 positions of selected mannoses in high-mannose type oligosaccharides of the hydrolases. Then, the uncovering enzyme removes the terminal G1cNAc, exposing the M6P recognition signal. At the trans-Golgi network, the M6P signal allows the segregation of lysosomal hydrolases from all other types of proteins through selective binding to the M6P receptors. The clathrin-coated vesicles produced bud off from the trans-Golgi network and

fuse with late endosomes. At the low pH of the late endosome, the hydrolases dissociate from the M6P receptors and the empty receptors are recycled to the Golgi apparatus for further rounds of transport.

[0010] With the exception of β -glucocerebrosidase, which is delivered via the mannose receptor, recombinant lysosomal enzymes comprise M6P glycosylation and are delivered to the lysosome primarily via CI-MPR/IGF2R. Glycosylation/CI-MPR-mediated enzyme replacement delivery however does not reach all clinically relevant tissues (Table 2). Improvement to enzyme replacement therapy have centered on improving CI-MPR delivery by (i) increasing surface expression of CI-MPR using the β2-agonist clenbuterol (Koeberl *et al.*, "Enhanced efficacy of enzyme replacement therapy in Pompe disease through mannose-6-phosphate receptor expression in skeletal muscle," 103(2) Mol. Genet. Metab. 107-12, 2011), (ii) increasing the amount of M6P residues on enzyme (Zhu *et al.*, "Conjugation of mannose-6-phosphate-containing oligosaccharides to acid alpha-glucosidase improves the clearance of glycogen in Pompe mice," 279(48) J. Biol. Chem. 50336-41, 2004), or (iii) fusing an IGF-II domain to the enzyme (Maga *et al.*, "Glycosylation-independent lysosomal targeting of acid alpha-glucosidase enhances muscle glycogen clearance in Pompe mice," 288(3) J. Biol. Chem. 1428-38, 2013).

[0011] A large number of lysosomal storage diseases are inadequately treated by enzyme replacement therapy or gene therapy mainly due to poor targeting of the replacement enzyme to the relevant tissue or organ, negative immunological reactions in the recipient host, and low serum half-life. A need exists for improved enzyme replacement therapies that enhance and promote better tissue biodistribution and lysosomal uptake of the enzyme. Applicant has developed an improved enzyme replacement therapy using antibody-guided delivery of enzymes to the lysosome of target affected tissues, which occurs independent of CI-MPR.

SUMMARY

[0012] Applicants have discovered that replacement enzymes can be effectively delivered into a target cell when associated with a delivery domain as part of a multidomain therapeutic protein. The multidomain therapeutic protein can be delivered to the cell, which can be ex vivo or in vivo, via a gene therapy vector, e.g., a viral vector, naked polynucleotide, polynucleotide complex, etc, comprising the coding sequence of the multidomain therapeutic protein.

[0013] In one aspect, the invention provides a polynucleotide containing a nucleic acid sequence encoding a multidomain therapeutic protein. In one embodiment, the multidomain therapeutic protein contains an enzyme domain and a delivery domain. In one embodiment, the polynucleotide also contains a viral vector nucleic acid sequence. In a specific embodiment, the polynucleotide also contains an adeno-associated virus (AAV) nucleic acid sequence.

[0014] In one embodiment, the enzyme domain has hydrolase activity, such as a glycosylase, such as a glycosidase, such as an alpha-glucosidase (GAA) or alpha-galacosidase A (GLA). In some embodiments, the enzyme domain comprises the amino acid sequence set forth as SEQ ID NO:1 or a biologically active portion tehreof. In some embodiments, the enzyme domain comprises the amino acid sequence set forth as SEQ ID NO:78. In some embodiments, the enzyme domain consists essentially of the amino acid sequence set forth as SEQ ID NO:78. In some embodiments, the enzyme domain consists of the amino acid sequence set forth as SEQ ID NO:78. In one embodiment, the delivery domain is an antigen-binding protein that binds to an internalization effector. In one embodiment, the internalization effector is a cell-surface molecule that is endocytosed and trafficked to the lysosome. In a specific embodiment, the internalization effector is a CD63 molecule. In one embodiment, the internalization effector is a ITGA7 molecule. In a specific embodiment, the delivery domain is an antibody, an antibody fragment, or a single-chain variable fragment (scFv), such as an scFv that binds CD63 or ITGA7 (*e.g.,* Figure 1A, panel G).

[0015] In one embodiment, the multidomain therapeutic protein contains two delivery domains (*e.g.,* Figure 1A, panel H). In one embodiment, the first delivery domain binds an internalization effector to facilitate delivery of the enzyme to the proper target cell or target subcellular compartment; and the second delivery domain binds a transcytosis effector to facilitate the transport of the multidomain therapeutic protein across a physiological barrier such as the alveolar membrane, the liver endothelium, the blood-brain barrier, or the like. In a specific embodiment, the second delivery domain is an anti-transferrin receptor (TfR) scFv domain. In a specific embodiment, the multidomain therapeutic protein comprises a GAA enzyme domain, an anti-CD63 scFv first delivery domain, and an anti-TfR scFv second delivery domain. In another embodiment, the multidomain therapeutic protein comprises a GAA enzyme domain, an anti-ITGA7 scFv first delivery domain, and an anti-CD63 scFv second delivery domain.

[0016] In one aspect, the invention provides a gene therapy vector, such as an AAV vector, naked polynucleotide, polynucleotide complex, etc., that comprises a nucleic acid sequence encoding a multidomain therapeutic protein containing an enzyme domain and a delivery domain.

[0017] In one embodiment, the enzyme domain has hydrolase activity, such as a glycosylase, such as a glycosidase, such as an alpha-glucosidase or alpha-galacosidase A. In one embodiment, the delivery domain is an antigen-binding protein that binds to an internalization effector. In one embodiment, the internalization effector is a cell-surface molecule that is endocytosed and trafficked to the lysosome. In a specific embodiment, the internalization effector is a CD63 molecule. In one embodiment, the internalization effector is an ITGA7 molecule. In a specific embodiment, the delivery domain is an antibody, an antibody fragment, or a single-chain variable fragment (scFv), such as an scFv that binds CD63 or ITGA7.

[0018] In one aspect, the invention provides a multidomain therapeutic protein containing an enzyme domain and a delivery domain. In one embodiment, the enzyme domain has hydrolase activity, such as a glycosylase, such as a glycosidase, such as an alpha-glucosidase or alpha-galacosidase A. In one embodiment, the delivery domain is an antigen-binding protein that binds to an internalization effector. In one embodiment, the internalization effector is a cell-surface molecule that is endocytosed and trafficked to the lysosome. In a specific embodiment, the internalization effector is a CD63 molecule. In another embodiment, the internalization effector is a ITGA7 molecule. In a specific embodiment, the delivery domain is an antibody, an antibody fragment, or a single-chain variable fragment (scFv), such as an scFv that binds CD63 or ITGA7.

[0019] In one embodiment, the multidomain therapeutic protein is used to treat a patient in need of enzyme replacement therapy.

[0020] In one aspect, the invention provides a method of producing a multidomain therapeutic protein containing an enzyme domain and a delivery domain in a cell. In one embodiment, the multidomain therapeutic protein is produced by contacting the cell with a gene therapy vector comprising a nucleic acid sequence encoding the multidomain therapeutic protein. The a nucleic acid sequence subsequently integrates into a genomic locus of the cell, from which the nucleic acid sequence is transcribed and translated in the case that the nucleic acid sequence is DNA, or translated in the case that the nucleic acid is RNA, and the multidomain therapeutic protein is produced. In one embodiment, the gene therapy vector is one that is commonly used in cell

transfection, such as an adeno-associated virus (AAV) vector. In one embodiment, the gene therapy vector is a naked polynucleotide. In one embodiment, the gene therapy vector is a polynucleotide complex, e.g., a lipid nanoparticle. In one embodiment, the genomic locus is a safe harbor locus, which enables high expression of the multidomain therapeutic protein, while not interfering with the expression of essential genes or promoting the expression of oncogenes or other deleterious genes. In one embodiment, the genomic locus is an adeno-associated virus site.

[0021] In one embodiment, the cell is a mammalian cell, such as a human cell or a mouse cell. In one embodiment, the cell is ex vivo, such as a HEK293 cell line. In another embodiment, the cell is in vivo and the gene therapy vector containing the multidomain therapeutic protein-coding nucleic acid sequence is administered to a human or non-human subject.

[0022] In one embodiment, the enzyme domain has hydrolase activity, such as a glycosylase, such as a glycosidase, such as an alpha-glucosidase (GAA) or alpha-galacosidase A (GLA). In one embodiment, the delivery domain is an antigen-binding protein that binds to an internalization effector. In one embodiment, the internalization effector is a cell-surface molecule that is endocytosed and trafficked to the lysosome. In a specific embodiment, the internalization effector is a CD63 molecule. In a specific embodiment, the delivery domain is an antibody, an antibody fragment, or a single-chain variable fragment (scFv), such as an scFv that binds CD63 (*e.g.,* SEQ ID NO:2). In some embodiments the multidomain therapeutic polypeptide comprises an scFv that binds CD63 (e.g., an scFv comprising the amino acid sequence set forth as SEQ ID NO:2) operably linked to GAA (set forth as SEQ ID NO:1) or a biologically active portion thereof (set forth as SEQ ID NO:78). In some embodiments, the multidomain therapeutic polypeptide comprises the sequence set forth as SEQ ID NO:10 or SEQ ID NO:79.

[0023] In a specific embodiment, an AAV vector containing a polynucleotide encoding an scFv-hydrolase fusion protein is administered to a human or non-human subject. The polynucleotide subsequently integrates at a genomic locus and the encoded fusion protein is produced. In a specific embodiment, the fusion protein is an anti-CD63scFv-GAA fusion protein (e.g., as set forth in SEQ ID NO: 10 or SEQ ID NO:79) or an anti-ITGA7scFv-GAA fusion protein, the human or non-human subject lacks endogenous GAA activity, and the GAA activity is effectively restored in the subject.

[0024] In one aspect, the invention provides a method of treating a patient (human or non-human) with an enzyme deficiency by administering to the patient a gene therapy vector containing a nucleic acid sequence encoding a multidomain therapeutic protein comprising a delivery domain and an enzyme domain. In one embodiment, the enzyme domain comprises a glycosidase, such as GAA (*e.g.,* SEQ ID NO:1) or a biologically active portion thereof (*e.g.*, SEQ ID NO:78) or GLA (*e.g.,* UniProtKB No. P06280, aa32-429, SEQ ID NO:13), and the patient has Pompe disease or Fabry disease. In one embodiment, the delivery domain of the multidomain therapeutic protein is an antigen-binding protein that binds to an internalization effector, such as CD63 or ITGA7. In one embodiment, the delivery domain is an scFv molecule that binds CD63. In one embodiment, the delivery domain is an scFv molecule that binds ITGA7. In another embodiment, the gene therapy vector comprises an AAV vector containing a nucleic acid sequence that encodes an anti-CD63-GAA fusion multidomain therapeutic protein (*e.g.*, SEQ ID NO:10 or SEQ ID NO:79). In another embodiment, the gene therapy vector is an AAV vector comprising a nucleic acid sequence that encodes an anti-ITGA7-GAA fusion multidomain therapeutic protein. In another embodiment, the gene therapy vector comprises a naked polynucleotide comprising a nucleic acid sequence that encodes an anti-CD63-GAA fusion multidomain therapeutic protein (*e.g.*, SEQ ID NO:10 or SEQ ID NO:79). In another embodiment, the gene therapy vector comprises a naked polynucleotide comprising a nucleic acid sequence that encodes an anti-ITGA7-GAA fusion multidomain therapeutic protein. In another embodiment, the gene therapy vector comprises a polynucleotide complex comprising a nucleic acid sequence that encodes an anti-CD63-GAA fusion multidomain therapeutic protein (*e.g.*, SEQ ID NO:10 or SEQ ID NO:79). In another embodiment, the gene therapy vector comprises a polynucleotide complex comprising a nucleic acid sequence that encodes an anti-ITGA7-GAA fusion multidomain therapeutic protein.

[0025] In one aspect, the present invention relates to a composition according to the invention for use in medicine. In one embodiment the composition, e.g., a pharmaceutical composition, may comprise a gene therapy vector comprising a nucleic acid sequence encoding a multidomain therapeutic protein comprising a delivery domain and an enzyme domain. In one embodiment, the delivery domain of the multidomain therapeutic protein is an antigen-binding protein that binds to an internalization effector, such as CD63 or ITGA7. In one embodiment, the delivery domain is an scFv molecule that binds CD63. In one embodiment, the delivery domain is an scFv molecule that binds ITGA7. In another embodiment, the gene therapy vector is an AAV vector containing a

polynucleotide that encodes an anti-CD63-GAA fusion multidomain therapeutic protein. In another embodiment, the gene therapy vector is an AAV vector containing a polynucleotide that encodes an anti-ITGA7-GAA fusion multidomain therapeutic protein. In some embodiments, the polynucleotide, e.g., a gene therapy vector, comprises a tissue specific regulatory element. In some embodiments, the tissue specific regulatory element comprises the sequence set forth as SEQ ID NO:8, SEQ ID NO:9, or both. In some embodiments, the polynucleotide, e.g., gene therapy vector comprises the nucleic acid sequence of SEQ ID NO:11.

[0026] In one aspect, described is a composition comprising a gene therapy vector containing a gene encoding a multidomain therapeutic protein comprising a delivery domain and an enzyme domain for use in treatment of a patient (human or non-human) with an enzyme deficiency, and/or for use in reducing glycogen accumulation in a tissue in a human or non-human subject. In one embodiment, the delivery domain of the multidomain therapeutic protein is an antigen-binding protein that binds to an internalization effector, such as CD63 or ITGA7. In one embodiment, the delivery domain is an scFv molecule that binds CD63. In one embodiment, the delivery domain is an scFv molecule that binds ITGA7. In another embodiment, the gene therapy vector is an AAV vectors containing a polynucleotide that encodes an anti-CD63-GAA fusion multidomain therapeutic protein. In another embodiment, the gene therapy vector is an AAV vectors containing a polynucleotide that encodes an anti-ITGA7-GAA fusion multidomain therapeutic protein. The clinical indications entailing an enzyme deficiency may be, but not limited to, e.g. Pompe's Disease or Fabry's disease. In some embodiments, the polynucleotide, e.g., a gene therapy vector, comprises a tissue specific regulatory element. In some embodiments, the tissue specific regulatory element comprises the sequence set forth as SEQ ID NO:8, SEQ ID NO:9, or both. In some embodiments, the polynucleotide, e.g., gene therapy vector comprises the nucleic acid sequence of SEQ ID NO:11.

[0027] In one aspect, described is use of a pharmaceutical composition according to the invention for the manufacture of a medicament for therapeutic application such as e.g. treatment of a patient (human or non-human) with an enzyme deficiency and/or reducing glycogen accumulation in a tissue in a human or non-human subject. The composition may comprise e.g. a gene therapy vector containing a gene encoding a multidomain therapeutic protein comprising a delivery domain and an enzyme domain. In one embodiment, the delivery domain of the multidomain therapeutic protein is an antigen-binding protein that binds to an internalization effector, such as CD63 or ITGA7. In one

embodiment, the delivery domain is an scFv molecule that binds CD63. In one embodiment, the delivery domain is an scFv molecule that binds ITGA7. In another embodiment, the gene therapy vector is an AAV vectors containing a polynucleotide that encodes an anti-CD63-GAA fusion multidomain therapeutic protein. In another embodiment, the gene therapy vector is an AAV vectors containing a polynucleotide that encodes an anti-ITGA7-GAA fusion multidomain therapeutic protein. The clinical indications entailing an enzyme deficiency may be, but not limited to, e.g. Pompe's Disease or Fabry's disease.

[0028] In one embodiment, the multidomain therapeutic protein comprises a GAA enzyme domain, and high serum levels of GAA are maintained in the serum of the patient for at least 12 weeks after administering the gene therapy vector. In one embodiment, the multidomain therapeutic protein comprises a GAA enzyme domain, and glycogen levels in heart, skeletal muscle, and liver tissue in the patient are maintained at wildtype levels 3 months after administration of the gene therapy vector. In one embodiment, the multidomain therapeutic protein comprises a GAA enzyme domain, and the muscle strength of the patient after treatment is restored to wildtype levels.

[0029] In one aspect, the invention provides a method of reducing glycogen accumulation in a tissue in a human or non-human subject by administering a gene therapy vector containing a polynucleotide that encodes a multidomain therapeutic protein. In one embodiment, the tissue is liver, heart, or skeletal muscle. In one embodiment, the human or non-human subject has Pompe disease. In one embodiment, the multidomain therapeutic protein comprises an anti-CD63 scFv-GAA fusion protein. In another embodiment, the multidomain therapeutic protein comprises an anti-ITGA7 scFv-GAA fusion protein.

[0030] In one aspect, described herein is a method of reducing cross-reactive immunological material against an enzyme in a patient (human or non-human) with a deficiency in the enzyme, the method comprising administering to the patient a gene therapy vector containing a gene encoding the replacement enzyme only or a multidomain therapeutic protein comprises a delivery domain and an enzyme domain. In some embodiments, the gene therapy vector is an AAV vector, which may be a chimeric AAV vector (e.g., an AAV2/8). In some embodiments, the enzyme domain comprises a glycosidase, such as GAA (*e.g.,* SEQ ID NO:1) or GLA (*e.g.,* UniProtKB No. P06280, aa32-429, SEQ ID NO:13), and the patient has Pompe disease or Fabry disease, and the delivery domain is an antigen-binding protein that binds to an internalization effector. In some

embodiments, the gene therapy vector is administered in sufficient amounts to increase serum levels of GAA such that cross-reactive immunological material is reduced or no cross-reactive immunological material is generated in detectable amounts. In some embodiments, the gene therapy vector is administered in sufficient amounts to increase serum levels of GAA over a period of time. In some embodiments, the patient is infected with a virus (e.g., an AAV) comprising the gene therapy vector, optionally wherein the virus is pseudotyped to specifically target a tissue or cell selected from the group consisting of the GALT, liver, hematopoietic stem cell, red blood cell or a combination thereof and/or wherein the gene therapy vector comprises a cell or tissue specific enhancer and/or promoter, e.g., a liver specific enhancer (e.g., serpina 1) and/or a liver specific promoter (e.g., TTR). In one embodiment, the internalization effector is a cell-surface molecule that is endocytosed and trafficked to the lysosome. In a specific embodiment, the internalization effector is a CD63 molecule. In a specific embodiment, the delivery domain is an antibody, an antibody fragment, or a single-chain variable fragment (scFv), such as an scFv that binds CD63 (*e.g.,* SEQ ID NO:2). In some embodiments, the method comprises administering to the patient a gene therapy vector as described herein in combination with at least one immunosuppressive agent, wherein the gene therapy vector and immunosuppressive agent are administered simultaneously and/or sequentially. In some embodiments, the patient maintains a constant level of the immunosuppressive agent.

[0031] In one aspect, described herein is a method of inducing tolerance to an enzyme in a patient (human or non-human) with a deficiency in the enzyme (i.e., tolerizing the patient to the enzyme, the method comprising reducing cross-reactive immunological material in the patient, e.g., administering to the patient a gene therapy vector containing a gene encoding the replacement enzyme only or a multidomain therapeutic protein comprising a delivery domain and an enzyme domain. In some embodiments, disclosed is a method of inducing tolerance to an enzyme in a patient with a deficiency in the enzyme, the method comprising administering to the patient a gene therapy vector containing a gene encoding the replacement enzyme only, or a multidomain therapeutic protein comprising a delivery domain and an enzyme domain, over a period of time such that no increase in detectable cross-reactive immunological material in the patient is generated. In some embodiments, the multidomain therapeutic protein comprises a delivery domain and an enzyme domain. In some embodiments, the gene therapy vector is an AAV vector, which may be a chimeric AAV vector (e.g., an AAV2/8) and/or an engineered AAV vector (e.g., a

tropism modified recombinant viral vector useful for targeting, e.g., a receptor or marker preferentially or exclusively expressed by a cell or tissue, e.g., hepatocyte or liver, mucosal tissue, red blood cells, hematopoietic stem cells, etc.), and optionally wherein the gene is expressed under the control of an enhancer and/or promoter specific for the cell or tissue, e.g., a liver specific promoter, etc. In some embodiments, the enzyme domain comprises a glycosidase, such as GAA (*e.g.,* SEQ ID NO:1) or GLA (*e.g.,* UniProtKB No. P06280, aa32-429, SEQ ID NO:13), and the patient has Pompe disease or Fabry disease, and the delivery domain is an antigen-binding protein that binds to an internalization effector. In one embodiment, the internalization effector is a cell-surface molecule that is endocytosed and trafficked to the lysosome. In a specific embodiment, the internalization effector is a CD63 molecule. In a specific embodiment, the delivery domain is an antibody, an antibody fragment, or a single-chain variable fragment (scFv), such as an scFv that binds CD63 (*e.g.,* SEQ ID NO:2). In some embodiments, a method of treating a patient with an enzyme deficiency comprises administering to the patient a recombinant form and/or isozyme of the enzyme (e.g., GAA in a patient with Pompe, e.g., an scFv63::GAA, GAA, optimized GAA, or combination thereof), wherein the patient is tolerant to the enzyme, e.g., wherein the patient has been tolerized to the enzyme according to a method disclosed herein. Accordingly, in some embodiments, a method of administering an enzyme to a patient deficient thereof comprises tolerizing the patient to the enzyme, e.g., by administering a gene therapy vector encoding the enzyme or multidomain therapeutic protein comprising the enzyme, preferably in sufficient amounts to increase serum levels of the enzyme such that cross-reactive immunological material is at a level comparable to that found in a patient not deficient of the enzyme, optionally wherein the gene therapy vector is specifically targeted to the liver and/or comprises a liver specific enhancer and/or promoter. In some embodiments, the method further comprises, after administration of the gene targeting vector, further administering the enzyme and/or a recombinant variant thereof, including a multidomain therapeutic protein comprising the enzyme.

[0032] In another aspect, the present invention provides antibodies and antigen-binding fragments thereof that bind to human CD63. The antibodies according to this aspect of the invention are useful, *inter alia*, for specifically directing the internalization and/or lysosomal trafficking of an enzyme, e.g., GAA or GLA. As such, this aspect of the invention also provides bispecific antibodies, antigen-binding fragments thereof that bind human CD63, and antibody-protein fusion constructs (see, e.g., Figure 1A).

[0033] Exemplary anti-CD63 antibodies of the present invention are listed in Table 11. Table 11sets forth the amino acid and nucleic acid sequence identifiers of the heavy chain variable regions (HCVRs) and light chain variable regions (LCVRs), as well as heavy chain complementarity determining regions (HCDR1, HCDR2 and HCDR3), and light chain complementarity determining regions (LCDR1, LCDR2 and LCDR3) of the exemplary anti-CD63 antibodies.

[0034] The present invention provides antibodies, or antigen-binding fragments thereof, comprising an HCVR comprising an amino acid sequence selected from any of the HCVR amino acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0035] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising an LCVR comprising an amino acid sequence selected from any of the LCVR amino acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0036] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising an HCVR and an LCVR amino acid sequence pair (HCVR/LCVR) comprising any of the HCVR amino acid sequences listed in Table 11 paired with any of the LCVR amino acid sequences listed in Table 11. According to certain embodiments, the present invention provides antibodies, or antigen-binding fragments thereof, comprising an HCVR/LCVR amino acid sequence pair contained within any of the exemplary anti-CD63 antibodies listed in Table 11. In certain embodiments, the HCVR/LCVR amino acid sequence pair is selected from the group consisting of SEQ ID NOs: 14/22, SEQ ID NOs: 30/38, SEQ ID NOs: 46/54, and SEQ ID NOs: 62/70.

[0037] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a heavy chain CDR1 (HCDR1) comprising an amino acid sequence selected from any of the HCDR1 amino acid sequences listed in Table 11 or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

[0038] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a heavy chain CDR2 (HCDR2) comprising an amino acid sequence selected from any

of the HCDR2 amino acid sequences listed in Table 11 or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

[0039] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a heavy chain CDR3 (HCDR3) comprising an amino acid sequence selected from any of the HCDR3 amino acid sequences listed in Table 11 or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

[0040] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a light chain CDR1 (LCDR1) comprising an amino acid sequence selected from any of the LCDR1 amino acid sequences listed in Table 11 or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

[0041] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a light chain CDR2 (LCDR2) comprising an amino acid sequence selected from any of the LCDR2 amino acid sequences listed in Table 11 or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

[0042] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a light chain CDR3 (LCDR3) comprising an amino acid sequence selected from any of the LCDR3 amino acid sequences listed in Table 11or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

[0043] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising an HCDR3 and an LCDR3 amino acid sequence pair (HCDR3/LCDR3) comprising any of the HCDR3 amino acid sequences listed in Table 11 paired with any of the LCDR3 amino acid sequences listed in Table 11. According to certain embodiments, the present invention provides antibodies, or antigen-binding fragments thereof, comprising an HCDR3/LCDR3 amino acid sequence pair contained within any of the exemplary anti-CD63 antibodies listed in Table 11. In certain embodiments, the HCDR3/LCDR3 amino acid sequence pair is selected from the group consisting of SEQ ID NOs: SEQ ID NOs: 20/28, SEQ ID NOs: 36/44, SEQ ID NOs: 52/60, and SEQ ID NOs: 68/76.

[0044] The present invention also provides antibodies, or antigen-binding fragments thereof, comprising a set of six CDRs (*i.e.*, HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3) contained

within any of the exemplary anti-CD63 antibodies listed in Table 11. In certain embodiments, the HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3 amino acid sequences set is selected from the group consisting of SEQ ID NOs:16-18-20-24-26-28, SEQ ID NOs: 32-34-36-40-42-44, SEQ ID NOs:48-50-52-56-58-60, and SEQ ID NOs: 64-66-68-72-74-76.

[0045] In a related embodiment, the present invention provides antibodies, or antigen-binding fragments thereof, comprising a set of six CDRs (*i.e.*, HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3) contained within an HCVR/LCVR amino acid sequence pair as defined by any of the exemplary anti-CD63 antibodies listed in Table 11. For example, the present invention includes antibodies, or antigen-binding fragments thereof, comprising the HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3 amino acid sequences set contained within an HCVR/LCVR amino acid sequence pair selected from the group consisting of SEQ ID NOs:14/22, SEQ ID NOs: 30/38, SEQ ID NOs: 46/54, and SEQ ID NOs: 62/70. Methods and techniques for identifying CDRs within HCVR and LCVR amino acid sequences are well known in the art and can be used to identify CDRs within the specified HCVR and/or LCVR amino acid sequences disclosed herein. Exemplary conventions that can be used to identify the boundaries of CDRs include, *e.g.*, the Kabat definition, the Chothia definition, and the AbM definition. In general terms, the Kabat definition is based on sequence variability, the Chothia definition is based on the location of the structural loop regions, and the AbM definition is a compromise between the Kabat and Chothia approaches. *See*, *e.g.*, Kabat, "Sequences of Proteins of Immunological Interest," National Institutes of Health, Bethesda, Md. (1991); Al-Lazikani *et al.*, *J. Mol. Biol. 273*:927-948 (1997); and Martin *et al.*, *Proc. Natl. Acad. Sci. USA 86*:9268-9272 (1989). Public databases are also available for identifying CDR sequences within an antibody.

[0046] The present invention also provides nucleic acid molecules encoding anti-CD63 antibodies or portions thereof. For example, the present invention provides nucleic acid molecules encoding any of the HCVR amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the HCVR nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0047] The present invention also provides nucleic acid molecules encoding any of the LCVR amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule

comprises a polynucleotide sequence selected from any of the LCVR nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0048] The present invention also provides nucleic acid molecules encoding any of the HCDR1 amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the HCDR1 nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0049] The present invention also provides nucleic acid molecules encoding any of the HCDR2 amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the HCDR2 nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0050] The present invention also provides nucleic acid molecules encoding any of the HCDR3 amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the HCDR3 nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0051] The present invention also provides nucleic acid molecules encoding any of the LCDR1 amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the LCDR1 nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0052] The present invention also provides nucleic acid molecules encoding any of the LCDR2 amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the LCDR2 nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0053] The present invention also provides nucleic acid molecules encoding any of the LCDR3 amino acid sequences listed in Table 11; in certain embodiments the nucleic acid molecule comprises a polynucleotide sequence selected from any of the LCDR3 nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto.

[0054] The present invention also provides nucleic acid molecules encoding an HCVR, wherein the HCVR comprises a set of three CDRs (*i.e.*, HCDR1-HCDR2-HCDR3), wherein the HCDR1-HCDR2-HCDR3 amino acid sequence set is as defined by any of the exemplary anti-CD63 antibodies listed in Table 11.

[0055] The present invention also provides nucleic acid molecules encoding an LCVR, wherein the LCVR comprises a set of three CDRs (*i.e.*, LCDR1-LCDR2-LCDR3), wherein the LCDR1-LCDR2-LCDR3 amino acid sequence set is as defined by any of the exemplary anti-CD63 antibodies listed in Table 11.

[0056] The present invention also provides nucleic acid molecules encoding both an HCVR and an LCVR, wherein the HCVR comprises an amino acid sequence of any of the HCVR amino acid sequences listed in Table 11, and wherein the LCVR comprises an amino acid sequence of any of the LCVR amino acid sequences listed in Table 11. In certain embodiments, the nucleic acid molecule comprises a polynucleotide sequence selected from any of the HCVR nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto, and a polynucleotide sequence selected from any of the LCVR nucleic acid sequences listed in Table 11, or a substantially similar sequence thereof having at least 90%, at least 95%, at least 98% or at least 99% sequence identity thereto. In certain embodiments according to this aspect of the invention, the nucleic acid molecule encodes an HCVR and LCVR, wherein the HCVR and LCVR are both derived from the same anti-CD63 antibody listed in Table 11.

[0057] The present invention also provides recombinant expression vectors capable of expressing a polypeptide comprising a heavy or light chain variable region of an anti-CD63 antibody. For example, the present invention includes recombinant expression vectors comprising any of the nucleic acid molecules mentioned above, *i.e.*, nucleic acid molecules encoding any of the HCVR, LCVR, and/or CDR sequences as set forth in Table 11. Also included within the scope of the

present invention are host cells into which such vectors have been introduced, as well as methods of producing the antibodies or portions thereof by culturing the host cells under conditions permitting production of the antibodies or antibody fragments, and recovering the antibodies and antibody fragments so produced.

[0058] In some aspects, the present invention includes antibodies or antigen-binding fragments thereof, such as anti-CD63 antibodies, having a modified glycosylation pattern. In some embodiments, modification to remove undesirable glycosylation sites may be useful, or an antibody lacking a fucose moiety present on the oligosaccharide chain, for example, to increase antibody dependent cellular cytotoxicity (ADCC) function (see Shields et al. (2002) JBC 277:26733), where cytotoxicity is desireable. In other applications, modification of galactosylation can be made in order to modify complement dependent cytotoxicity (CDC).

[0059] In another aspect, the invention provides a pharmaceutical composition comprising a recombinant human antibody or fragment thereof which specifically binds CD63 and a pharmaceutically acceptable carrier. In a related aspect, the invention features a composition which is a combination of an anti-CD63 antibody and a second therapeutic agent. In one embodiment, the second therapeutic agent is any agent that is advantageously combined with an anti-CD63 antibody. Additional combination therapies and co-formulations involving the anti-CD63 antibodies of the present invention are disclosed elsewhere herein.

DRAWINGS

[0060] **Figure 1A** schematically represents multidomain therapeutic proteins. Panel A depicts a multidomain therapeutic protein comprising a bispecific antibody (ii) and a replacement enzyme (i). Panel B depicts an enzyme-Fc fusion polypeptide (i) associating with an internalization effector-specific half-body (ii) to form the multidomain therapeutic protein. Panel C depicts a replacement enzyme (hexagon) covalently linked to the C-terminus of the heavy chain of an anti-internalization effector antibody. Panel D depicts a replacement enzyme (hexagon) covalently linked to the N-terminus of the heavy chain of an anti-internalization effector antibody. Panel E depicts a replacement enzyme (hexagon) covalently linked to the C-terminus of the light chain of an anti-internalization effector antibody. Panel F depicts a replacement enzyme (hexagon) covalently linked to the N-terminus of the light chain of an anti-internalization effector antibody. Panel G depicts a replacement enzyme (hexagon) covalently linked to the C-terminus of a single-

chain variable fragment (scFv) containing a VH region (shaded bar) and a VL region (open bar). Panel H depicts a replacement enzyme (hexagon) covalently linked to two scFv domains, the first scFv (i) which serves as a first delivery domain, and the second scFv (ii) which serves as a second delivery domain.

[0061] Figure 1B is a non-limiting exemplary illustration of an AAV gene therapy vector that encodes a multidomain therapeutic protein represented in panel, wherein the scFv is an anti-human CD63 scFv and the replacement enzyme is GAA (e.g., anti-hCD63scFv::hGAA; see, e.g., the amino acid sequence set forth as SEQ ID NO:10). Amino acids 1-119 of SEQ ID NO:10 provide the amino acid sequence of the heavy chain variable domain (V_H) of the H5C6 antibody; amino acids 120-134 of SEQ ID NO:10 provide an amino acid linker sequence between the heavy and light chain variable domains of H5C6; amino acids 135-245 of SEQ ID NO:10 provide the amino acid sequence of the light chain variable domain (V_L) of the H5C6 antibody; amino acids 136-250 of SEQ ID NO:10 provides an amino acid linker sequence between the anti-hCD63scFv and GAA; and amino acids 251-1133 of SEQ ID NO:10 provides the amino acid sequence of GAA. Exemplary 5'ITR and 3' ITR sequences are respectively set forth as SEQ ID NO:6 and SEQ ID NO:7. An exemplary liver specific enhancer (serpina 1) is set forth as SEQ ID NO:9. An exemplary liver specific promoter (TTR) is set forth as SEQ ID NO:8. Additional exemplary anti-CD63 V_H and V_L amino acid sequences (and nucleotide sequences encoding same) that may be used to construct a multidomain therapeutic protein comprising an anti-CD63 antibody or antigen binding portion thereof are provided in Table 11.

[0062] Figure 2 is a bar graph depicting the amount of stored glycogen in micrograms per milligram of tissue as a function of delivered enzyme. The X-axis depicts tissues from a $CD63^{hu/hu}$; $GAA^{-/-}$ mouse from left to right: heart, quadriceps, gastrocnemius, diaphragm, soleus, and extensor digitorum longus (EDL) muscle. Black boxes ("1") depict the amount of stored glycogen in an untreated mouse Pompe disease model. Orange boxes ("2") depict the amount of stored glycogen in an untreated wildtype mouse model. Lime green boxes ("3") depict the amount of stored glycogen in a mouse Pompe disease model treated with AAV-hGAA (adeno-associated virus vector containing gene encoding human GAA) at a dose of 10^{10} vg. Forest green boxes ("4") depict the amount of stored glycogen in a mouse Pompe disease model treated with AAV-hGAA at a dose of 10^{11} vg. Light blue boxes ("5") depict the amount of stored glycogen in a mouse Pompe disease model treated with AAV-anti-hCD63scFv::hGAA (adeno-associated virus vector containing gene

encoding an anti-human CD63 scFv domain linked to human GAA) at a dose of 10^{10} vg. Dark blue boxes ("6") depict the amount of stored glycogen in a mouse Pompe disease model treated with AAV-anti-hCD63scFv::hGAA at a dose of 10^{11} vg.

[0063] Figure 3 is a bar graph depicting the average glycogen measured (μg/mg) in skeletal muscle tissue in each mouse at 3 months post-AAV injection. Each measurement is plotted as a function of GAA exposure (*i.e.*, serum levels) per mouse treated with a particular enzyme construct at a particular dosage. Filled squares represent AAV-hGAA at a dose of 10^{10} vg. Filled pyramids represent AAV-hGAA at a dose of 10^{11} vg. Filled inverse pyramids represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{10} vg. Filled diamonds represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{11} vg.

[0064] Figure 4 is a dot plot depicting the average cardiac muscle glycogen measured (μg/mg) in heart tissue at 3 months post-AAV injection as a function of GAA exposure (*i.e.*, serum levels), per mouse treated with a particular enzyme construct at a particular dosage. Filled squares represent AAV-hGAA at a dose of 10^{10} vg. Filled pyramids represent AAV-hGAA at a dose of 10^{11} vg. Filled inverse pyramids represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{10} vg. Filled diamonds represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{11} vg.

[0065] Figure 5 is a dot plot depicting anti-GAA antibody titers at 3 months post-AAV injection as a function of GAA exposure (i.e. serum levels), per mouse treated with a particular enzyme construct at a particular dosage. Open squares represent AAV-hGAA at a dose of 10^{10} vg. Open circles represent AAV-hGAA at a dose of 10^{11} vg. Open diamonds represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{10} vg. Hexagons represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{11} vg.

[0066] Figure 6 is a dot plot depicting anti-GAA antibody titers at 3 months post-AAV injection as a function of enzyme construct and dose. Circles represent control mice receiving empty AAV vector. Squares represent AAV-hGAA at a dose of 10^{10} vg. Pyramids represent AAV-hGAA at a dose of 10^{11} vg. Inverse pyramids represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{10} vg. Diamonds represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{11} vg.

[0067] Figure 7A is a line graph depicting serum levels of GAA (arbitrary units "a.u."; y-axis) as a function of time in weeks after gene therapy vector injection. Squares represent AAV-hGAA at a dose of 10^{10} vg. Pyramids represent AAV-hGAA at a dose of 10^{11} vg. Inverse pyramids represent

AAV-anti-hCD63scFv::hGAA at a dose of 10^{10} vg. Diamonds represent AAV-anti-hCD63scFv::hGAA at a dose of 10^{11} vg.

[0068] Figure 7B is a bar graph depicting mRNA ratios (hGAA mRNA relative to mGADPH mRNA) following administration of AAV constructs in CD63 HumIn GAA KO mice (GAA-/-,CD63hu/hu mice) or GAA+/+,CD63hu/hu mice, as such: (1) untreated control, (2) AAV-liver-specific promoter-hGAA (1e10vg), (3) AAV-liver-specific promoter-hGAA (1e11vg), (4) AAV-liver-specific promoter-anti-hCD63::hGAA (1e10vg), (5) AAV-liver-specific promoter- anti-hCD63::hGAA (1e11vg), or (6) untreated control (GAA+/+,CD63hu/hu). Liver expression of GAA was detected for all injections of AAV construct.

[0069] Figure 7C is a plot graph comparing serum GAA level to RNA expression level of GAA for mice receiving the AAV encoding the fusion protein (squares) and mice receiving the AAV encoding GAA (both constructs provided a liver-specific promoter (LSP) to drive expression).

[0070] Figure 7D is a bar graph showing Huh-7 human hepatocytes transiently transfected with liver-specific promoter driven constructs encoding for hGAA, anti-hCD63 scFv::GAA (fusion construct), or a non-binding fusion construct scFv::GAA control. Both scFv::GAA fusion constructs had a higher ratio of protein in the secreted supernatant than hGAA alone 3 days after transfection. Addition of M6P into the supernatant during the experimental period to mitigate CI-MPR-mediated uptake did not affect the ratio. (* = $p< 0.05$, n=3).

[0071] Figure 8 are fluorescent micrographs depicting the lamp1-stained lysosomes in mouse muscle fibers counter-stained with DAPI to reveal nuclei. Panels A and A1 depict quadriceps cells derived from an untreated wildtype ($GAA^{+/+}$) mouse and stained for lamp1 (panel A), and nuclei (panel A1). Panels B and B1 depict quadriceps cells derived from an untreated GAA null ($GAA^{-/-}$) mouse and stained for lamp1 (panel B), and nuclei (panel B1). Panels C and C1 depict quadriceps cells derived from a $GAA^{-/-}$ mouse treated with an AAV-hGAA construct and stained for lamp1 (panel C), and nuclei (panel C1). Panels D and D1 depict quadriceps cells derived from a $GAA^{-/-}$ mouse treated with an AAV-hCD63scFv::hGAA construct and stained for lamp1 (panel D), and nuclei (panel D1).

[0072] Figure 9 depict line graphs showing grip strength and Rotarod test performance of mice treated with either AAV-LSP hGAA or AAV-LSP anti-hCD63::hGAA. Accelerating Rotarod measurements (**A**) and forelimb grip strength measurements (**B**) of wild-type GAA mice (inverted

triangle), untreated control (square), AAV-LSP-hGAA treatment (1e11vg/mouse) (triangle) or AAV-LSP-anti-hCD63::hGAA treatment (1e11vg/mouse) (circle) were taken at monthly intervals for 6 months. Error bars are +/- SD. N=8-10 for all groups.

[0073] Figure 10A and Figure 10B depict the use of other membrane proteins as guides, such as anti-ITGA7 (Integrin alpha-7) scFv fusion proteins to guide GAA. **Figure 10A** shows GAA activity (y-axis) of of C2C12 mouse myoblasts incubated overnight with anti-mouse CD63-GAA or anti-moue ITGA7-GAA with or without the presence of 5mM M6P. **Figure 10B** shows GAA KO mice humanized for CD63 (GAA-/-;CD63hu/hu) that were given plasmids encoding an scFv::GAA format of anti-hCD63::GAA (2) or a full-length IgG4::GAA format of anti-mouse integrin alpha-7 (3) by hydrodynamic delivery (HDD), then tissue glycogen levels were measured 3 weeks post-HDD. Untreated control mice, GAA-/-;CD63hu/hu (1) and untreated wild-type GAA control mice, GAA+/+;CD63hu/hu (4) were also tested for glycogen levels in the same tissues.

[0074] Figure 11 is a dot plot depicting serum levels of GAA (arbitrary units "a.u."; y-axis) at one-month post-AAV injection as a function of as a function of enzyme construct and dose. Squares represent AAV-LSP-Δ8GAA. Pyramids represent AAV-anti-hCD63scFv::GAA. Both constructs provided a liver-specific promoter (LSP) to drive expression). Dose is provided as viral genome (vg) per kilogram (kg) of the mouse.

[0075] Figure 12 provides dot blots depicting the levels of glycogen in micrograms per milligram of tissue (heart, quadricep, diaphragm, or tricep) as a function of GAA serum levels. Squares represent AAV-LSP-Δ8GAA. Pyramids represent AAV-anti-hCD63scFv::GAA. Both constructs provided a liver-specific promoter (LSP) to drive expression).

DESCRIPTION

[0076] This invention is not limited to particular embodiments, compositions, methods and experimental conditions described, as such embodiments, compositions, methods and conditions may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0077] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some preferred methods and materials are

now described. All publications cited herein are incorporated herein by reference to describe in their entirety. Unless defined otherwise, 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.

[0078] "Enzyme-deficiency diseases" include non-lysosomal storage disease such as Krabbe disease (galactosylceramidase), phenylketonuria, galactosemia, maple syrup urine disease, mitochondrial disorders, Friedreich ataxia, Zellweger syndrome, adrenoleukodystrophy, Wilson disease, hemochromatosis, ornithine transcarbamylase deficiency, methylmalonic academia, propionic academia, and lysosomal storage diseases. "Lysosomal storage diseases" include any disorder resulting from a defect in lysosome function. Currently, approximately 50 lysosomal storage disorders have been identified, the most well-known of which include Tay-Sachs, Gaucher, and Niemann-Pick disease. The pathogeneses of the diseases are ascribed to the buildup of incomplete degradation products in the lysosome, usually due to loss of protein function. Lysosomal storage diseases are caused by loss-of-function or attenuating variants in the proteins whose normal function is to degrade or coordinate degradation of lysosomal contents. The proteins affiliated with lysosomal storage diseases include enzymes, receptors and other transmembrane proteins (*e.g.*, NPC1), post-translational modifying proteins (*e.g.*, sulfatase), membrane transport proteins, and non-enzymatic cofactors and other soluble proteins (*e.g.*, GM2 ganglioside activator). Thus, lysosomal storage diseases encompass more than those disorders caused by defective enzymes *per se*, and include any disorder caused by any molecular defect. Thus, as used herein, the term "enzyme" is meant to encompass those other proteins associated with lysosomal storage diseases.

[0079] The nature of the molecular lesion affects the severity of the disease in many cases, *i.e.* complete loss-of-function tends to be associated with pre-natal or neo-natal onset, and involves severe symptoms; partial loss-of-function is associated with milder (relatively) and later-onset disease. Generally, only a small percentage of activity needs to be restored to have to correct metabolic defects in deficient cells. Table 1 lists some of the more common lysosomal storage diseases and their associated loss-of-function proteins. Lysosomal storage diseases are generally described in Desnick and Schuchman, 2012.

[0080] Lysosomal storage diseases can be categorized according to the type of product that accumulates within the defective lysosome. Sphingolipidoses are a class of diseases that affect the metabolism of sphingolipids, which are lipids containing fatty acids linked to aliphatic amino alcohols (reviewed in S. Hakomori, "Glycosphingolipids in Cellular Interaction, Differentiation, and Oncogenesis," 50 Annual Review of Biochemistry 733-764, July 1981). The accumulated products of sphingolipidoses include gangliosides (*e.g.*, Tay-Sachs disease), glycolipids (*e.g.*, Fabry's disease), and glucocerebrosides (*e.g.*, Gaucher's disease).

[0081] Mucopolysaccharidoses are a group of diseases that affect the metabolism of glycosaminoglycans (GAGS or mucopolysaccharides), which are long unbranched chains of repeating disaccharides that help build bone, cartilage, tendons, corneas, skin and connective tissue (reviewed in J. Muenzer, "Early initiation of enzyme replacement therapy for the mucopolysaccharidoses," 111(2) Mol. Genet. Metab. 63-72 (Feb. 2014); Sasisekharan *et al.*, "Glycomics approach to structure-function relationships of glycosaminoglycans," 8(1) Ann. Rev. Biomed. Eng. 181-231 (Dec. 2014)). The accumulated products of mucopolysaccharidoses include heparan sulfate, dermatan sulfate, keratin sulfate, various forms of chondroitin sulfate, and hyaluronic acid. For example, Morquio syndrome A is due to a defect in the lysosomal enzyme galactose-6-sulfate sulfatase, which results in the lysosomal accumulation of keratin sulfate and chondroitin 6-sulfate.

[0082] Glycogen storage diseases (a.k.a., glycogenosis) result from a cell's inability to metabolize (make or break-down) glycogen. Glycogen metabolism is moderated by various enzymes or other proteins including glucose-6-phosphatase, acid alpha-glucosidase, glycogen de-branching enzyme, glycogen branching enzyme, muscle glycogen phosphorylase, liver glycogen phosphorylase, muscle phosphofructokinase, phosphorylase kinase, glucose transporter, aldolase A, beta-enolase, and glycogen synthase. An exemplar lysosomal storage/glycogen storage disease is Pompe's disease, in which defective acid alpha-glucosidase causes glycogen to accumulate in lysosomes. Symptoms include hepatomegaly, muscle weakness, heart failure, and in the case of the infantile variant, death by age 2 (see DiMauro and Spiegel, "Progress and problems in muscle glycogenosis," 30(2) Acta Myol. 96-102 (Oct. 2011)).

[0083] "Multidomain therapeutic protein" includes (i) a single protein that contains more than one functional domain, (ii) a protein that contains more than one polypeptide chain, and (iii) a mixture

of more than one protein or more than one polypeptide. The term polypeptide is generally taken to mean a single chain of amino acids linked together via peptide bonds. The term protein encompasses the term polypeptide, but also includes more complex structures. That is, a single polypeptide is a protein, and a protein can contain one or more polypeptides associated in a higher order structure. For example, hemoglobin is a protein containing four polypeptides: two alpha globin polypeptides and two beta globin polypeptides. Myoglobin is also a protein, but it contains only a single myoglobin polypeptide.

[0084] The multidomain therapeutic protein comprises one or more polypeptide(s) and at least two domains providing two functions. One of those domains is the "enzyme domain" which provides the replacement of a defective protein activity associated with an enzyme deficiency disease. The other of those domains is the "delivery domain" which provides binding to an internalization effector. Thus, a single polypeptide that provides an enzyme replacement activity and the ability to bind to an internalization effector (a.k.a. internalization effector-binding protein (delivery domain activity) is a multidomain therapeutic protein. Also, a mixture of proteins, wherein one protein provides the enzyme function, and another protein provides the internalization effector binding activity, is a multidomain therapeutic protein. Figure 1A depicts various exemplars of multidomain therapeutic proteins. In one example (Figure 1A, panel A), the multidomain therapeutic protein contains an enzyme (represented by the hexagon) and a bispecific antibody (the IE-BP) that binds the enzyme (hashed lines) and an internalization effector (solid lines). Here, one arm of the bispecific antibody binds non-covalently to the enzyme, and the other arm binds non-covalently to the internalization effector, thereby enabling the internalization of the replacement enzyme into the cell or subcellular compartment. In another example (panel B), the multidomain therapeutic protein comprises a single protein containing two polypeptides, one polypeptide having enzyme function and the other having delivery domain function. Here, the enzyme is fused to an immunoglobulin Fc domain or heavy chain constant region, which associates with the Fc domain of the enzyme half-antibody to form the bifunctional multidomain therapeutic protein. The embodiment depicted in panel B is similar to that in panel A, except that the enzyme is covalently attached to one of the half-antibodies, rather than through antigen-antibody interaction at the immunoglobulin variable domain of the half-antibody.

[0085] In other examples, the multidomain therapeutic protein consists of the enzyme covalently linked (directly or indirectly through a linker) to the delivery domain. In one embodiment, the

enzyme is attached to the C-terminus of an immunoglobulin molecule (*e.g.*, the heavy chain or alternatively the light chain). In another embodiment, the enzyme is attached to the N-terminus of the immunoglobulin molecule (*e.g.*, the heavy chain or alternatively the light chain). In these exemplars, the immunoglobulin molecule is the delivery domain. In yet another embodiment, the enzyme is attached to the C-terminus of a scFv molecule that binds the internalization effector.

[0086] In one embodiment, the multidomain therapeutic protein comprises two delivery domains. In one embodiment, the first delivery domain binds to a lysosomal trafficking molecule or other internalization effector (*e.g.*, CD63). In another embodiment, the second delivery domain binds to a transcytosis effector to facilitate transcellular transport of the multidomain therapeutic protein. In one embodiment, the transcytosis effector is *inter alia* an LDL receptor, an IgA receptor, a transferrin receptor, or a neonatal Fc receptor (FcRn). In a specific embodiment, the transcytosis delivery domain comprises a molecule that binds to a transferrin receptor, such as *e.g.*, an anti-transferrin receptor antibody or an anti-transferrin receptor scFv molecule. Tuma and Hubbard, "Transcytosis: Crossing Cellular Barriers," Physiological Reviews, 83(3): 871-935 (1 July 2003) is incorporated herein by reference for cell surface receptors that mediate transcytosis that are useful in the practice of the subject invention.

[0087] "Enzyme domain" or "enzyme" denotes any protein associated with the etiology or physiological effect of an enzyme deficiency disease. An enzyme includes the actual enzyme, transport protein, receptor, or other protein that is defective and which is attributed as the molecular lesion that caused the disease. An enzyme also includes any protein that can provide a similar or sufficient biochemical or physiological activity that replaces or circumvents the molecular lesion of the disease. For example, an "isozyme" may be used as an enzyme. Examples of lysosomal storage disease-related proteins include those listed in Table 1 as "Involved Enzyme/Protein" and any known or later discovered protein or other molecule that circumvents the molecular defect of the enzyme-deficiency disease.

[0088] In some embodiments, the enzyme is a hydrolase, including esterases, glycosylases, hydrolases that act on ether bonds, peptidases, linear amidases, diphosphatases, ketone hydrolases, halogenases, phosphoamidases, sulfohydrolases, sulfinases, desulfinases, and the like. In some embodiments, the enzyme is a glycosylase, including glycosidases and *N*-glycosylases. In some embodiments, the enzyme is a glycosidase, including alpha-amylase, beta-amylase, glucan 1,4-

alpha-glucosidase, cellulose, endo-1,3(4)-beta-glucanase, inulinase, endo-1,4-beta-xylanase, endo-1,4-b-xylanase, dextranase, chitinase, polygalacturonidase, lysozyme, exo-alpha-sialidase, alpha-glucosidase, beta-glucosidase, alpha-galactosidase, beta-galactosidase, alpha-mannosidase, beta-mannosidase, beta-fructofuranosidase, alpha,alpha-trehalose, beta-glucuronidase, xylan endo-1,3-beta-xylosidase, amylo-alpha-1,6-glucosidase, hyaluronoglucosaminidase, hyaluronoglucuronidase, and the like.

[0089] In the case of Pompe disease, in which the molecular defect is a defect in α-glucosidase activity, enzymes include human alpha-glucosidase, and "isozymes" such as other alpha-glucosidases, engineered recombinant alpha-glucosidase, other glucosidases, recombinant glucosidases, any protein engineered to hydrolyze a terminal non-reducing 1-4 linked alpha-glucose residue to release a single alpha-glucose molecule, any EC 3.2.1.20 enzyme, natural or recombinant low pH carbohydrate hydrolases for glycogen or starches, and glucosyl hydrolases such as sucrase isomaltase, maltase glucoamylase, glucosidase II, and neutral alpha-glucosidase.

[0090] An "internalizing effector" includes a protein that is capable of being internalized into a cell or that otherwise participates in or contributes to retrograde membrane trafficking. In some instances, the internalizing effector is a protein that undergoes transcytosis; that is, the protein is internalized on one side of a cell and transported to the other side of the cell (*e.g.*, apical-to-basal). In many embodiments, the internalizing effector protein is a cell surface-expressed protein or a soluble extracellular protein. However, the present invention also contemplates embodiments in which the internalizing effector protein is expressed within an intracellular compartment such as the endosome, endoplasmic reticulum, Golgi, lysosome, *etc*. For example, proteins involved in retrograde membrane trafficking (*e.g.*, pathways from early/recycling endosomes to the trans-Golgi network) may serve as internalizing effector proteins in various embodiments of the present invention. In any event, the binding of the delivery domain to an internalizing effector protein causes the entire multidomain therapeutic protein, and any molecules associated therewith (*e.g.*, enzyme), to also become internalized into the cell. As explained below, internalizing effector proteins include proteins that are directly internalized into a cell, as well as proteins that are indirectly internalized into a cell.

[0091] Internalizing effector proteins that are directly internalized into a cell include membrane-associated molecules with at least one extracellular domain (*e.g.*, transmembrane proteins, GPI-

anchored proteins, etc.), which undergo cellular internalization, and are preferably processed via an intracellular degradative and/or recycling pathway. Specific non-limiting examples of internalizing effector proteins that are directly internalized into a cell include, *e.g.*, CD63, MHC-I (*e.g.*, HLA-B27), Kremen-1, Kremen-2, LRP5, LRP6, LRP8, transferrin receptor, LDL-receptor, LDL-related protein 1 receptor, ASGR1, ASGR2, amyloid precursor protein-like protein-2 (APLP2), apelin receptor (APLNR), MAL (Myelin And Lymphocyte protein, a.k.a. VIP17), IGF2R, vacuolar-type H+ ATPase, diphtheria toxin receptor, folate receptor, glutamate receptors, glutathione receptor, leptin receptors, scavenger receptors (*e.g.*, SCARA1-5, SCARB1-3, CD36), and the like.

[0092] In certain embodiments, the internalizing effector is prolactin receptor (PRLR). It was discovered that PRLR is, not only a target for certain therapeutic applications, but also an effective internalizing effector protein on the basis of its high rate of internalization and turn-over. The potential for PRLR as an internalizing effector protein, for example, is illustrated in W02015/026907, where it is demonstrated, *inter alia*, that anti-PRLR antibodies are effectively internalized by PRLR-expressing cells in vitro.

[0093] In certain embodiments, the internalization effector is a kidney specific internalizer, such as CDH16 (Cadheri-16), CLDN16 (Claudn-16), KL (Klotho), PTH1R (parathyroid hormone receptor), SLC22A13 (Solute carrier family 22 member 13), SLC5A2 (Sodium/glucose cotransporter 2), and UMOD (Uromodulin). In other certain embodiments, the internalization effector is a muscle specific internalizer, such as BMPR1A (Bone morphogenetic protein receptor 1A), m-cadherin, CD9, MuSK (muscle-specific kinase), LGR4/GPR48 (G protein-coupled receptor 48), cholinergic receptor (nicotinic) alpha 1, CDH15 (Cadheri-15), ITGA7 (Integrin alpha-7), CACNG1 (L-type calcium channel subunit gamma-1), CACNAlS (L-type calcium channel subunit alpha-15), CACNG6 (L-type calcium channel subunit gamma-6), SCN1B (Sodium channel subunit beta-1), CHRNA1 (ACh receptor subunit alpha), CHRND (ACh receptor subunit delta), LRRC14B (Leucine-rich repeat-containing protein 14B), dystroglycan (DAG1), and POPDC3 (Popeye domain-containing protein 3). In some specific embodiments, the internalization effector is ITGA7, CD9, CD63, ALPL2, ASGR1, ASGR2, or PRLR.

[0094] In those embodiments in which the internalization effector (IE) is directly internalized into a cell, the delivery domain can be, *e.g.*, an antibody or antigen-binding fragment of an antibody that specifically binds the IE, or a ligand or portion of a ligand that specifically interacts with the IE.

For example, if the IE is Kremen-1 or Kremen-2, the delivery domain can comprise or consist of a Kremen ligand (*e.g.*, DKK1) or Kremen-binding portion thereof. As another example, if the IE is a receptor molecule such as ASGR1, the delivery domain can comprise or consist of a ligand specific for the receptor (*e.g.*, asialoorosomucoid [ASOR] or Beta-GalNAc) or a receptor-binding portion thereof.

[0095] Internalizing effector proteins that are indirectly internalized into a cell include proteins and polypeptides that do not internalize on their own, but become internalized into a cell after binding to or otherwise associating with a second protein or polypeptide that is directly internalized into the cell. Proteins that are indirectly internalized into a cell include, *e.g.*, soluble ligands that are capable of binding to an internalizing cell surface-expressed receptor molecule. A non-limiting example of a soluble ligand that is (indirectly) internalized into a cell via its interaction with an internalizing cell surface-expressed receptor molecule is transferrin. In embodiments, wherein the IE is transferrin (or another indirectly internalized protein), the binding of the delivery domain to the IE, and the interaction of IE with transferrin receptor (or another internalizing cell-surface expressed receptor molecule), causes the entire delivery domain, and any molecules associated therewith (*e.g.*, the enzyme), to become internalized into the cell concurrent with the internalization of the IE and its binding partner.

[0096] In those embodiments in which the IE is indirectly internalized into a cell, the delivery domain can be, *e.g.*, an antibody, antigen-binding fragment of an antibody, or an scFv that specifically binds IE, or a receptor or portion of a receptor that specifically interacts with the soluble effector protein. For example, if the IE is a cytokine, the delivery domain can comprise or consist of the corresponding cytokine receptor or ligand-binding portion thereof.

[0097] An exemplar IE is CD63, which is a member of the tetraspanin superfamily of cell surface proteins that span the cell membrane four times. CD63 is expressed in virtually all tissues and is thought to be involved in forming and stabilizing signaling complexes. CD63 localizes to the cell membrane, lysosomal membrane, and late endosomal membrane. CD63 is known to associate with integrins and may be involved in epithelial-mesenchymal transitioning. See H. Maecker *et al.*, "The tetraspanin superfamily: molecular facilitators," 11(6) FASEB J. 428-42, May 1997; and M. Metzelaar *et al.*, "CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells," 266 J. Biol. Chem. 3239-3245, 1991.

[0098] Another exemplar IE is amyloid beta (A4) precursor-like protein 2 ("APLP2"), a ubiquitously expressed member of the APP (amyloid precursor protein) family. APLP2 is a membrane-bound protein known to interact with major histocompatibility complex (MHC) class I molecules (*e.g.*, Kd). It binds Kd at the cell surface and is internalized in a clathrin-dependent manner with Kd in tow. See Tuli *et al.*, "Mechanism for amyloid precursor-like protein 2 enhancement of major histocompatibility complex class I molecule degradation," 284 The Journal of Biological Chemistry 34296 -34307 (2009).

[0099] Another IE exemplar is the prolactin receptor (PRLR). The prolactin receptor is a member of the type I cytokine receptor family and upon ligand binding and subsequent dimerization activates "the tyrosine kinases Jak2, Fyn and Tec, the phosphatase SHP-2, the guanine nucleotide exchange factor Vav, and the signaling suppressor SOCS," (see Clevenger and Kline, "Prolactin receptor signal transduction," 10(10) Lupus 706-18 (2001), abstract). The prolactin receptor undergoes endocytotic recycling and can be found in lysosomal fractions. See Genty *et al.*, "Endocytosis and degradation of prolactin and its receptor in Chinese hamster ovary cells stably transfected with prolactin receptor cDNA," 99(2) Mol. Cell Endocrinol. 221-8 (1994); and Ferland *et al.*, "The effect of chloroquine on lysosomal prolactin receptors in rat liver," 115(5) Endocrinology 1842-9 (1984).

[0100] As used herein, "immunological reaction" generally means a patient's immunological response to an outside or "non-self' protein. This immunological response includes an allergic reaction and the development of antibodies that interfere with the effectiveness of the replacement enzyme. Some patients may not produce any of the non-functioning protein, thus rendering the replacement enzyme a "foreign" protein. For example, repeated injection of recombinant GLA (rGLA) to those Fabry patients who lack GLA frequently results in an allergic reaction. In other patients, the production of antibodies against rGLA has been shown to decrease the effectiveness of the replacement enzyme in treating the disease. See for example Tajima *et al.* ("Use of a Modified α-*N*-Acetylgalactosaminidase (NAGA) in the Development of Enzyme Replacement Therapy for Fabry Disease," 85(5) Am. J. Hum. Genet. 569-580 (2009)), which discusses the use of modified NAGA as the "isozyme" to replace GLA. The modified NAGA has no immunological cross-reactivity with GLA, and "did not react to serum from a patient with Fabry disease recurrently treated with a recombinant GLA." *Id,* abstract.

[0101] An "immunosuppressive agent" includes drugs and/or proteins that result in general immunosuppression, and may be used to prevent cross-reactive immunological materials (CRIM) against replacement enzymes, e.g., GAA or GLA respectively in a patient with Pompe or Fabry's disease. Non-limiting examples of an immunosuppressive agent include methotrexate, mycophenolate mofetil, cyclophosphamide, rapamycin DNA alkylating agents, anti-CD20 antibody, anti-BAFF antibody, anti-CD3 antibody, anti-CD4 antibody, and any combination thereof.

[0102] Regulatory elements, e.g., promoters, that are specific to a tissue, e.g., liver, enhance expression of nucleic acid sequences, e.g., genes, under the control of such regulatory element in the tissue for which the regulatory element is specific. Non-limiting examples of a liver specific regulatory element, e.g., liver specific promoters, may be found in Chuah et al. (2014) *Mol. Ther.* 22:1605-13.

[0103] The term "protein" means any amino acid polymer having more than about 20 amino acids covalently linked via amide bonds. Proteins contain one or more amino acid polymer chains, generally known in the art as "polypeptides". Thus, a polypeptide may be a protein, and a protein may contain multiple polypeptides to form a single functioning biomolecule. Disulfide bridges (*i.e.*, between cysteine residues to form cystine) may be present in some proteins. These covalent links may be within a single polypeptide chain, or between two individual polypeptide chains. For example, disulfide bridges are essential to proper structure and function of insulin, immunoglobulins, protamine, and the like. For a recent review of disulfide bond formation, see Oka and Bulleid, "Forming disulfides in the endoplasmic reticulum," 1833(11) Biochim Biophys Acta 2425-9 (2013).

[0104] As used herein, "protein" includes biotherapeutic proteins, recombinant proteins used in research or therapy, trap proteins and other Fc-fusion proteins, chimeric proteins, antibodies, monoclonal antibodies, human antibodies, bispecific antibodies, antibody fragments, nanobodies, recombinant antibody chimeras, scFv fusion proteins, cytokines, chemokines, peptide hormones, and the like. Proteins may be produced using recombinant cell-based production systems, such as the insect bacculovirus system, yeast systems (*e.g.*, *Pichia* sp.), mammalian systems (*e.g.*, CHO cells and CHO derivatives like CHO-K1 cells). For a recent review discussing biotherapeutic proteins and their production, see Ghaderi *et al.*, "Production platforms for biotherapeutic

glycoproteins. Occurrence, impact, and challenges of non-human sialylation," 28 Biotechnol Genet Eng Rev. 147-75 (2012).

[0105] The term "antibody", as used herein, includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (heavy chain CDRs may be abbreviated as HCDR1, HCDR2 and HCDR3; light chain CDRs may be abbreviated as LCDR1, LCDR2 and LCDR3. The term "high affinity" antibody refers to those antibodies having a binding affinity to their target of at least 10^{-9} M, at least 10^{-10} M; at least 10^{-11} M; or at least 10^{-12} M, as measured by surface plasmon resonance, *e.g.*, BIACORE™ or solution-affinity ELISA. The term "antibody" may encompass any type of antibody, such as e.g. monoclonal or polyclonal. Moreover, the antibody may be or any origin, such as e.g. mammalian or non-mammalian. In one embodiment, the antibody may be mammalian or avian. In a further embodiment, the antibody may be or human origin and may further be a human monoclonal antibody.

[0106] The phrase "bispecific antibody" includes an antibody capable of selectively binding two or more epitopes. Bispecific antibodies generally comprise two different heavy chains, with each heavy chain specifically binding a different epitope—either on two different molecules (*e.g.*, antigens) or on the same molecule (*e.g.*, on the same antigen). If a bispecific antibody is capable of selectively binding two different epitopes (a first epitope and a second epitope), the affinity of the first heavy chain for the first epitope will generally be at least one to two or three or four orders of magnitude lower than the affinity of the first heavy chain for the second epitope, and vice versa. The epitopes recognized by the bispecific antibody can be on the same or a different target (*e.g.*, on the same or a different protein). Bispecific antibodies can be made, for example, by combining heavy chains that recognize different epitopes of the same antigen. For example, nucleic acid

sequences encoding heavy chain variable sequences that recognize different epitopes of the same antigen can be fused to nucleic acid sequences encoding different heavy chain constant regions, and such sequences can be expressed in a cell that expresses an immunoglobulin light chain. A typical bispecific antibody has two heavy chains each having three heavy chain CDRs, followed by (N-terminal to C-terminal) a CH1 domain, a hinge, a CH2 domain, and a CH3 domain, and an immunoglobulin light chain that either does not confer antigen-binding specificity but that can associate with each heavy chain, or that can associate with each heavy chain and that can bind one or more of the epitopes bound by the heavy chain antigen-binding regions, or that can associate with each heavy chain and enable binding or one or both of the heavy chains to one or both epitopes.

[0107] The phrase "heavy chain," or "immunoglobulin heavy chain" includes an immunoglobulin heavy chain constant region sequence from any organism, and unless otherwise specified includes a heavy chain variable domain. Heavy chain variable domains include three heavy chain CDRs and four FR regions, unless otherwise specified. Fragments of heavy chains include CDRs, CDRs and FRs, and combinations thereof. A typical heavy chain has, following the variable domain (from N-terminal to C-terminal), a CH1 domain, a hinge, a CH2 domain, and a CH3 domain. A functional fragment of a heavy chain includes a fragment that is capable of specifically recognizing an antigen (*e.g.*, recognizing the antigen with a KD in the micromolar, nanomolar, or picomolar range), that is capable of expressing and secreting from a cell, and that comprises at least one CDR.

[0108] The phrase "light chain" includes an immunoglobulin light chain constant region sequence from any organism, and unless otherwise specified includes human kappa and lambda light chains. Light chain variable (VL) domains typically include three light chain CDRs and four framework (FR) regions, unless otherwise specified. Generally, a full-length light chain includes, from amino terminus to carboxyl terminus, a VL domain that includes FR1-CDR1- FR2-CDR2-FR3-CDR3-FR4, and a light chain constant domain. Light chains that can be used with this invention include *e.g.*, those, that do not selectively bind either the first or second antigen selectively bound by the antigen-binding protein. Suitable light chains include those that can be identified by screening for the most commonly employed light chains in existing antibody libraries (wet libraries or *in silico*), where the light chains do not substantially interfere with the affinity and/or selectivity of the antigen-binding domains of the antigen-binding proteins. Suitable light chains include those that

can bind one or both epitopes that are bound by the antigen-binding regions of the antigen-binding protein.

[0109] The phrase "variable domain" includes an amino acid sequence of an immunoglobulin light or heavy chain (modified as desired) that comprises the following amino acid regions, in sequence from N-terminal to C-terminal (unless otherwise indicated): FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. A "variable domain" includes an amino acid sequence capable of folding into a canonical domain (VH or VL) having a dual beta sheet structure wherein the beta sheets are connected by a disulfide bond between a residue of a first beta sheet and a second beta sheet.

[0110] The phrase "complementarity determining region," or the term "CDR," includes an amino acid sequence encoded by a nucleic acid sequence of an organism's immunoglobulin genes that normally (*i.e.*, in a wildtype animal) appears between two framework regions in a variable region of a light or a heavy chain of an immunoglobulin molecule (*e.g.*, an antibody or a T cell receptor). A CDR can be encoded by, for example, a germline sequence or a rearranged or unrearranged sequence, and, for example, by a naive or a mature B cell or a T cell. In some circumstances (*e.g.*, for a CDR3), CDRs can be encoded by two or more sequences (*e.g.*, germline sequences) that are not contiguous (*e.g.*, in an unrearranged nucleic acid sequence) but are contiguous in a B cell nucleic acid sequence, *e.g.*, as the result of splicing or connecting the sequences (*e.g.*, V-D-J recombination to form a heavy chain CDR3).

[0111] The term "antibody fragment", refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. Examples of binding fragments encompassed within the term "antibody fragment" include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.* (1989) Nature 241:544-546), which consists of a VH domain, (vi) an isolated CDR, and (vii) an scFv, which consists of the two domains of the Fv fragment, VL and VH, joined by a synthetic linker to form a single protein chain in which the VL and VH regions pair to form monovalent molecules. Other forms of single chain antibodies, such as diabodies are also encompassed under the term "antibody" (see *e.g.*, Holliger *et al.* (1993) PNAS USA 90:6444-6448; Poljak *et al.* (1994) Structure 2:1121-1123).

[0112] The phrase "Fc-containing protein" includes antibodies, bispecific antibodies, immunoadhesins, and other binding proteins that comprise at least a functional portion of an immunoglobulin CH2 and CH3 region. A "functional portion" refers to a CH2 and CH3 region that can bind a Fc receptor (*e.g.*, an FcyR; or an FcRn, *i.e.*, a neonatal Fc receptor), and/or that can participate in the activation of complement. If the CH2 and CH3 region contains deletions, substitutions, and/or insertions or other modifications that render it unable to bind any Fc receptor and also unable to activate complement, the CH2 and CH3 region is not functional.

[0113] Fc-containing proteins can comprise modifications in immunoglobulin domains, including where the modifications affect one or more effector function of the binding protein (*e.g.*, modifications that affect FcyR binding, FcRn binding and thus half-life, and/or CDC activity). Such modifications include, but are not limited to, the following modifications and combinations thereof, with reference to EU numbering of an immunoglobulin constant region: 238, 239, 248, 249, 250, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 297, 298, 301, 303, 305, 307, 308, 309, 311, 312, 315, 318, 320, 322, 324, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 337, 338, 339, 340, 342, 344, 356, 358, 359, 360, 361, 362, 373, 375, 376, 378, 380, 382, 383, 384, 386, 388, 389, 398, 414, 416, 419, 428, 430, 433, 434, 435, 437, 438, and 439.

[0114] For example, and not by way of limitation, the binding protein is an Fc-containing protein and exhibits enhanced serum half-life (as compared with the same Fc-containing protein without the recited modification(s)) and have a modification at position 250 (*e.g.*, E or Q); 250 and 428 (*e.g.*, L or F); 252 (*e.g.*, L/Y/F/W or T), 254 (*e.g.*, S or T), and 256 (*e.g.*, S/R/Q/E/D or T); or a modification at 428 and/or 433 (*e.g.*, L/R/SI/P/Q or K) and/or 434 (*e.g.*, H/F or Y); or a modification at 250 and/or 428; or a modification at 307 or 308 (*e.g.*, 308F, V308F), and 434. In another example, the modification can comprise a 428L (*e.g.*, M428L) and 434S (*e.g.*, N434S) modification; a 428L, 2591 (*e.g.*, V259I), and a 308F (*e.g.*, V308F) modification; a 433K (*e.g.*, H433K) and a 434 (*e.g.*, 434Y) modification; a 252, 254, and 256 (*e.g.*, 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.*, T250Q and M428L); a 307 and/or 308 modification (*e.g.*, 308F or 308P).

[0115] The term "antigen-binding protein," as used herein, refers to a polypeptide or protein (one or more polypeptides complexed in a functional unit) that specifically recognizes an epitope on an

antigen, such as a cell-specific antigen and/or a target antigen of the present invention. An antigen-binding protein may be multi-specific. The term "multi-specific" with reference to an antigen-binding protein means that the protein recognizes different epitopes, either on the same antigen or on different antigens. A multi-specific antigen-binding protein of the present invention can be a single multifunctional polypeptide, or it can be a multimeric complex of two or more polypeptides that are covalently or non-covalently associated with one another. The term "antigen-binding protein" includes antibodies or fragments thereof of the present invention that may be linked to or co-expressed with another functional molecule, *e.g.*, another peptide or protein. For example, an antibody or fragment thereof can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, non-covalent association or otherwise) to one or more other molecular entities, such as a protein or fragment thereof to produce a bispecific or a multi-specific antigen-binding molecule with a second binding specificity.

[0116] As used herein, the term "epitope" refers to the portion of the antigen which is recognized by the multi-specific antigen-binding polypeptide. A single antigen (such as an antigenic polypeptide) may have more than one epitope. Epitopes may be defined as structural or functional. Functional epitopes are generally a subset of structural epitopes and are defined as those residues that directly contribute to the affinity of the interaction between the antigen-binding polypeptide and the antigen. Epitopes may also be conformational, that is, composed of non-linear amino acids. In certain embodiments, epitopes may include determinants that are chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl groups, or sulfonyl groups, and, in certain embodiments, may have specific three-dimensional structural characteristics, and/or specific charge characteristics. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents.

[0117] The term "domain" refers to any part of a protein or polypeptide having a particular function or structure. Preferably, domains of the present invention bind to cell-specific or target antigens. Cell-specific antigen- or target antigen-binding domains, and the like, as used herein, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen.

[0118] The term "half-body" or "half-antibody", which are used interchangeably, refers to half of an antibody, which essentially contains one heavy chain and one light chain. Antibody heavy chains can form dimers, thus the heavy chain of one half-body can associate with heavy chain associated with a different molecule (*e.g.*, another half-body) or another Fc-containing polypeptide. Two slightly different Fc-domains may "heterodimerize" as in the formation of bispecific antibodies or other heterodimers, -trimers, -tetramers, and the like. See Vincent and Murini, "Current strategies in antibody engineering: Fc engineering and pH-dependent antigen binding, bispecific antibodies and antibody drug conjugates," 7 Biotechnol. J. 1444-1450 (20912); and Shimamoto *et al.*, "Peptibodies: A flexible alternative format to antibodies," 4(5) MAbs 586-91 (2012).

[0119] In one embodiment, the half-body variable domain specifically recognizes the internalization effector and the half body Fc-domain dimerizes with an Fc-fusion protein that comprises a replacement enzyme (*e.g.*, a peptibody) Id, 586.

[0120] The term "single-chain variable fragment" or "scFv" includes a single chain fusion polypeptide containing an immunoglobulin heavy chain variable region (VH) and an immunoglobulin light chain variable region (VL). In some embodiments, the VH and VL are connect by a linker sequence of 10 to 25 amino acids. ScFv polypeptides may also include other amino acid sequences, such as CL or CH1 regions. ScFv molecules can be manufactured by phage display or made by directly subcloning the heavy and light chains from a hybridoma or B-cell. Ahmad *et al.,* Clinical and Developmental Immunology, volume 2012, article ID 98025 is incorporated herein by reference for methods of making scFv fragments by phage display and antibody domain cloning.

[0121] "Alpha-glucosidase" (or "α-glucosidase"), "α-glucosidase activity", "GAA", and "GAA activity" are used interchangeably and refer to any protein that facilitates the hydrolysis of 1,4-alpha bonds of glycogen and starch into glucose. GAA is also known *inter alia* as EC 3.2.1.20, maltase, glucoinvertase, glucosidosucrase, maltase-glucoamylase, alpha-glucopyranosidase, glucosidoinvertase, alpha-D-glucosidase, alpha-glucoside hydrolase, alpha-1,4-glucosidase, and alpha-D-glucoside glucohydrolase. GAA can be found in the lysosome and at the brush border of the small intestine. Patients who suffer from Pompe disease lack functioning lysosomal α-glucosidase. See S. Chiba, "Molecular mechanism in alpha-glucosidase and glucoamylase,"

61(8) Biosci. Biotechnol. Biochem. 1233-9 (1997); and Hesselink *et al.*, "Lysosomal dysfunction in muscle with special reference to glycogen storage disease type II," 1637(2) Biochim. Biophys. Acta. 164-70 (2003).

[0122] "Alpha-galactosidase A"(or "α-galactosidase A"), "α-galactosidase A activity", "α-galactosidase", "α-galactosidase activity", "GLA", and "GLA activity" are used interchangeably and refer to any protein that facilitates the hydrolysis of terminal α-galactosyl moieties from glycolipids and glycoproteins, and also hydrolyses α-D-fucosides. GLA is also known *inter alia* as EC 3.2.1.22, melibiase, α-D-galactosidase, α-galactosidase A, α-galactoside galactohydrolase, α-D-galactoside galactohydrolase. GLA is a lysosomal enzyme encoded by the X-linked GLA gene. Defects in GLA can lead to Fabry Disease, in which the glycolipid known as globotriaosylceramide (a.k.a. Gb3, GL-3, or ceramide trihexoside) accumulates within blood vessels (*i.e.*, prominent vasculopathy), resulting in pain and impairment in the function of kidney, heart, skin, and/or cerebrovascular tissues. and other tissues, and organs. See for example Prabakaran *et al.* "Mannose 6-phosphate receptor and sortilin mediated endocytosis of α-galactosidase A in kidney endothelial cells," 7(6) PLoS One e39975 pp. 1-9 (2012).

[0123] In one aspect, the invention provides a method of treating a patient (or subject) suffering from a lysosomal storage disease by administering to the patient a "multidomain therapeutic protein". The multidomain therapeutic protein enters the cells of the patient and delivers to the lysosomes an enzyme or enzymatic activity that (*i.e.*, "replacement enzyme") that replaces the enzyme (i.e, "endogenous enzyme") or enzymatic activity that is associated with the LSD. In one embodiment, the multidomain therapeutic protein is delivered to the patient via a gene therapy vector that contains a polynucleotide that encodes the multidomain therapeutic protein.

[0124] LSDs include sphingolipidoses, a mucopolysaccharidoses, and glycogen storage diseases. In some embodiments, the LSD is any one or more of Fabry disease, Gaucher disease type I, Gaucher disease type II, Gaucher disease type III, Niemann-Pick disease type A, Niemann-Pick disease type BGM1-gangliosidosis, Sandhoff disease, Tay-Sachs disease, GM2- activator deficiency, GM3-gangliosidosis, metachromatic leukodystrophy, sphingolipid-activator deficiency, Scheie disease, Hurler-Sceie disease, Hurler disease, Hunter disease, Sanfilippo A, Sanfilippo B, Sanfilippo C, Sanfilippo D, Morquio syndrome A, Morquio syndrome B, Maroteaux-Lamy disease, Sly disease,

MPS IX, and Pompe disease. In a specific embodiment, the LSD is Fabry disease. In another embodiment, the LSD is Pompe disease.

[0125] In some embodiments, the multidomain therapeutic protein comprises (a) the replacement enzyme, and (b) a molecular entity that binds an internalization effector (delivery domain). In some cases, the replacement enzyme is any one or more of α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, saposin-C activator, ceramidase, sphingomyelinase, β-hexosaminidase, GM2 activator, GM3 synthase, arylsulfatase, sphingolipid activator, α-iduronidase, iduronidase-2-sulfatase, heparin *N*-sulfatase, *N*-acetyl-α-glucosaminidase, α-glucosamide *N*-acetyltransferase, *N*-acetylglucosamine-6-sulfatase, *N*-acetylgalactosamine-6-sulfate sulfatase, *N*-acetylgalactosamine-4-sulfatase, β-glucuronidase, and hyaluronidase.

[0126] In some cases, the patient may not make sufficient protein such that a replacement enzyme is recognized by the patient as "non-self" and an immunological reaction ensues after administering a replacement enzyme. This is not desirable. Therefore, in some embodiments, the replacement enzyme is designed or produced in such a way as to avoid inducing an immunological reaction in the subject. One such solution is to use an "isozyme" as a replacement enzyme. An isozyme is sufficiently close to a "self" protein of the patient, but has the replacement enzyme activity sufficient to ameliorate the symptoms of the LSD.

[0127] In one particular embodiment, in which the LSD is Pompe disease and the endogenous enzyme is α-glucosidase (GAA), the isozyme can be any one of acid α-glucosidase, sucrase-isomaltase (SI), maltase-glucoamylase (MGAM), glucosidase II (GANAB), and neutral α-glucosidase (C GNAC). In another particular embodiment, in which the LSD is Fabry disease and the endogenous enzyme is α-galactosidase A (GLA), the isozyme can be an α-*N*-acetylgalactosaminidase engineered to have GLA activity.

[0128] Provided herein are methods, other than to use of a isozyme, to reduce cross-reactive immunological materials (CRIM) against the replacement enzyme. As demonstrated in Figures 5 and 6, administration of a multidomain therapeutic protein (e.g., via a gene therapy vector) comprising an internalizing effector binding domain and the enzyme domain reduces the level of CRIM against the replacement enzyme comprised to administration of a control therapeutic protein (lacking the internalizing effector domain and comprising an enzyme domain). As such, in one embodiment or reducing CRIM against an enzyme in a patient with a deficiency in the enzyme

comprises administering to the patient the patient a multidomain therapeutic protein (or nucleic acid encoding same, e.g., a gene therapy vector containing a gene encoding the multidomain therapeutic protein, wherein the multidomain therapeutic protein comprises a delivery domain (e.g., internalization effector binding protein) and an enzyme domain.

[0129] The multidomain therapeutic protein has an internalization effector binding protein component that enables the uptake of the replacement enzyme into the cell. Thus, in some embodiments, the internalization effector can be CD63, MHC-I, Kremen-1, Kremen-2, LRP5, LRP6, LRP8, transferrin receptor, LDL-receptor, LDL-related protein 1 receptor, ASGR1, ASGR2, amyloid precursor protein-like protein-2 (APLP2), apelin receptor (APLNR), PRLR (prolactin receptor), MAL (Myelin And Lymphocyte protein, a.k.a. VIP17), IGF2R, vacuolar-type H+ ATPase, diphtheria toxin receptor, folate receptor, glutamate receptors, glutathione receptor, leptin receptor, scavenger receptor, SCARA1-5, SCARB1-3, and CD36. In certain embodiments, the internalization effector is a kidney specific internalizer, such as CDH16 (Cadheri-16), CLDN16 (Claudn-16), KL (Klotho), PTH1R (parathyroid hormone receptor), SLC22A13 (Solute carrier family 22 member 13), SLC5A2 (Sodium/glucose cotransporter 2), and UMOD (Uromodulin). In other certain embodiments, the internalization effector is a muscle specific internalizer, such as BMPR1A (Bone morphogenetic protein receptor 1A), m-cadherin, CD9, MuSK (muscle-specific kinase), LGR4/GPR48 (G protein-coupled receptor 48), cholinergic receptor (nicotinic) alpha 1, CDH15 (Cadheri-15), ITGA7 (Integrin alpha-7), CACNG1 (L-type calcium channel subunit gamma-1), CACNAlS (L-type calcium channel subunit alpha-15), CACNG6 (L-type calcium channel subunit gamma-6), SCN1B (Sodium channel subunit beta-1), CHRNA1 (ACh receptor subunit alpha), CHRND (ACh receptor subunit delta), LRRC14B (Leucine-rich repeat-containing protein 14B), dystroglycan (DAG1), and POPDC3 (Popeye domain-containing protein 3). In some specific embodiments, the internalization effector is ITGA7, CD9, CD63, APLP2, ASGR1, ASGR2, or PRLR.

[0130] In some embodiments, the internalization effector-binding protein comprises an antigen-binding protein, which includes for example a receptor-fusion molecule, a trap molecule, a receptor-Fc fusion molecule, an antibody, an Fab fragment, an F(ab')2 fragment, an Fd fragment, an Fv fragment, a single-chain Fv (scFv) molecule, a dAb fragment, an isolated complementarity determining region (CDR), a CDR3 peptide, a constrained FR3-CDR3-FR4 peptide, a domain-specific antibody, a single domain antibody, a domain-deleted antibody, a chimeric antibody, a

CDR-grafted antibody, a diabody, a triabody, a tetrabody, a minibody, a nanobody, a monovalent nanobody, a bivalent nanobody, a small modular immunopharmaceutical (SMIP), a camelid antibody (VHH heavy chain homodimeric antibody), and a shark variable IgNAR domain.

[0131] In one embodiment, the molecular entity that binds the internalization effector is an antibody, an antibody fragment, or other antigen-binding protein. For example, the molecular entity can be a bispecific antibody, in which one arm binds the internalization effector (*e.g.*, ITGA7, CD9, CD63, PRLR, APLP2. ASGR1, ASGR2), and the other arm binds the replacement enzyme. Here, the multidomain therapeutic protein comprises the bispecific antibody and the replacement enzyme (Fig. 1A). In a specific embodiment, the disease treated is Fabry disease, and the multidomain therapeutic protein comprises GLA and a bispecific antibody that binds GLA and CD63. In a specific embodiment, the disease treated is Fabry disease, and the multidomain therapeutic protein comprises GLA and a bispecific antibody that binds GLA and ITGA7. In another specific embodiment, the disease treated is Pompe disease, and the multidomain therapeutic protein comprises GAA and a bispecific antibody that binds GAA and CD63. In another specific embodiment, the disease treated is Pompe disease, and the multidomain therapeutic protein comprises GAA and a bispecific antibody that binds GAA and ITGA7.

[0132] In another embodiment, the molecular entity that binds the internalization effector comprises a half-antibody, and the replacement enzyme contains an Fc domain (enzyme-Fc fusion polypeptide). In one embodiment, the Fc domain of the enzyme-Fc fusion polypeptide associates with the Fc domain of the internalization effector-specific half-body to form the multidomain therapeutic protein (Fig. 1B).

[0133] In other embodiments, the replacement enzyme is covalently linked to internalization effector-binding protein. The enzyme-Fc fusion:half-body embodiment described in the previous paragraph (see also Fig. 1B) falls into this class, since the Fc dimer can be secured via one or more disulfide bridges. The covalent linkage between the enzyme activity domain or polypeptide and the internalization-binding domain or polypeptide may be any type of covalent bond, *i.e.*, any bond that involved sharing of electrons. In some cases, the covalent bond is a peptide bond between two amino acids, such that the replacement enzyme and the internalization effector-binding protein in whole or in part form a continuous polypeptide chain, as in a fusion protein. In some cases, the replacement enzyme portion and the internalization effector-binding protein are directly linked. In

other cases, a linker is used to tether the two portions. See Chen *et al.*, "Fusion protein linkers: property, design and functionality," 65(10) Adv Drug Deliv Rev. 1357-69 (2013).

[0134] In a particular embodiment, the replacement enzyme is covalently linked to the C- terminus of the heavy chain of an anti-internalization effector antibody (see Fig. 1C) or to the C-terminus of the light chain (Fig. 1E). In another particular embodiment, the replacement enzyme is covalently linked to the N-terminus of the heavy chain of an anti-internalization effector antibody (see Fig. 1D) or to the N-terminus of the light chain (Fig. 1F). In another particular embodiment, the enzyme is linked to the C-terminus of an anti-internalization effector scFv domain (Fig. 1G).

[0135] In some cases, especially where the replacement enzyme is not normally proteolytically processed in the lysosome, a cleavable linker is added to those embodiments of the multidomain therapeutic protein that comprise an antibody-enzyme fusion. In some embodiments, a cathepsin cleavable linker is inserted between the antibody and the replacement enzyme to facilitate removal of the antibody in the lysosome in order to a) possibly help preserve enzymatic activity by removing the sterically large antibody and b) possibly increase lysosomal half-life of the enzyme.

[0136] In one particular embodiment, the multidomain therapeutic protein is delivered to the patient or cell in a gene therapy vector that contains a polynucleotide that encodes the multidomain therapeutic protein. In one embodiment, the multidomain therapeutic protein comprises a delivery domain and an enzyme domain. In a specific embodiment, the delivery domain binds to an internalizing effector, such as CD63, MHC-I, Kremen-1, Kremen-2, LRP5, LRP6, LRP8, transferrin receptor, LDL-receptor, LDL-related protein 1 receptor, ASGR1, ASGR2, amyloid precursor protein-like protein-2 (APLP2), apelin receptor (APLNR), MAL (myelin and lymphocyte protein (MAL), IGF2R, vacuolar-type H+ ATPase, diphtheria toxin receptor, folate receptor, glutamate receptors, glutathione receptor, leptin receptors, scavenger receptor A1-5 (SCARA1-5), SCARB1-3, or CD36. In one embodiment, the delivery domain is a single-chain variable fragment (scFv) that binds to CD63 (*i.e.,* anti-CD63 scFv). In another embodiment, the delivery domain is a single-chain variable fragment (scFv) that binds to ITGA7 (*i.e.,* anti-ITGA7 scFv).

[0137] In one particular embodiment, the enzyme domain of the multidomain therapeutic protein comprises a hydrolase. In a specific embodiment, the enzyme domain comprises a hydrolase that is a glycosylase. In a more specific embodiment, the enzyme domain comprises a glycosylase that is a

glycosidase. In a more specific embodiment, the enzyme domain is a glycosidase that is alpha-glucosidase.

[0138] Generally, disclosed herein are compositions comprising and use of polynucleotides, e.g., (m)RNA, DNA, and modified forms thereof, that encode a multidomain therapeutic protein comprising an internalizing effector domain and an enzyme domain in the treatment of lysosomal storage diseases, e.g., for the reduction of glycogen and/or the enhancement of immune tolerance for GAA in a patient with Pompe disease.

[0139] The term "polynucleotide" includes a polymer of nucleotides (e.g., RNA or DNA) that encodes at least one polypeptide, including fusion polypeptides, e.g., a multidomain therapeutic polypeptide comprising an internalizing effector domain and an enzyme domain. Polynucleotide as used herein encompasses polymers comprising both modified and unmodified nucleotides. A polynucleotide may contain one or more coding and non-coding regions. A polynucleotide can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, etc. Where appropriate, e.g., in the case of chemically synthesized molecules, A polynucleotide can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, backbone modifications, etc. A polynucleotide sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, a polynucleotide is or comprises natural nucleosides (e.g., adenosine, guanosine, cytidine, uridine); nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

[0140] In some embodiments, a polynucleotide comprises one or more nonstandard nucleotide residues. The nonstandard nucleotide residues may include, e.g., 5-methyl-cytidine ("5mC"), pseudouridine (".psi.U"), and/or 2-thio-uridine ("2sU"). See, e.g., U.S. Pat. No. 8,278,036 or WO2011012316, each of which is incorporated in its entirety by reference for a discussion of such

residues and their incorporation into a polynucleotide. The presence of nonstandard nucleotide residues may render a polynucleotide more stable and/or less immunogenic than a control a polynucleotide with the same sequence but containing only standard residues. In further embodiments, a polynucleotide may comprise one or more nonstandard nucleotide residues chosen from isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine and 2-chloro-6-aminopurine cytosine, as well as combinations of these modifications and other nucleobase modifications. Certain embodiments may further include additional modifications to the furanose ring or nucleobase. Additional modifications may include, for example, sugar modifications or substitutions (e.g., one or more of a 2'-O-alkyl modification, a locked nucleic acid (***LNA***)). In some embodiments, the polynucleotide may be complexed or hybridized with additional polynucleotides and/or peptide polynucleotides (***PNA***). In embodiments where the sugar modification is a 2'-O-alkyl modification, such modification may include, but are not limited to a 2'-deoxy-2'-fluoro modification, a 2'-O-methyl modification, a 2'-O-methoxyethyl modification and a 2'-deoxy modification. In certain embodiments, any of these modifications may be present in 0-100% of the nucleotides--for example, more than 0%, 1%, 10%, 25%, 50%, 75%, 85%, 90%, 95%, or 100% of the constituent nucleotides individually or in combination. In some embodiments, a polynucleotide comprises messenger RNA (mRNA) molecules, which may or may not be modified, e.g., which may or may not comprise a modified nucleotide, by well-known methods to increase their stability and/or decrease their immunogenicity. In some embodiments, a polynucleotide comprises DNA molecules, which may which may or may not be modified, e.g., which may or may not comprise a modified nucleotide, by well-known methods to increase their stability and/or decrease their immunogenicity.

[0141] In some embodiments, the polynucleotide also includes a "locus-targeting nucleic acid sequence". The locus targeting sequence enables the integration of the multidomain therapeutic protein-encoding polynucleotide into the genome of the recipient host cell. In some embodiments, the locus targeting sequence include flanking homology arms to enable homologous recombination. In some embodiments, the locus targeting sequence includes guide RNA sequences and a type II Cas enzyme to facilitate integration (*i.e.,* the CRISPR-Cas9 method). In some embodiments, the locus targeting sequence includes guide zinc-finger nuclease (ZFN) recognition sequences to facilitate integration. In some embodiments, the locus targeting sequence includes transcription activator-like effector nuclease (TALEN) recognition sequences to facilitate integration. In still

other embodiments, the locus targeting sequence includes a single residue-to-nucleotide code used by BuD-derived nucleases to facilitate integration.

[0142] In some embodiments, the genomic locus into which the multidomain therapeutic protein-encoding polynucleotide is integrated is a "safe harbor locus". In one embodiment, a "safe harbor locus" enables high expression of the multidomain therapeutic protein, while not interfering with the expression of essential genes or promoting the expression of oncogenes or other deleterious genes. In one embodiment, the genomic locus is at or proximal to the liver-expressed albumin (Alb) locus, a EESYR locus, a SARS locus, position 188,083,272 of human chromosome 1 or its non-human mammalian orthologue, position 3,046,320 of human chromosome 10 or its non-human mammalian orthologue, position 67, 328,980 of human chromosome 17 or its non-human mammalian orthologue, an adeno-associated virus site 1 (AAVS1) on chromosome, a naturally occurring site of integration of AAV virus on human chromosome 19 or its non-human mammalian orthologue, a chemokine receptor 5 (CCR5) gene, a chemokine receptor gene encoding an HIV-1 coreceptor, or a mouse Rosa26 locus or its non-murine mammalian orthologue. In one embodiment, the genomic locus is an adeno-associated virus site. In one embodiment, the genomic locus for integration is selected according to the method of Papapetrou and Schambach, J. Molecular Therapy, vol. 24 (4):678-684, April 2016, which is herein incorporated by reference for the step-wise selection of a safe harbor genomic locus for gene therapy vector integration; see also Barzel et al. Nature, vol. 517:360-364, incorporated herein by reference in its entirety, for the promoterless gene targeting into the liver-expressed albumin (*Alb*) locus.

[0143] In some embodiments, the polynucleotide, e.g., DNA, also contains a promoter operably linked to the multidomain therapeutic protein-encoding nucleic acid sequence. In a specific embodiment, the promoter is a tissue-specific promotor that drives gene expression in a particular tissue. In one embodiment, the tissue specific promoter is a liver-specific enhancer/promoter derived from serpina1 (*e.g.,* SEQ ID NO:9) and/or is a TTR promoter (SEQ ID NO:8). In other embodiments, the promoter is a CMV promoter. In other embodiments, the promoter is a ubiquitin C promoter

[0144] In one embodiment, the multidomain therapeutic protein-encoding "gene therapy vector" is any vector capable of delivering the polynucleotide encoding the multidomain therapeutic protein to a host, e.g., a patient. In some embodiments the gene therapy vector targets a specific host cell

or organ, e.g., for local delivery, e.g., tissue specific delivery. Typically, local delivery requires a protein (e.g., a multidomain therapeutic protein) encoded by mRNAs be translated and expressed mainly in and/or by an organ, e.g., a liver, whereby thereby forming a depot, e.g., a liver depot for production (and secretion) of the protein. In some embodiments, a gene therapy vector delivers a multidomain therapeutic protein polynucleotide to the liver in a patient to form a liver depot. See, e.g., DeRosa et al. *Gene Therapy*, vol. 10:699-707, incorporated herein by reference in its entirety. In some embodiments, a gene therapy vector delivers a polynucleotide encoding a multidomain therapeutic protein to muscle tissue in a patient. In some embodiments, a gene therapy vector delivers a polynucleotide encoding a multidomain therapeutic protein to the brain of a patient.

[0145] Any now-known or future-developed gene therapy delivery vector, natural or engineered, can be used in the practice of this invention. In some embodiments, the gene therapy vector is a viral vector, e.g., comprises a virus, viral capsid, viral genome etc. In some embodiments, the gene therapy vector is a naked polynucleotide, e.g., an episome. In some embodiments, the gene therapy vector comprises a polynucleotide complex. Exemplary non-limiting polynucleotide complexes for use as a gene therapy vector include lipoplexes, polymersomes, polypexes, dendrimers, inorganic nanoparticles (e.g., polynucleotide coated gold, silica, iron oxide, calcium phosphate, etc.). In some embodiments, a gene therapy vector as described herein comprises a combination of a viral vector, naked polynucleotides, and polynucleotide complexes.

[0146] In one embodiment, the gene therapy vector is a virus, including a retrovirus, adenovirus, herpes simplex virus, pox virus, vaccinia virus, lentivirus, or an adeno-associated virus. In one embodiment, the gene therapy vector is an adeno-associated virus (AAV), including serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and AAV11, or engineered or naturally selected variants thereof.

[0147] In one embodiment, the polynucleotide also contains adeno-associated virus (AAV) nucleic acid sequence. In one embodiment, the gene therapy vector is a chimeric adeno-associated virus containing genetic elements from two or more serotypes. For example, an AAV vector with rep genes from AAV1 and cap genes from AAV2 (designated as AAV1/2 or AAV RC1/2) may be used as a gene therapy vector to deliver the multidomain therapeutic protein polynucleotide to a cell or a cell of a patient in need. In one embodiment, the gene therapy vector is an AAV1/2, AAV1/3, AAV1/4, AAV1/5, AAV1/6, AAV1/7, AAV1/8, AAV1/9, AAV1/10, AAV1/11, AAV2/1,

AAV2/3, AAV2/4, AAV2/5, AAV2/6, AAV2/7, AAV2/8, AAV2/9, AAV2/10, AAV2/11, AAV3/1, AAV3/2, AAV3/4, AAV3/5, AAV3/6, AAV3/7, AAV3/8, AAV3/9, AAV3/10, AAV3/10, AAV4/1, AAV4/2, AAV4/3, AAV4/5, AAV4/6, AAV4/7, AAV4/8, AAV4/9, AAV4/10, AAV4/11, AAV5/1, AAV5/2, AAV5/3, AAV5/4, AAV5/6, AAV5/7, AAV5/8, AAV5/9, AAV5/10, AAV5/11, AAV6/1, AAV6/2, AAV6/3, AAV6/4, AAV6/5, AAV6/7, AAV6/8, AAV6/9, AAV6/10, AAV6/10, AAV7/1, AAV7/2, AAV7/3, AAV7/4, AAV7/5, AAV7/6, AAV7/8, AAV7/9, AAV7/10, AAV7/11, AAV8/1, AAV8/2, AAV8/3, AAV8/4, AAV8/5, AAV8/6, AAV8/7, AAV8/9, AAV8/10, AAV8/11, AAV9/1, AAV9/2, AAV9/3, AAV9/4, AAV9/5, AAV9/6, AAV9/7, AAV9/8, AAV9/10, AAV9/11, AAV10/1, AAV10/2, AAV10/3, AAV10/4, AAV10/5, AAV10/6, AAV10/7, AAV10/8, AAV10/9, AAV10/11, AAV11/1, AAV11/2, AAV11/3, AAV11/4, AAV11/5, AAV11/6, AAV11/7, AAV11/8, AAV11/9, AAV11/10, chimeric virion or derivatives thereof. Gao *et al.,* "Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy," PNAS 99(18): 11854–11859, Sep. 3, 2002, is incorporated herein by reference for AAV vectors and chimeric virions useful as gene therapy vectors, and their construction and use.

[0148] In a more specific embodiment, the gene therapy vector is a chimeric AAV vector with a serotype 2 rep gene sequence and a serotype 8 cap sequence ("AAV2/8" or "AAV RC2/8).

[0149] In some embodiments, the gene therapy vector is a viral vector that has been pseudotyped (e.g., engineered) to target a specific cell, e.g., a hepatocyte. Many of the advances in targeted gene therapy using viral vectors may be summarized as non-recombinatorial (non-genetic) or recombinatorial (genetic) modification of the viral vector, which result in the pseudotyping, expanding, and/or retargeting of the natural tropism of the viral vector. (Reviewed in Nicklin and Baker (2002) *Curr. Gene Ther.* 2:273-93; Verheiji and Rottier (2012) *Advances Virol* 2012:1-15). Non-genetic approaches typically utilize an adaptor, which recognizes both a wildtype (non-modified) virus surface protein and a target cell. Soluble pseudo-receptors (for the wildtype virus), polymers such as polyethylene glycol, and antibodies or portions thereof, have been used as the virus binding domain of the adaptors, while natural peptide or vitamin ligands, and antibodies and portions thereof have been used for the cell binding domain of the adaptors described above. For example, retargeting of the viral vector to a target cell may be accomplished upon binding of the vector:adaptor complex to a protein expressed on the surface of the target cell, e.g., a cell surface protein. Such approach has been used for AAV (Bartlett et al. (1999) *Nat. Biotechnol.* 74: 2777-

2785), adenoviruses (Hemminki et al. (2001) *Cancer Res.* 61: 6377–81; van Beusechem et al. (2003) *Gene Therapy* 10:1982–1991; Einfeld, et al. (2001) *J. Virol.* 75:11284–91; Glasgow et al. (2009) *PLOS One* 4:e8355), herpesviruses (Nakano et al. (2005) *Mol. Ther.* 11:617-24), and paramyxoviruses (Bian et al. (2005) *Cancer Gene Ther.* 12:295-303; Bian et al. (2005) *Int. J. Oncol.* 29:1359-69), Coronaviruses (Haijema et al. (2003) *J. Virol.* 77:4528–4538; Wurdinger et al. (2005) *Gene Therapy* 12:1394–1404).

[0150] A more popular approach has been the recombinatorial genetic modification of viral capsid proteins, and thus, the surface of the viral capsid. In indirect recombinatorial approaches, a viral capsid is modified with a heterologous "scaffold", which then links to an adaptor. The adaptor binds to the scaffold and the target cell. (Arnold et al. (2006) *Mol. Ther.* 5:125-132; Ponnazhagen et al. (2002) *J. Virol.* 76:12900–907; *see also* WO 97/05266) Scaffolds such as (1) Fc binding molecules (e.g., Fc receptors, Protein A, etc.), which bind to the Fc of antibody adaptors, (2) (strept)avidin, which binds to biotinylated adaptors, (3) biotin, which binds to adaptors fused with (strept)avidin, and (4) protein:protein binding pairs that form isometric peptide bonds such as SpyCatcher, which binds a SpyTagged adaptor, have been incorporated into Ad (Pereboeva et al. (2007) *Gene Therapy* 14: 627–637; Park et al. (2008) *Biochemical and Biophysical Research Communications* 366: 769–774; Henning et al. (2002) *Human Gene Therapy* 13:1427–1439; Banerjee et al. (2011) *Bioorganic and Medicinal Chemistry Letters* 21:4985–4988), AAV (Gigout et al. (2005) *Molecular Therapy* 11:856–865; Stachler et al. (2008) *Molecular Therapy* 16:1467–1473), and togavirus (Quetglas et al. (2010) *Virus Research* 153:179–196; Ohno et al. (1997) *Nature Biotechnology* 15:763–767; Klimstra et al. (2005) *Virology* 338:9–21).

[0151] In a direct recombinatorial targeting approach, a targeting ligand is directly inserted into, or coupled to, a viral capsid, i.e., protein viral capsids are modified to express a heterologous ligand. The ligand than redirects, e.g., binds, a receptor or marker preferentially or exclusively expressed on a target cell. (Stachler et al. (2006) *Gene Ther.* 13:926–931; White et al. (2004) *Circulation* 109:513–519.). Direct recombinatorial approaches have been used in AAV (Park et al., (2007) *Frontiers in Bioscience* 13:2653–59; Girod et al. (1999) *Nature Medicine* 5:1052–56; Grifman et al. (2001) *Molecular Therapy* 3:964–75; Shi et al. (2001) *Human Gene Therapy* 12:1697–1711; Shi and Bartlett (2003) *Molecular Therapy* 7:515–525), retrovirus (Dalba et al. *Current Gene Therapy* 5:655–667; Tai and Kasahara (2008) *Frontiers in Bioscience* 13:3083-3095; Russell and Cosset (1999) *Journal of Gene Medicine* 1:300–311; Erlwein et al. (2002) *Virology* 302:333–341;

Chadwick et al. (1999) *Journal of Molecular Biology* 285:485–494; Pizzato et al. (2001) *Gene Therapy* 8:1088–1096), poxvirus (Guse et al. (2011) *Expert Opinion on Biological Therapy* 11:595–608; Galmiche et al. (1997) *Journal of General Virology* 78:3019–3027; Paul et al. (2007) *Viral Immunology* 20:664–671), paramyxovirus (Nakamura and Russell (2004) *Expert Opinion on Biological Therapy* 4:1685–1692; Hammond et al. (2001) *Journal of Virology* 75:2087–2096; Galanis (2010) *Clinical Pharmacology and Therapeutics* 88:620–625; Blechacz and Russell (2008) *Current Gene Therapy* 8:162–175; Russell and Peng (2009) *Current Topics in Microbiology and Immunology* 330:213–241), and herpesvirus (Shah and Breakefield (2006) *Current Gene Therapy* 6:361-370; Campadelli-Fiume et al. (2011) *Reviews in Medical Virology* 21:213–226).

[0152] In some embodiments, a gene therapy vector as described herein is pseudotyped to those tissues that are particularly suited for generating a regulatory response, e.g., tolerance toward, e.g., the replacement enzyme. Such tissues include, but are not limited to mucosal tissue, e.g., gut-associated lymphoid tissue (GALT), hematopoietic stem cells, and the liver. In some embodiments, the gene therapy vector, or gene encoding a multidomain therapeutic protein as described herein is expressed under the control of promoters specific for those tissues, e.g., a liver specific promoter.

[0153] In some embodiments, a gene therapy vector as described herein comprises a naked polynucleotide. For example, in some embodiments, a polynucleotide encoding a multidomain therapeutic polypeptide may be injected, e.g., intramuscularly, directly into an organ for the formation of a depot, intravenously, etc. Additional methods well-known for the enhanced delivery of naked polynucleotides include but are not limited to electroporation, sonoporation, use of a gene gun to shoot polynucleotides coated gold particles, magnetofection, and hydrodynamic delivery.

[0154] In some embodiments, a gene therapy vector as described herein comprises polynucleotide complexes, such as, but not limited to, nanoparticles (e.g., polynucleotide self-assembled nanoparticles, polymer-based self-assembled nanoparticles, inorganic nanoparticles, lipid nanoparticles, semiconductive/metallic nanoparticles), gels and hydrogels, polynucleotide complexes with cations and anions, microparticles, and any combination thereof.

[0155] In some embodiments, the polynucleotides disclosed herein may be formulated as self-assembled nanoparticles. As a non-limiting example, polynucleotides may be used to make nanoparticles which may be used in a delivery system for the polynucleotides (See e.g.,

International Pub. No. WO2012125987; herein incorporated by reference in its entirety). In some embodiments, the polynucleotide self-assembled nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

[0156] In some embodiment, these self-assembled nanoparticles may be microsponges formed of long polymers of polynucleotide hairpins which form into crystalline `pleated` sheets before self-assembling into microsponges. These microsponges are densely-packed sponge like microparticles which may function as an efficient carrier and may be able to deliver cargo to a cell. The microsponges may be from 1 μm to 300 nm in diameter. The microsponges may be complexed with other agents known in the art to form larger microsponges. As a non-limiting example, the microsponge may be complexed with an agent to form an outer layer to promote cellular uptake such as polycation polyethyleneime (PEI). This complex can form a 250-nm diameter particle that can remain stable at high temperatures (150 °C.) (Grabow and Jaegar, Nature Materials 2012, 11:269-269; herein incorporated by reference in its entirety). Additionally these microsponges may be able to exhibit an extraordinary degree of protection from degradation by ribonucleases. In another embodiment, the polymer-based self-assembled nanoparticles such as, but not limited to, microsponges, may be fully programmable nanoparticles. The geometry, size and stoichiometry of the nanoparticle may be precisely controlled to create the optimal nanoparticle for delivery of cargo such as, but not limited to, polynucleotides.

[0157] In some embodiments, polynucleotides may be formulated in inorganic nanoparticles (U.S. Pat. No. 8,257,745, herein incorporated by reference in its entirety). The inorganic nanoparticles may include, but are not limited to, clay substances that are water swellable. As a non-limiting example, the inorganic nanoparticle may include synthetic smectite clays which are made from simple silicates (See e.g., U.S. Pat. Nos. 5,585,108 and 8,257,745 each of which are herein incorporated by reference in their entirety).

[0158] In some embodiments, a polynucleotide may be formulated in water-dispersible nanoparticle comprising a semiconductive or metallic material (U.S. Pub. No. 20120228565; herein incorporated by reference in its entirety) or formed in a magnetic nanoparticle (U.S. Pub. No. 20120265001 and 20120283503; each of which is herein incorporated by reference in its entirety).

The water-dispersible nanoparticles may be hydrophobic nanoparticles or hydrophilic nanoparticles.

[0159] In some embodiments, the polynucleotides disclosed herein may be encapsulated into any hydrogel known in the art which may form a gel when injected into a subject. Hydrogels are a network of polymer chains that are hydrophilic, and are sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content. The hydrogel described herein may be used to encapsulate lipid nanoparticles which are biocompatible, biodegradable and/or porous.

[0160] As a non-limiting example, the hydrogel may be an aptamer-functionalized hydrogel. The aptamer-functionalized hydrogel may be programmed to release one or more polynucleotides using polynucleotide hybridization. (Battig et al., J. Am. Chem. Society. 2012 134:12410-12413; herein incorporated by reference in its entirety). In some embodiment, the polynucleotide may be encapsulated in a lipid nanoparticle and then the lipid nanoparticle may be encapsulated into a hyrdogel.

[0161] In some embodiments, the polynucleotides disclosed herein may be encapsulated into a fibrin gel, fibrin hydrogel or fibrin glue. In another embodiment, the polynucleotides may be formulated in a lipid nanoparticle or a rapidly eliminated lipid nanoparticle prior to being encapsulated into a fibrin gel, fibrin hydrogel or a fibrin glue. In yet another embodiment, the polynucleotides may be formulated as a lipoplex prior to being encapsulated into a fibrin gel, hydrogel or a fibrin glue. Fibrin gels, hydrogels and glues comprise two components, a fibrinogen solution and a thrombin solution which is rich in calcium (See e.g., Spicer and Mikos, Journal of Controlled Release 2010. 148: 49-55; Kidd et al. Journal of Controlled Release 2012. 157:80-85; each of which is herein incorporated by reference in its entirety). The concentration of the components of the fibrin gel, hydrogel and/or glue can be altered to change the characteristics, the network mesh size, and/or the degradation characteristics of the gel, hydrogel and/or glue such as, but not limited to changing the release characteristics of the fibrin gel, hydrogel and/or glue. (See e.g., Spicer and Mikos, Journal of Controlled Release 2010. 148: 49-55; Kidd et al. Journal of Controlled Release 2012. 157:80-85; Catelas et al. Tissue Engineering 2008. 14:119-128; each of which is herein incorporated by reference in its entirety). This feature may be advantageous when

used to deliver the polynucleotide disclosed herein. (See e.g., Kidd et al. Journal of Controlled Release 2012. 157:80-85; Catelas et al. Tissue Engineering 2008. 14:119-128; each of which is herein incorporated by reference in its entirety).

[0162] In some embodiments, a polynucleotide disclosed herein may include cations or anions. In one embodiment, the formulations include metal cations such as, but not limited to, Zn2+, Ca2+, Cu2+, Mg+ and combinations thereof. As a non-limiting example, formulations may include polymers and a polynucleotide complexed with a metal cation (See e.g., U.S. Pat. Nos. 6,265,389 and 6,555,525, each of which is herein incorporated by reference in its entirety).

[0163] In some embodiments, a polynucleotide may be formulated in nanoparticles and/or microparticles. These nanoparticles and/or microparticles may be molded into any size shape and chemistry. As an example, the nanoparticles and/or microparticles may be made using the PRINT® technology by LIQUIDA TECHNOLOGIES.RTM. (Morrisville, N.C.) (See e.g., International Pub. No. WO2007024323; herein incorporated by reference in its entirety).

[0164] In some embodiments, the polynucleotides disclosed herein may be formulated in NanoJackets and NanoLiposomes by Keystone Nano (State College, Pa.). NanoJackets are made of compounds that are naturally found in the body including calcium, phosphate and may also include a small amount of silicates. Nanojackets may range in size from 5 to 50 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, polynucleotides, primary constructs and/or polynucleotide. NanoLiposomes are made of lipids such as, but not limited to, lipids which naturally occur in the body. NanoLiposomes may range in size from 60-80 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, polynucleotides, primary constructs and/or polynucleotide. In one aspect, the polynucleotides disclosed herein are formulated in a NanoLiposome such as, but not limited to, Ceramide NanoLiposomes.

[0165] In one embodiment, the multidomain therapeutic protein is an anti-CD63 scFv-GAA fusion protein or an anti-ITGA7 scFv-GAA fusion protein. The administration of the anti-CD63 scFv-GAA fusion protein or the anti-ITGA7 scFv-GAA fusion protein via AAV-delivery provides long term stable production of GAA in the serum of the patient after administration of the multidomain therapeutic protein-harboring gene therapy vector. In one embodiment, the level of GAA in the serum of the recipient patient is $\geq$ 1.5 fold to 100 fold, $\geq$ 1.5 fold to 10 fold, $\geq$ 2.5 fold, 2.5 fold – 3

fold, 2.5 fold, 2.6 fold, 2.7 fold, 2.8 fold, 2.9 fold, 3.0 fold, 3.1 fold, 3.2 fold , 3.3 fold, 3.4 fold, 3.5 fold, 3.6 fold, 3.7 fold, 3.8 fold, 3.9 fold, 4 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, or 10 fold greater than the serum levels of a patient receiving GAA not linked to a delivery domain after 1 month, 3 months, 4 months, 5 months, or 6 months after administration of the multidomain therapeutic protein-harboring gene therapy vector.

[0166] In one embodiment, the administration of the anti-CD63 scFv-GAA fusion protein or the anti-ITGA7 scFv-GAA fusion protein via AAV-delivery provides long term stable reduction in stored glycogen levels in patients with Pompe disease. In one embodiment, the glycogen levels in heart, skeletal muscle, and liver tissue in the patient are reduced to wildtype (non-disease) levels. In one embodiment, the glycogen levels in heart, skeletal muscle, and liver tissue in the patient are maintained at wildtype levels 1 month, 2 months, 3 months, 4 months, 5 months, or 6 months after administration of the multidomain therapeutic protein-harboring gene therapy vector.

[0167] In one embodiment, the administration of the anti-CD63 scFv-GAA fusion protein or the anti-ITGA7 scFv-GAA fusion protein via AAV-delivery provides long term restoration of muscle strength in patients with Pompe disease. In one embodiment, the strength of the patient as measured by grip strength is restored to normal (*i.e.,* non-disease normal levels) 1 month, 2 months, 3 months, 4 months, 5 months, or 6 months after administration of the multidomain therapeutic protein-harboring gene therapy vector.

[0168] In another aspect, the invention provides a composition comprising an enzyme activity and an antigen-binding protein, wherein the enzyme is associated with an enzyme-deficiency disease (LSD) and internalization effector-binding protein. Enzymes (which include proteins that are not *per se* catalytic) associated with lysosomal storage diseases include for example any and all hydrolases, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, saposin-C activator, ceramidase, sphingomyelinase, β-hexosaminidase, GM2 activator, GM3 synthase, arylsulfatase, sphingolipid activator, α-iduronidase, iduronidase-2-sulfatase, heparin *N*-sulfatase, *N*-acetyl-α-glucosaminidase, α-glucosamide *N*-acetyltransferase, *N*-acetylglucosamine-6-sulfatase, *N*-acetylgalactosamine-6-sulfate sulfatase, *N*-acetylgalactosamine-4-sulfatase, β-glucuronidase, hyaluronidase, and the like.

[0169] Internalization effector-binding proteins for example include a receptor-fusion molecule, a trap molecule, a receptor-Fc fusion molecule, an antibody, an Fab fragment, an F(ab')2 fragment,

an Fd fragment, an Fv fragment, a single-chain Fv (scFv) molecule, a dAb fragment, an isolated complementarity determining region (CDR), a CDR3 peptide, a constrained FR3-CDR3-FR4 peptide, a domain-specific antibody, a single domain antibody, a domain-deleted antibody, a chimeric antibody, a CDR-grafted antibody, a diabody, a triabody, a tetrabody, a minibody, a nanobody, a monovalent nanobody, a bivalent nanobody, a small modular immunopharmaceutical (SMIP), a camelid antibody (VHH heavy chain homodimeric antibody), a shark variable IgNAR domain, other antigen-binding proteins, and the like.

[0170] Internalization effectors include for example CD63, MHC-I, Kremen-1, Kremen- 2, LRP5, LRP6, LRP8, transferrin receptor, LDL-receptor, LDL-related protein 1 receptor, ASGR1, ASGR2, amyloid precursor protein-like protein-2 (APLP2), apelin receptor (APLNR), PRLR (prolactin receptor), MAL (Myelin And Lymphocyte protein, a.k.a. VIP17), IGF2R, vacuolar-type H+ ATPase, diphtheria toxin receptor, folate receptor, glutamate receptors, glutathione receptor, leptin receptor, scavenger receptor, SCARA1-5, SCARB1-3, and CD36. In certain embodiments, the internalization effector is a kidney specific internalizer, such as CDH16 (Cadheri-16), CLDN16 (Claudn-16), KL (Klotho), PTH1R (parathyroid hormone receptor), SLC22A13 (Solute carrier family 22 member 13), SLC5A2 (Sodium/glucose cotransporter 2), and UMOD (Uromodulin). In other certain embodiments, the internalization effector is a muscle specific internalizer, such as BMPR1A (Bone morphogenetic protein receptor 1A), m-cadherin, CD9, MuSK (muscle-specific kinase), LGR4/GPR48 (G protein-coupled receptor 48), cholinergic receptor (nicotinic) alpha 1, CDH15 (Cadheri-15), ITGA7 (Integrin alpha-7), CACNG1 (L-type calcium channel subunit gamma-1), CACNAlS (L-type calcium channel subunit alpha-15), CACNG6 (L-type calcium channel subunit gamma-6), SCN1B (Sodium channel subunit beta-1), CHRNA1 (ACh receptor subunit alpha), CHRND (ACh receptor subunit delta), LRRC14B (Leucine-rich repeat-containing protein 14B), dystroglycan (DAG1), and POPDC3 (Popeye domain-containing protein 3). In some specific embodiments, the internalization effector is ITGA7, CD9, CD63, ALPL2, ASGR1, ASGR2 or PRLR.

[0171] In some embodiments, the enzyme is covalently linked (*i.e.*, electrons shared across atoms) to the antigen-binding protein. In one particular embodiment, the internalization effector-binding protein consists of or contains a half-body; the enzyme is fused to an Fc-fusion domain (*e.g.*, at the C-terminus); and the Fc-domain that is covalently linked to the enzyme associates with the Fc-

domain of the antigen-binding protein, such that the association contains one or more disulfide bridges. This particular embodiment is schematically depicted in Figure 1A, panel B.

[0172] In another particular embodiment, the internalization effector-binding protein (delivery domain) consists of or contains an antibody or an antibody fragment, and the enzyme is covalently linked to the antibody or antibody fragment. In a specific embodiment, the delivery domain is an antibody, and the enzyme is covalently linked (directly through a peptide bond, or indirectly via a linker) to the C-terminus of the heavy chain or the light chain of the antibody (Figure 1A, panels C or E, respectively). In another specific embodiment, the delivery domain is an antibody, and the enzyme is covalently linked (directly through a peptide bond, or indirectly via a linker) to the N-terminus of the heavy chain or the light chain of the antibody (Figure 1A, panels D or F, respectively).

[0173] In some embodiments, the enzyme and delivery domain are not covalently linked, but are combined in an admixture. The delivery domain and the enzyme can associate through non-covalent forces to form a complex. For example, in one particular embodiment, the delivery domain is a bispecific antibody in which one arm of the antibody binds the internalization effector and the other arm binds the enzyme. This embodiment is schematically depicted in Figure 1A, panel A.

[0174] In some embodiments, the enzyme is GAA or comprises GAA activity (*e.g.*, an isozyme with GAA activity), and the internalization effector is ITGA7, CDH15, CD9, CD63, APLP2, ASGR1, ASGR2 or PRLR. In a particular embodiment, the enzyme is GAA or comprises GAA activity, the internalization domain is CD63, and the delivery domain is a bispecific antibody with specificity for CD63 and GAA. In a particular embodiment, the enzyme is GAA or comprises GAA activity, the internalization domain is ITGA7, and the delivery domain is a bispecific antibody with specificity for ITGA7 and GAA.

[0175] In some embodiments, the enzyme is GLA or comprises GLA activity (*e.g.*, an isozyme with GAA activity), and the internalization effector is ITGA7, CD9, CD63, APLP2, ASGR1, ASGR2, or PRLR. In a particular embodiment, the enzyme is GLA or comprises GLA activity, the internalization domain is CD63, and the delivery domain is a bispecific antibody with specificity for CD63 and GLA. In a particular embodiment, the enzyme is GLA or comprises GLA activity,

the internalization domain is ITGA7, and the delivery domain is a bispecific antibody with specificity for ITGA7 and GLA.

Pharmaceutical Compositions and Administration thereof

[0176] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's The Science and Practice of Pharmacy, 21.sup.st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, Md., 2006; incorporated herein by reference in its entirety) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

[0177] In some embodiments, a pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use in humans and for veterinary use. In some embodiments, an excipient is approved by United States Food and Drug Administration. In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[0178] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical compositions.

[0179] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, etc., and/or combinations thereof.

[0180] Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, etc., and/or combinations thereof.

[0181] Exemplary surface active agents and/or emulsifiers include, but are not limited to, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [TWEEN® 20], polyoxyethylene sorbitan [TWEEN® 60], polyoxyethylene sorbitan monooleate [TWEEN® 80], sorbitan monopalmitate [SPAN® 40], sorbitan monostearate [SPAN® 60], sorbitan tristearate [SPAN® 65], glyceryl monooleate, sorbitan monooleate [SPAN® 80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ® 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ® 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLUORINC® F 68, POLOXAMER® 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

[0182] Exemplary binding agents include, but are not limited to, starch (e.g. cornstarch and starch paste); gelatin; sugars (e.g. sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol,); natural and synthetic gums (e.g. acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (Veegum®), and larch arabogalactan); alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; etc.; and combinations thereof.

[0183] Exemplary preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfate, sodium metabisulfite, and/or sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Exemplary antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Exemplary antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Exemplary alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Exemplary acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl

ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, GLYDANT PLUS® , PHENONIP® , methylparaben, GERMALL® 115, GERMABEN® II, NEOLONE.TM., KATHON.TM., and/or EUXYL® .

[0184] Exemplary buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, etc., and/or combinations thereof.

[0185] Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, etc., and combinations thereof.

[0186] Exemplary oils include, but are not limited to, almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macademia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and/or combinations thereof

[0187] Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

[0188] Delivery

[0189] The present disclosure encompasses the delivery of the gene therapy vector (e.g., the polynucleotides) by any appropriate route taking into consideration likely advances in the sciences of drug delivery. Delivery may be naked or formulated.

[0190] Naked Delivery

[0191] The polynucleotides of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering polynucleotides free from agents which promote transfection. For example, the polynucleotides delivered to the cell may contain no modifications. The naked polynucleotides may be delivered to the cell using routes of administration known in the art and described herein.

[0192] Formulated Delivery

[0193] The polynucleotides may be formulated, using the methods described herein. The formulations may contain polynucleotides and may further include, but are not limited to, cell penetration agents, a pharmaceutically acceptable carrier, a delivery agent, a bioerodible or biocompatible polymer, a solvent, and a sustained-release delivery depot. The formulated polynucleotides mRNA may be delivered to the cell using routes of administration known in the art and described herein.

[0194] Administration

[0195] The polynucleotides of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to enteral, gastroenteral, epidural, oral, transdermal, epidural (peridural), intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion,

intravitreal, (through the eye), intracavernous injection, (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), or in ear drops. In specific embodiments, compositions may be administered in a way which allows them cross the blood-brain barrier, vascular barrier, or other epithelial barrier. Non-limiting routes of administration for the polynucleotides, primary constructs or mRNA of the present invention are described below.

[0196] Parenteral and Injectible Administration

[0197] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR® , alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[0198] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland

fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[0199] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0200] In order to prolong the effect of an active ingredient, it is often desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

[0201] Depot Administration

[0202] As described herein, in some embodiments, the composition is formulated in depots for extended release. Generally, a specific organ or tissue (a "target tissue") is targeted for administration.

[0203] In some aspects of the invention, the polynucleotides are spatially retained within or proximal to a target tissue. Provided are method of providing a composition to a target tissue of a mammalian subject by contacting the target tissue (which contains one or more target cells) with the composition under conditions such that the composition, in particular the nucleic acid component(s) of the composition, is substantially retained in the target tissue, meaning that at least 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the composition is retained in the target tissue. Advantageously, retention is determined by measuring the amount of the nucleic acid present in the composition that enters one or more target cells. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or

greater than 99.99% of the nucleic acids administered to the subject are present intracellularly at a period of time following administration. For example, intramuscular injection to a mammalian subject is performed using an aqueous composition containing a polynucleotide and a transfection reagent, and retention of the composition is determined by measuring the amount of the ribonucleic acid present in the muscle cells.

[0204] Aspects of the invention are directed to methods of providing a composition to a target tissue of a mammalian subject, by contacting the target tissue (containing one or more target cells) with the composition under conditions such that the composition is substantially retained in the target tissue. The composition contains an effective amount of a polynucleotide such that the polypeptide of interest is produced in at least one target cell. The compositions generally contain a cell penetration agent, although "naked" nucleic acid (such as nucleic acids without a cell penetration agent or other agent) is also contemplated, and a pharmaceutically acceptable carrier.

[0205] In some circumstances, the amount of a protein produced by cells in a tissue is desirably increased. Preferably, this increase in protein production is spatially restricted to cells within the target tissue. Thus, provided are methods of increasing production of a protein of interest in a tissue of a mammalian subject. A composition is provided that contains polynucleotides characterized in that a unit quantity of composition has been determined to produce the polypeptide of interest in a substantial percentage of cells contained within a predetermined volume of the target tissue.

[0206] In some embodiments, the composition includes a plurality of different polynucleotides, where one or more than one of the polynucleotides encodes a polypeptide of interest. Optionally, the composition also contains a cell penetration agent to assist in the intracellular delivery of the composition. A determination is made of the dose of the composition required to produce the polypeptide of interest in a substantial percentage of cells contained within the predetermined volume of the target tissue (generally, without inducing significant production of the polypeptide of interest in tissue adjacent to the predetermined volume, or distally to the target tissue). Subsequent to this determination, the determined dose is introduced directly into the tissue of the mammalian subject.

[0207] In one embodiment, the invention provides for the polynucleotides to be delivered in more than one injection or by split dose injections.

[0208] In one embodiment, the invention may be retained near target tissue using a small disposable drug reservoir, patch pump or osmotic pump. Non-limiting examples of patch pumps include those manufactured and/or sold by BD® (Franklin Lakes, N.J.), Insulet Corporation (Bedford, Mass.), SteadyMed Therapeutics (San Francisco, Calif.), Medtronic (Minneapolis, Minn.) (e.g., MiniMed), UniLife (York, Pa.), Valeritas (Bridgewater, N.J.), and SpringLeaf Therapeutics (Boston, Mass.). A non-limiting example of an osmotic pump include those manufactured by DURECT® (Cupertino, Calif.) (e.g., DUROS® and ALZET®).

[0209] Dosing

[0210] The present invention provides methods comprising administering a gene therapy vector comprising polynucleotide encoding a multidomain therapeutic polypeptide, and optionally subsequently the multidomain therapeutic polypeptide to a subject in need thereof. In some embodiments, a method comprises administering a gene therapy vector comprising polynucleotide encoding a multidomain therapeutic polypeptide in a therapeutically effective amount to a patient in need thereof, wherein the therapeutically effective amount is sufficient to obviate the subsequent administration of the multidomain therapeutic polypeptide. Accordingly, in some embodiments, a method of treating a patient in need thereof lacking an enzyme, e.g., reducing glycogen levels and/or reducing CRIM to GAA in a patient with Pompe disease, comprises administering to the patient a gene therapy vector comprising a polynucleotide encoding a multidomain therapeutic protein comprising the replacement enzyme, e.g., an anti-CD63 scFv::GAA fusion protein, e.g., a multidomain therapeutic protein comprising the sequence set forth as SEQ ID NO:11, in a therapeutically effective amount, wherein the therapeutically effective amount negates the need for subsequent administration to the patient of the replacement enzyme, e.g., GAA or derivatives thereof. In some embodiments, a method of treating a patient lacking an enzyme and in need thereof, e.g., reducing glycogen levels and/or reducing CRIM to GAA in a patient with Pompe disease, comprises administering to the patient a gene therapy vector comprising a polynucleotide encoding a multidomain therapeutic protein comprising a replacement enzyme, e.g., an anti-CD63 scFv::GAA fusion protein, e.g., a multidomain therapeutic protein comprising the sequence set forth as SEQ ID NO:11, in a therapeutically effective amount, and further comprises administering to the patient a therapeutically effective amount of the replacement enzyme. Nucleic acids, proteins or complexes, or pharmaceutical, imaging, diagnostic, or prophylactic compositions thereof, may be administered to a subject using any amount and any route of administration

effective for preventing, treating, diagnosing, or imaging a disease, disorder, and/or condition (e.g., a disease, disorder, and/or condition relating to working memory deficits). The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. Compositions in accordance with the invention are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[0211] The following examples are provided to further illustrate the methods of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1: Construction of Anti-hCD63 ScFv::GAA Polynucleotide and Gene Therapy Vector

[0212] AAV2/8 viruses encoding for the expression of human GAA (hGAA; SEQ ID NO: 1; nucleic acid sequence represented by SEQ ID NO:12) or an anti-human CD63 single chain variable fragment (ScFv) fused on its C-terminus to human GAA (anti-hCD63 ScFv-hGAA; SEQ ID NO: 10; nucleic acid represented by SEQ ID NO:11) were generated using a standard triple transfection protocol (Gray et al. 2011; see also "Production of recombinant adeno-associated viral vectors and use *in vitro* and *in vivo* administration", Current Protocols in Neuroscience, John Wiley & Sons, New York (1999), pp. 4.17.1–4.17.25, Vol 1). For the production, 1×10^7 HEK293 cells were plated onto 15 cm plates. The following day the cells were transfected with (A) either 8 μg of a control

pAAV vector comprising a liver specific serpina 1 enhancer (SEQ ID NO:9) and encoding TTR driven human GAA or test pAAV comprising a liver specific serpina 1 enhancer (SEQ ID NO:9) and encoding a TTR driven hCD63 ScFv-hGAA (see **Figure 1B**) and (B) pAAV RC2/8-derived vector (Gao, 2002) and 16 μg of pHelper (Agilent, Cat #240074) using PEIpro (Polyplus transfection, New York, NY catalog# 115-100)-mediated transfection at ratio of 1:1 (1ul PEIpro : 1μg DNA). Seventy-two hours after transfection, the cells were collected and lysed in a buffer comprised of 20mM Tris-HCl, 1mM MgCl2, 2.5mM KCl, 100mM NaCl using a standard freeze-thaw method. Next, benzonase (Sigma, Cat# E1014-25KU) was added to the samples at a final concentration of 0.5 U/μL, and this was then incubated at 37°C for 60 minutes. Viruses were then purified using iodixanol gradient ultracentrifugation as described in (Zolotukhin et al., 1999, Gene Ther 1999;6:973–985) and were subsequently titrated by qPCR.

[0213] AAV samples were treated with DNaseI (Thermofisher Scientific, Cat #EN0525) at 37°C for one hour and lysed using DNA extract All Reagents (Thermofisher Scientific Cat# 4403319). Encapsidated viral genomes were quantified using an QuantStudio 3 Real-Time PCR System (Thermofisher Scientific) using primers directed to the AAV2 ITRs. The sequences of the AAV2 ITRs primers are 5′-GGAACCCCTAGTGATGGAGTT-3′ (fwd ITR; SEQ ID NO:3) and 5′-CGGCCTCAGTGAGCGA-3′ (rev ITR; SEQ ID NO:4) (Aurnhammer *et al.*, 2012), derived the left internal inverted repeat (ITR) sequence from of the AAV (SEQ ID NO:6) and the right internal inverted repeat (ITR) sequence from of the AAV (SEQ ID NO:7), respectively. The sequence of the AAV2 ITRs probe is 5′-6-FAM-CACTCCCTCTCTGCGCGCTCG-TAMRA-3′ (SEQ ID NO:5) (Aurnhammer C., Haase M., Muether N., et al., 2012, Hum. Gene Ther. Methods 23, 18–28). After a 95°C activation step for 10 min, a two-step PCR cycle was performed at 95°C for 15 seconds and 60°C for 30 seconds for 40 cycles. The TaqMan Universal PCR Master Mix (Thermofisher Scientific, Cat #4304437) was used in the qPCR. DNA plasmid (Agilent, Cat #240074) was used as standard to determine absolute titers.

[0214] Anti-human CD63 antibodies and their fusions used the H5C6 mouse anti-human CD63 variable domains (amino acids 1-119 of SEQ ID NO:10 provide the amino acid sequence of the heavy chain variable domain (V_H) of the H5C6 antibody and amino acids 135-245 of SEQ ID NO:10 provide the amino acid sequence of the light chain variable domain (V_L) of the H5C6 antibody). The anti-hCD63 ScFv used here (SEQ ID NO:2) was derived from the H5C6 clone, which is mouse-anti-hCD63 monoclonal IgG1, kappa light chain antibody (H5C6 was deposited to

the Developmental Studies Hybridoma Bank at the University of Iowa by August, J.T. / Hildreth, J.E.K. (DSHB Hybridoma Product H5C6; DSHB Cat# h5c6, RRID:AB_528158). ScFv versions of the antibodies were cloned with variable domains in heavy-light order with a glycine-serine linker in between (5'-VH-Gly-Ser-VL-3')

Example 2: Glycogen content in Murine Pompe Model post-AAV

[0215] To determine the effect of AAV delivered anti-hCD63 ScFv-GAA fusion versus AAV delivered GAA, in a relevant glycogen storage *in vivo* model, both therapies were delivered to a Pompe disease mouse model where mice were homozygous for the deletion of the mouse GAA gene and were homozygous for the expression of human CD63 in place of mouse CD63 with a strain background of 75% C57BL/6; 25% 129SvJ. These mice are herein referred to as CD63 HumIn GAA KO mice or alternatively as CD63hu/hu; $GAA^{-/-}$ mice.

[0216] For the experiment, 2-month-old CD63 HumIn GAA KO mice were administered via tail vein injection with either AAV2/8 virus containing a genome with either the TTR liver specific promoter driving human GAA (AAV-hGAA; described in Example 1) or the TTR liver specific promoter driving anti-human CD63 ScFv fused at its C-terminus with human GAA (AAV-anti-hCD63 ScFv-hGAA; described in Example 1). Both AAV2/8 viruses were delivered at either one of two doses, 1e10 vg/mouse or 1e11 vg/mouse. As controls, untreated CD63 HumIn GAA KO mice and untreated CD63 HumIn with the mouse GAA gene intact were included in the assay. Mice were housed for 3 months after treatment and bled incrementally (monthly) during this period for serum measurements of GAA levels and anti-GAA antibodies. After 3 months, all mice were sacrificed and individual tissues were harvested for glycogen measurements, PAS-H staining, quantification of central nuclei, measurement of lysosomal proliferation, and measurement of LC3b expression. Experimental dosing and treatment protocol for groups of mice are shown in Table 4.

Table 4: Experimental dosing and treatment protocol for groups of mice

Group	Mice	Number of Mice	Treatment	Dosage
1	CD63 HumIn GAA KO	4	None	N/A
2	CD63 HumIn GAA KO	4	AAV-hGAA	1e10 vg/mouse
3	CD63 HumIn GAA KO	4	AAV-hGAA	1e11 vg/mouse
4	CD63 HumIn GAA KO	5	AAV-anti-hCD63 ScFv-hGAA	1e10 vg/mouse

5	CD63 HumIn GAA KO	4	AAV-anti-hCD63 ScFv-hGAA	1e11 vg/mouse
6	CD63 HumIn GAA WT	2	None	N/A

[0217] The results are also depicted in Figure 2, which shows that anti-hCD63scFv::GAA brings glycogen down to wildtype levels in skeletal muscle, unlike GAA alone. Treatment with the two-domain anti-hCD63scFv::GAA multidomain therapeutic protein resulted in much greater reduction in stored glycogen compared to the single-domain GAA replacement enzyme. By plotting quadriceps glycogen levels (Figure 3) or heart glycogen levels (Figure 4) against the total serum expression of GAA or scfv-GAA over three months for individual mice, it was observed that the anti-hCD63scFv::GAA fusion protein removes more glycogen than the GAA enzyme alone, even at similar serum levels (Figures 3 and 4).

Example 3: Immunological Response to GAA

[0218] To measure anti-human GAA antibody serum levels, serum from all the treatment groups was separated from the blood collected during the terminal bleed using serum separator tubes (BD Biosciences, Cat#365967) as per the manufacturer's specifications. Separately, 96-well high protein binding plates (ThermoFisher, Cat#15041) were coated with 20µg of hGAA (R&D Systems, Cat#8329-GH-025) diluted in PBS overnight. Plates were washed with PBS + 0.05% Tween (PBS-T) 3 times. Plates were blocked with 0.5% BSA in PBS-T, and serial dilutions of mouse serum ranging from 1:300 to 1:5.1e7 were added to the plate overnight. Total anti-mouse IgG (subclasses 1 + 2a + 2b + 3) was measured using a HRP conjugated goat anti-mouse IgG antibody (Jackson Immuno Research, Cat# 115-035-164) and the BD Opt EIA substrate kit. The colormetric reactions were stopped using 1 N phosphoric acid. Absorbance was then read at 450nm on a Spectramax i3 plate reader (Molecular Devices). Dilution curves were fit to sigmoidal curves, and titers were calculated from the curves. The titers expressed as mean total IgG titer +/- SD are shown in Table 5.

[0219] As shown in Table 5, mice that did not receive treatment showed an average background titer with mean levels of 1.1E+03. Mice treated with the low dose of virus (1e10 vg/mouse) of either AAV-anti-hCD63 ScFv-hGAA or AAV-hGAA demonstrated high titers, whereas in mice treated with the high dose (1e11 vg/mouse), titers were lower. Mice treated with 1e11vg of AAV-

anti-hCD63 ScFv-hGAA that had the highest levels of GAA in serum had titers within the range of untreated mice.

Table 5: Serum anti-GAA antibody levels

	Total IgG anti-GAA titer				
	CD63 HumIn GAA KO + no treatment	CD63 HumIn GAA KO + AAV-hGAA (1e10vg)	CD63 HumIn GAA KO + AAV-hGAA (1e11vg)	CD63 HumIn GAA KO + AAV-anti-hCD63 ScFv-hGAA (1e10vg)	CD63 HumIn GAA KO + AAV-anti-hCD63 ScFv-hGAA (1e11vg)
Mean	1.1E+03	7.6E+06	2.4E+04	5.5E+04	4.0E+03
SD	1.3E+03	1.0E+07	2.5E+04	1.9E+04	4.9E+03

[0220] Higher levels of GAA or anti-hCD63scFv::GAA after AAV administration correspond with lower anti-GAA titers. The serum of GAA null mice treated with high or low titers of AAV-anti-hCD63scFv::GAA or AAV-GAA were assessed for anti-GAA antibodies over the course of the three months post-injection. **Figure 5** depicts serum anti-GAA antibody titers vs GAA exposure (*i.e.*, the total serum expression over 3 months of GAA or scfv-GAA) for individual mice, which demonstrates a negative correlation between antibody titer and serum exposure to GAA, demonstrating that mice with high GAA exposure were tolerized to GAA. Likewise, **Figure 6**, which plots anti-GAA antibody titers for various groups infected with AAV encoding GAA or an anti-hCD63scFv::GAA protein, demonstrates that higher doses of construct led to lower titers of anti-GAAs.

Example 4: Serum GAA

[0221] To measure human GAA serum levels over the course of the experiment, samples were collected at monthly time points via tail bleed. Serum was separated from the blood using serum separator tubes (BD Biosciences, Cat# 365967) as per the manufacturer's specifications. 1µL of isolated serum was then loaded onto a 4-20% Novex wedgewell pre-cast gel, run at 220V for 45 minutes and transferred to nitrocellulose membrane at 200mA for 1 hour using standard procedures. The nitrocellulose membrane was then probed with an anti-GAA primary antibody (Abcam, #ab137068) used at a dilution of 1:2000 and an anti-GAPDH antibody (Abcam,

#AB9484) used at a dilution of 1:1000 in 12mL and incubated overnight at 4 °C. After primary antibody incubation, the membrane was washed three times with 1 x TBST for 5 minutes per wash. Anti- rabbit IgG (LiCor, 926-32211) and anti- mouse IgG (LiCor, 925-68070) (LiCor, Lincoln, NE) secondary antibodies at a dilution of 1:15000 in 12mL were then added to the membrane and incubated for 1 hour at room temperature. After secondary antibody incubation, the membrane was washed two times with 1 x TBST for 5 minutes per wash and one time with 1 x TBS for 5 minutes. The membrane was then imaged and quantified using a LiCor Odyssey instrument (LI-COR Biotechnology). Serum levels of GAA expressed as mean +/- standard deviation (SD) in arbitrary units are shown in Table 6.

[0222] As shown in Table 6, CD63 HumIn GAA KO mice treated with the high dose (10^{11} vg/mouse) of AAV-anti-hCD63 ScFv-hGAA or AAV-hGAA tested demonstrated sustained levels of GAA in the serum over the course of the experiment, with serum levels of GAA somewhat higher in AAV-anti-hCD63 ScFv-hGAA treated mice than in the AAV-hGAA treated mice. In mice treated with the treated with the low dose (10^{10} vg/mouse) of either AAV-anti-hCD63 ScFv-hGAA or AAV-hGAA, the levels of GAA dropped over the course of the experiment, approaching negligible levels in some mice by the 12 week time point.

Table 6: Serum GAA levels

	AAV-anti-hCD63 ScFv-hGAA (10^{10}vg)		**AAV-hGAA (10^{10}vg)**		**AAV-anti-hCD63 ScFv-hGAA (10^{11}vg)**		**AAV-hGAA (10^{11}vg)**	
Week	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	0.21	0.17	0.04	0.03	2.36	1.78	0.77	0.53
2	0.19	0.16	0.07	0.07	2.02	1.18	1.15	0.56
4	0.17	0.15	0.01	0.02	2.80	1.16	1.22	0.75
8	0.29	0.33	0.03	0.05	2.58	1.18	0.62	0.60
12	0.12	0.19	0.00	0.01	2.61	1.53	0.77	0.86
Area under the curve	2.27	2.23	0.27	0.36	28.13	13.73	9.80	7.22

[0223] Expression of GAA or anti-hCD63scfv::GAA was maintained over time in mice receiving the high dose of AAV (10^{11} vg/mouse), but fell off in mice receiving the lower dose (10^{10}

vg/mouse). Figure 7A depicts a graph plotting serum levels of GAA, as probed by western blot, over time for various groups infected with AAV encoding GAA or an anti-hCD63 scfv fusion to GAA. The fusion protein (scFv::GAA) demonstrated consistently higher levels (*e.g.*, 2.5 to 3-fold) of serum GAA than the GAA enzyme without the delivery domain (Figure 7A).

[0224] Real-time PCR quantifications of expression in liver, heart, and quadriceps lysates 3 months after injection are shown in Figure 7B. Liver expression was detected for all injections of AAV construct, with highest levels for the 1e11vg/mouse injections for both AAV-hGAA and AAV-anti-hCD63::hGAA (both driven by liver-specific promoter, LSP). A comparison of serum GAA level to RNA expression level of GAA was also made (Figure 7C) and the results show that mice receiving the AAV encoding the fusion protein presented lower GAA RNA expression localized to the liver at 3 months however GAA serum levels were high in that particular mouse. AAV-LSP-hGAA injections did not present high serum levels of GAA when RNA levels were low in the liver. See Figure 7C. This data suggests that the AAV encoding the fusion protein (and expression is driven by a liver-specific promoter) attains an improved secretion profile for GAA.

[0225] A higher secreted to intracellular ratio of antibody::hGAA versus hGAA alone in Huh-7 hepatocytes was also observed. In one experiment, Huh-7 human hepatocytes were transiently transfected with liver-specific promoter driven constructs encoding for hGAA, anti-hCD63 scFv::GAA fusion, or a non-binding scFv::GAA fusion control. Both scFv::GAA fusion constructs had a higher ratio of protein in the secreted supernatant than hGAA alone 3 days after transfection (statistically significant to $p < 0.05$, $n=3$). Addition of M6P into the supernatant during the experimental period to mitigate CI-MPR-mediated uptake did not affect the ratio.

Example 5: Tissue Measurement of Glycogen and Histological Characterization of Muscle Tissue

[0226] Tissue measurements of glycogen: To measure the glycogen content in individual tissues, heart, quadriceps, gastrocnemius, diaphragm, soleus, and EDL tissue were dissected from mice from all groups immediately after CO_2 asphyxiation, and were then snap frozen in liquid nitrogen, and stored at -80°C. ~50mg of each tissue was lysed on a benchtop homogenizer with stainless steel beads in distilled water at a ratio of 1mg to 25µL water for glycogen measurements. Glycogen analysis lysates were heated at 105 ° for 15 minutes and centrifuged at 21000 x g to clear debris. Glycogen measurements were performed using a Glycogen Assay Kit (Sigma-Aldrich, #MAK016)

according to manufacturer's instructions for fluorometric assays. The fluorescence of each sample was measured at 535nm excitation and 587nm emission on a fluorescence plate reader (Molecular Devices, Spectramax i3). The calculated amount of glycogen was calculated using the following formula provided by the manufacturer. The calculated amount of glycogen from each tissue in each treatment group was then averaged and is expressed as mean +/- standard deviation (SD) in Table 7.

[0227] As shown in Table 7, loss of *Gaa* causes a large increase in mean glycogen levels across all tissues measured, as compared to GAA WT mice. Treatment with AAV-anti-hCD63 ScFv-hGAA at 10^{11} vg/mouse reduced glycogen to WT- or near-WT levels in all tissues tested, unlike treatment with AAV-GAA which only partially reduced stored glycogen. The low doses of either virus also reduced glycogen, but to a lesser extent that the high doses. The 10^{10} vg/mouse dose of AAV-anti-hCD63 ScFv-hGAA reduced glycogen levels in a similar manner as the 10^{11} vg/mouse dose of AAV-GAA.

Table 7: Mean +/- SD glycogen level measured in heart, quadriceps, gastrocnemius, diaphragm, soleus, and EDL

	no treatment		**AAV-hGAA (10^{10} vg)**		**AAV-hGAA (10^{11} vg)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Heart	27.798	3.013	17.246	4.375	1.770	2.279
Quadricep	14.650	1.783	11.012	0.528	5.878	3.504
Gastrocnemius	14.295	0.480	10.990	0.868	6.073	3.080
Diaphragm	15.463	1.173	11.446	1.237	3.995	3.395
Soleus	17.260	2.262	13.684	2.506	6.533	5.201
EDL	13.588	0.498	11.178	1.760	6.275	3.159
	AAV-anti-hCD63 ScFv-hGAA (10^{10} vg)		**AAV-anti-hCD63 ScFv-hGAA (10^{11} vg)**		**CD63 HumIn GAA WT mice (control)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Heart	2.190	2.678	0.058	0.010	0.085	0.007
Quadricep	4.485	3.147	0.798	0.251	0.440	0.042
Gastrocnemius	5.198	2.516	0.825	0.461	0.790	0.014
Diaphragm	3.083	2.968	0.388	0.121	0.385	0.007
Soleus	5.268	2.786	1.040	0.896	0.545	0.049
EDL	2.495	1.750	0.313	0.099	0.260	0.141

[0228] Quadricep harvest for histopathology and quantification: Quadricep tissue samples from mice from each group besides the low dose (1e10 vg/mouse) treatment group were either snap frozen immediately after dissection in liquid nitrogen and stored at -80°C for quantification of LC3b expression or were placed onto blocks containing O.C.T medium (Tissue-Tek, #4583).

[0229] Tissues samples in O.C.T medium were sent to Histoserv, Inc (Germantown, MD) for sectioning and periodic acid Schiff (PAS) staining to detect polysaccharides. Additional sections were prepared and returned for staining of central nuclei and lysosomal proliferation.

[0230] PAS staining: PAS stain sections were imaged using a Leica slide scanner at 20x magnification. The resulting images from representative mice for each treatment group are shown in Figure 8.

[0231] As shown in Figure 8, CD63 HumIn GAA KO that were treated with AAV-anti-hCD63 ScFv-hGAA at 3 months demonstrated a marked decrease in staining as compared to both the CD63 HumIn GAA KO mice with no treatment and the CD63 HumIn GAA KO mice treated with AAV-hGAA, which displayed high levels of PAS staining. This further indicates that the treatment with AAV-anti-hCD63 ScFv-hGAA can reduce polysaccharides accumulation in CD63 HumIn GAA KO mice, and can do so in a uniform manner across muscle fibers.

[0232] Quantification of central nuclei and lysosomal proliferation: Unstained sections from Histoserv were removed from the freezer and then fixed with 4% paraformaldehyde in PBS for 15 minutes in a staining chamber. The fixed slides were then washed twice for 5 minutes in PBS and subsequently incubated with blocking buffer (eBiosciences, 00-4953-54) for 1 hour at room temperature. Slides were then either stained with either a rat anti-Lamp-1 antibody (Abcam, #AB25245) at a dilution of 1:50 in blocking buffer, a rabbit anti-Laminin antibody (Sigma, #L9393) at a dilution of 1:1000 in blocking buffer, or blocking buffer with no added antibody while in a humidified staining chamber and then transferred to 4°C for overnight incubation. The following day, slides were then washed twice for 5 minutes in PBS and subsequently stained with either goat anti-rabbit IgG (H+L) superclonal secondary antibody conjugated with Alexa Fluor 647 (Life Tech Thermo, #A27040) or goat anti-rat IgG (H+L) cross-adsorbed secondary antibody conjugated with Alexa Fluor 555 (Life Tech Thermo, #A21434) in a staining chamber then allowed to incubate for 1 hour at room temperature. Stained slides were then washed twice for 5 minutes in PBS before they were mounted with Fluoromount-G with DAPI (Life Tech Thermo, #00-4959-52)

and imaged on a Zeiss LSM710 instrument (Carl Zeiss Microscopy GmbH). Number of centralized nuclei was quantified using Halo software (Indica Labs, NM) and is expressed as percentage of fibers showing central nuclei +/- standard deviation are shown in Table 8. Lysosomal proliferation is depicted in Figure 8.

Table 8: Quantification of central nuclei

	no treatment		AAV-hGAA (1e11vg)		AAV-anti-hCD63 ScFv-hGAA (1e11vg)		CD63 HumIn GAA WT mice (control)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
% of Fiber with central nuclei	22.00	10.10	33.00	4.36	12.75	6.29	8.50	7.78

[0233] Quantification of LC3b expression: For quantification of LC3b expression, snap frozen samples were thawed, homogenized and then lysed in RIPA buffer at a 1mg tissue to 25µL RIPA buffer ratio (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Sigma Aldrich, R0278) by bead impaction for 45 seconds (MP Biomedical). Lysates were cleared of insoluble material by centrifugation at 21,000 x g and then 300µg of lysate in RIPA buffer was loaded on a 4-20% Novex wedgewell pre-cast gel, transferred to a nitrocellulose membrane and analyzed by western blot using a similar protocol as previously described for the analysis of serum GAA levels, substituting the use of primary antibody that recognizes mouse LC3b-I and LC3b-II (Sigma, #L7543) in place of the primary antibody against GAA. The membrane was then imaged and quantified using a LiCor Odyssey instrument (LI-COR Biotechnology). LC3b-I and LC3b-II levels expressed as (mean +/- standard deviation) in arbitrary units are shown in Table 9.

[0234] As shown in Table 9, there was a significant increase in both mean LC3b-I and LC3b-II levels in mice lacking GAA as compared to CD63 HumIn GAA WT mice. Treatment with AAV-anti-hCD63 ScFv-hGAA decreased mean LC3b-I and LC3b-II levels in CD63 HumIn GAA KO to the WT- or near-WT levels. CD63 HumIn GAA KO treated with AAV-hGAA demonstrated

slightly decreased mean LC3b-I and LC3b-II levels as compared to CD63 HumIn GAA KO mice, but this decrease was not as pronounced as with AAV-anti-hCD63 ScFv-hGAA treatment.

Table 9: LC3b-I and LC3b-II levels in the quadriceps of mice

LC3b-I levels (arbitrary units)				
treatment	untreated	AAV-hGAA	AAV-anti-hCD63 ScFv-hGAA	CD63 HumIn GAA WT (control)
average	833	628	403	282
SD	109	139	33	49
LC3b-II levels (arbitrary units)				
treatment	untreated	AAV-hGAA	AAV-anti-hCD63 ScFv-hGAA	CD63 HumIn GAA WT (control)
average	3308	2888	445	369
SD	582	1282	398	33

Example 6: AAV anti-hCD63::GAA treatment leads to significant gains in tests of muscle strength and coordination

[0235] Grip strength and Rotarod test performance of mice treated (see above) with either AAV-LSP hGAA or AAV-LSP anti-hCD63::hGAA. Accelerating Rotarod measurements (Figure 9A) and forelimb grip strength measurements (Figure 9B) of wild-type GAA mice, untreated control, AAV-LSP-hGAA (1e11vg/mouse) or AAV-LSP-anti-hCD63:: hGAA treatment (1e11vg/mouse) were taken at monthly intervals for 6 months. Error bars are +/- SD. N=8-10 for all groups.

Example 7: Other membrane proteins as "guides" directing GAA to tissues

[0236] Other membrane proteins were tested, such as anti-ITGA7 (Integrin alpha-7) fusion proteins, to guide GAA to tissues to replace GAA in enzyme-deficient mice. C2C12 mouse myoblasts were incubated overnight with anti-mCD63-GAA or anti-ITGA7-GAA with or without the presence of 5mM M6P. Active GAA enzyme was detected in myoblast lysates over time for both fusion proteins (Figure 10A). In further experiments, GAA KO mice humanized for CD63 (GAA-/-;CD63hu/hu) were given plasmids encoding an scFv::GAA format of anti-hCD63::GAA or a full-length IgG4::GAA format of anti-integrin alpha-7 by hydrodynamic delivery (HDD), and mice were sacrificed 3 weeks post-HDD. Tissue glycogen levels were measured in heart,

quadriceps, gastrocnemius and diaphragm. Untreated control mice, $GAA^{-/-}$xCD63hu/hu and untreated wild-type GAA control mice, GAA+/+;CD63hu/hu (4) were also tested under the same conditions. Glycogen levels were at very low levels in both anti-hCD63::GAA treated mice and anti-ITGA7::GAA treated mice groups, as in the wild-type mice. **See Figure 10B**.

Example 8: At comparable serum levels, AAV anti-CD63::GAA treatment is more effective than AAV with optimized GAA construct

[0237] CD63 HumIn GAA KO mice ($GAA^{-/-}$ x $CD63^{hu/hu}$) infected with AAVs containing a liver specific enhancer (serpina 1; SEQ ID NO:9) and a liver specific promoter (LSP; TTR; SEQ ID NO:8) driving the expression of an anti-hCD63::GAA multidomain therapeutic (SEQ ID NO:10), , which uses a chymotrypsinogen B2 signal peptide (SP7) and contains amino acids 36-952 of human GAA (Δ8GAA; SEQ ID NO:78) exhibited significant gains in tests of muscle strength and coordination. Three different doses were given for each virus: 5e11vg/kg, 2e12vg/kg, and 4e12vg/kg. Serum was collected by submandibular bleeds on a regular basis. One month post-AAV infection, mice were sacrificed. Cardiac and skeletal muscle tissue samples were collected and snap frozen in liquid nitrogen and kept at -80° C for storage. Glycogen in tissues were measured by homogenizing tissues by bead impaction in distilled water. Samples were boiled and centrifuged, and the supernatants were used in a commercial glycogen assay kit. Serum was quantified using western blot with an antibody against human GAA as described in previous examples. For each mouse, the glycogen level in each tissue was plotted against the serum level of the construct at 1 month. 4-parameter curve fits were used to determine the EC50 of the two treatments in each tissue.

[0238] Infection with AAVs containing a liver specific promoter (LSP) encoding either anti-hCD63::GAA or sp7-Δ8GAA provided comparable serum levels of GAA at each infection dose. **Figure 11**. However, in every muscle tissue assayed, an ~2.2 fold reduction in EC50 was observed when using anti-hCD63::GAA vs. sp7-Δ8GAA, demonstrating that at equivalent serum levels, anti-CD63::GAA clears glycogen more efficiently than a modified GAA expression construct that is not fused to an antibody. **See Figure 12** and **Table 10.**

Table 10: EC_{50} (95% Confidence Interval) of AAV anti-hCD63::GAA and AAV sp7-Δ8GAA in heart, diaphragm, quadricep, and tricep

Tissue	**Construct**	**EC_{50} of glycogen clearance** (arbitrary units)	**95% Confidence Interval for EC_{50}**
Heart	sp7-Δ8GAA	0.038	0.00036 – 0.064
	anti-hCD63::GAA	0.017	0.0061 – 0.029
Diaphragm	sp7-Δ8GAA	0.135	wide
	anti-hCD63::GAA	0.057	0.034 – 0.080
Quadriceps	sp7-Δ8GAA	0.187	0.14-0.31
	anti-hCD63::GAA	0.080	wide
Triceps	sp7-Δ8GAA	0.19	0.13 - 0.41
	anti-hCD63::GAA	0.083	0.069 to 0.11

Example 9: Exemplary CD63 antibodies

[0239] *Generation of anti-human CD63 antibodies*

[0240] Anti-human CD63 antibodies were obtained by immunizing a mouse (e.g., an engineered mouse comprising DNA encoding human immunoglobulin heavy and human kappa light chain variable regions), with human CD63.

[0241] Following immunization, splenocytes were harvested from each mouse and either (1) fused with mouse myeloma cells to preserve their viability and form hybridoma cells and screened for human CD63 specificity, or (2) B-cell sorted (as described in US 2007/0280945A1) using a either a human CD63 fragment as the sorting reagent that binds and identifies reactive antibodies (antigen-positive B cells).

[0242] Chimeric antibodies to human CD63 were initially isolated having a human variable region and a mouse constant region using, *e.g.*, VELOCIMMUNE technology as described in US Patent No. 7,105,348; US Patent No. 8,642,835; and US 9,622,459, each of which is incorporated herein by reference.

[0243] In some antibodies, for testing purposes, mouse constant regions were replaced with a desired human constant region, for example wild-type human CH or modified human CH (*e.g.* IgG1, IgG2 or IgG4 isotypes), and light chain constant region (CL), to generate a fully human anti-hCD63, including a fully human bispecific antibody comprising an anti-hCD63antibody or antigen binding portion thereof. While the constant region selected may vary according to specific use, high affinity antigen-binding and target specificity characteristics reside in the variable region.

[0244] Certain biological properties of the exemplary bispecific antibodies comprising an anti-human CD63 binding arm generated in accordance with the methods of this Example are described in detail in the Examples set forth below.

[0245] *Heavy and Light Chain Variable Region Amino Acid and Nucleic Acid Sequences of anti-CD63 antibodies*

[0246] Table 11 sets forth sequence identifiers of a nucleic acid (NA) sequence encoding, and in parentheses an amino acid (AA) sequence of, a heavy or light chain variable region (HCVR or LCVR, respectively), or a heavy or light chain CDR (HCDR and LCDR, respectively) of selected anti-CD63 antibodies used to generate the multidomain therapeutic anti-CD63::GAA proteins disclosed herein.

Table 11: anti-CD63 Sequence Identifiers

	SEQ ID NOs:							
Antibody Designation	HCVR NA (AA)	HCDR1 NA (AA)	HCDR2 NA (AA)	HCDR3 NA (AA)	LCVR NA (AA)	LCDR1 NA (AA)	LCDR2 NA (AA)	LCDR3 NA (AA)
H1M12451N	13 (14)	15 (16)	17 (18)	19 (20)	21 (22)	23 (24)	25 (26)	27 (28)
H2M12395N	29 (30)	31 (32)	33 (34)	35 (36)	37 (38)	39 (40)	41 (42)	43 (44)
H4H12450N	45 (46)	47 (48)	49 (50)	51 (52)	53 (54)	55 (56)	57 (58)	59 (60)
H2M12450N	61 (62)	63 (64)	65 (66)	67 (68)	69 (70)	71 (72)	73 (74)	74 (76)

[0247] *Binding by parental anti-human CD63 antibodies*

[0248] Relative cell surface binding of the anti-CD63 antibodies to human CD63 expressing cells was accessed via flow cytometry using CD63 positive HEK293 cells (ATCC, Cat # CRL-1573),

which endogenously express human CD63, and CD63 negative HEK293/CD63 knock out cells. For the assay, cells were plated in PBS without calcium and magnesium (VWR, Cat# 45000-446), containing 2% FBS (Saradigm Cat# 1500-500) (Staining Buffer) in 96 well V-bottom plates (Axygen Scientific, Cat# P-96-450-V-C-S). Cells were then incubated with anti-CD63 antibodies or isotype control antibodies at concentrations ranging from 100 nM to 1.7 pM for 30 minutes on ice. Wells containing no antibody were used as controls. HEK293/CD63KO cells were stained with only the highest concentration (100 nM) of the antibodies. The cells were then washed once with staining buffer and were incubated with a PE conjugated anti-mouse Fc secondary antibody (Jackson ImmunoResearch, Cat# 115-115-164) at 100 nM for 30 minutes at 4°C. Cells were then washed and fixed using a 50% solution of Cytofix (BD Biosciences, Cat# 554655) diluted in PBS. Samples were run on the Intellicyte Hypercyt flow cytometer and results were analyzed in ForeCyt software (Intellicyte) to calculate the mean fluorescent intensity (MFI). Measured values were analyzed using a four parameter logistic equation over a 12-point response curve using GraphPad Prism and the resulting EC_{50} values are reported (Table 12). The signal to noise ratio (S/N) was determined by calculating the ratio of the anti-CD63 antibodies or the control antibodies MFI to the wells containing no antibodies (Table 12).

[0249] As shown in Table 12, three of the anti-CD63 antibodies of the invention demonstrated binding to HEK293 cells with S/N values ranging from 21.0 to 31.6 and EC_{50} values ranging from 0.5 nM to 1.9 nM. The non-binding controls did not demonstrate binding to HEK293 cells (S/N $\leq$ 1.5). Both the anti-CD63 antibodies and the isotype control antibodies demonstrated weak to little binding to the HEK293/CD63KO cells (S/N $\leq$ 4.4).

Table 12: Binding of anti-CD63 antibodies to HEK293 and HEK293/CD63 KO cells as measured by flow cytometry

Antibody	HEK293 EC_{50} (nM)	HEK293 (S/N)	HEK293/CD63 KO (S/N)
H2M12450N	0.5	26.7	2.9
H1M12451N	1.8	31.6	4.1
H2M12395N	1.9	21.0	2.9
Isotype Control 1	ND	1.4	4.4
Isotype Control 2	ND	1.5	1.6

ND = Not determined

[0250] The ability of the anti-CD63 monoclonal antibodies of the invention to bind to human CD63 expressing cells was also determined using an electrochemiluminescence (ECL) based detection assay.

[0251] To generate overexpressing cells, mouse embryonic fibroblast NIH3T3 cells (ATCC, Cat# CRL-1658) were transfected to form a cell line "NIH3T3/hCD63" that stably expresses human CD63 (hCD63; amino acids M1-M238 of accession number NP_001771; SEQ ID NO:77). Expression levels of human CD63 in endogenously expressing cells, a human androgen-sensitive prostate adenocarcinoma cell line, LNCAP (ATCC, Cat# CRL-1740), and human primary glioblastoma cell line, U87MG (ATCC, Cat# HTB-14) were analyzed with a Quantum™ Alexa Fluor® 647 MESF (Bangs Laboratories, Cat# 647B) and a Simply Cellular® anti-Mouse IgG (Bangs Laboratories Inc, Cat# 815) following the manufacturer's instructions. LNCAP cells were determined to have a lower human CD63 copy number than U87MG cells. Non-transfected NIH3T3 cells, which have no detectable expression of human CD63 by fluorescence activated cell sorting (FACS), were included as a negative control.

[0252] Briefly, cell lines were rinsed once in PBS buffer without Ca^{2+}/Mg^{2+} and incubated for 10 minutes at 37°C with Enzyme Free Cell Dissociation Solution (Millipore, Cat# S-004-C) to detach the cells. Cells were then washed once with PBS with Ca^{2+}/Mg^{2+} and counted with a Cellometer™ Auto T4 cell counter (Nexcelom Bioscience, LLC). Approximately $2.0x10^4$ NIH3T3/hCD63, LNCAP, U87MG or NIH3T3 cells were seeded separately onto 96-well carbon electrode plates (Meso Scale Discovery, Cat# L15XB-6) and were then incubated for one hour at 37°C. Nonspecific binding sites were blocked with 2% BSA (w/v) in PBS with Ca^{2+}/Mg^{2+} for one hour at room temperature (RT). Solutions containing anti-CD63 antibodies or isotype control antibodies at a range of concentrations (1.7pM to 100nM) in 0.5% BSA (w/v) in PBS with Ca^{2+}/Mg^{2+}, as well as control buffer alone, were then added in duplicate to the plate-bound NIH3T3/hCD63, LNCAP, U87MG or NIH3T3 cells and incubated for one hour at RT. Plates were subsequently washed to remove unbound antibodies using an AquaMax2000 plate washer with a cell washing head (MDS Analytical Technologies). The plate-bound antibodies were detected with 1μg/mL of either a SULFO-TAG™-conjugated goat polyclonal anti-human IgG antibody specific for Fcγ fragment (Jackson Immunoresearch, Cat# 109-005-098) or a SULFO-TAG™-conjugated goat polyclonal anti-mouse IgG antibody specific for Fcγ fragment (Jackson Immunoresearch, Cat#

115-005-164) for one hour at RT. Plates were washed and then incubated with Read Buffer (MSD, Cat# R92TD-2) according to the manufacturer's instructions. Luminescent signals were measured using a SECTOR Imager (MSD). Luminescence intensity, measured in relative light units (RLU), was recorded to indicate the binding intensity of each antibody at the range of concentrations tested. The ratio of signal detected with 3.7nM antibody binding to human CD63 expressing cells compared to the same concentration of antibody binding to negative cells was reported as an indication of specificity of CD63 binding. Antibodies with the binding ratio of greater than 3 were classified as specific binders and antibodies with the binding ratio less than or equal to 3 were classified as non-binders and marked as NB in Table 13. In addition, the direct binding signals (in RLU) were analyzed as a function of the antibody concentration and the data were fitted with a sigmoidal (four-parameter logistic) dose-response model using GraphPad Prism™ software. The EC_{50} value for binding to human CD63 expressing cells, defined as the concentration of antibody at which 50% of the maximal binding signal is detected, was determined to indicate potency of each antibody and reported in Table 13 only for specific binders.

[0253] As shown in Table 13, the four anti-CD63 antibodies generated as described in Example 9 and a Comparator Ab (Comparator 1) bound specifically to human CD63 expressed on engineered NIH3T3/hCD63 cells as well as that endogenously expressed on LNCAP and U87MG cell lines. The four anti-CD63 antibodies described in Example 9 bound to NIH3T3/hCD63 cells with EC_{50} values ranging from 280pM to 970pM and binding ratios over the negative cell line ranging from 91 to 281-fold. The four anti-CD63 antibodies of the invention bound to U87MG cells with EC_{50} values ranging from 500pM to 1.4nM and binding ratios over the negative cell line ranging from 52 to 272-fold. The four anti-CD63 antibodies generated as described in Example 9 bound to LNCAP cells with EC_{50} values ranging from 210pM to 1.7nM and binding ratios over the negative cell line ranging from 7 to 20-fold. The lower binding ratios on LNCAP cells are in agreement with lower CD63 copy number on these cells compared to U87MG cells. The isotype control antibodies were non-binders, as expected, with cell binding ratios less than or equal to 3.

Table 13. Anti-CD63 Antibodies Binding to Human CD63 expressing cells as measured by Electrochemiluminescence based detection

Antibody	Cell Binding Potency, EC_{50} (M)			Ratio at 3.7nM Ab concentration of Cell Binding Signal (RLU) to human CD63 cells relative to negative NIH3T3		
	NIH3T3/hCD63	U87MG	LNCAP	NIH3T3/hCD63	U87MG	LNCAP
H1M12451N	5.7E-10	9.5E-10	1.7E-09	91	52	7
H2M12395N	2.9E-10	7.9E-10	3.5E-10	256	171	18
H4H12450N	9.7E-10	1.4E-09	1.4E-09	281	272	20
H2M12450N	2.8E-10	5.0E-10	2.1E-10	230	175	20
CONTROLS						
Comparator Ab 1	5.7E-10	6.7E-10	2.0E-09	253	271	16
Human IgG4 isotype control	NB	NB	NB	1	1	1
Mouse IgG2 isotype control	NB	NB	NB	3	2	2

NB - non-binder; antibodies with a binding ratio of less than or equal to 3 were classified as non-binders.

[0254] *Cytotoxicity mediated by parental anti-human CD63 antibodies*

[0255] In order to assess the ability of an anti-CD63 antibody described herein to internalize in CD63 expressing cells, an *in vitro* indirect cytotoxicity assay was performed. Human CD63 positive T47D cells (ATCC, Cat #HTB-133) and human CD63 negative NIH3T3 cells (ATCC, Cat #CRL-1658) were respectively seeded in PDL-coated 96-well plates (BD Biocoat, Cat #356461) at either 6,000 cells per well in RPMI (Irvine Scientific, Cat #9160) containing 10% FBS (ATCC, Cat# 30-2020), pencillin/streptomycin/L-glutamine (Gibco, Cat #10378-016), 50uM Beta-Mercaptoethanol (Sigma, Cat #M7522) (growth media), Sodium Pyruvate 100mM (Millipore, Cat #TMS-005-C), HEPES 1M (Irvine Scientific, Cat #9319), and Insulin bovine 10 ug/mL (Gemini BioProducts, Cat #700-912P) or 2,000 cells per well in DME high glucose (Irvine Scientific, Cat #9033), 10% Bovine calf serum (Hyclone, Cat #SH30072.03), plus pencillin/streptomycin/L-glutamine (Gibco, Cat #10378-016) and grown overnight at 37°C in 5% CO_2. For cell viability curves, cells were incubated for 5 minutes at 37°C with a serially diluted anti-CD63 antibody

(H2M12450N) or a non-binding isotype control antibody at concentrations ranging from 3.0 pM to 2.2 nM. A Fab anti-mFc secondary antibody conjugated to the cytotoxic payload MMAF (Moradec, Cat #AM-201AF-50) was then added at 20 nM to each well. Media alone served as a negative control, and 33 µM of digitonin (Promega, Cat #G9441) was used to determine the maximum cytotoxicity. Following a 72 hour incubation, cell viability was measured using Cell Counting Kit-8 (Dojindo, Cat #CK04) as per manufacturer's protocols with an incubation time range of 1-3 hours. The absorbance at 450nm (OD_{450}) was measured on an Envision plate reader (PerkinElmer). Background OD_{450} levels from digitonin treated cells were subtracted from all wells and viability was expressed as a percentage of the untreated controls (% viability). IC_{50} values were determined from a four- parameter logistic equation over an 8-point response curve (GraphPad Prism). All IC_{50} values are expressed in nM concentration and the minimum % viable cells remaining after treatment is reported.

[0256] As summarized in Table 14, the anti-CD63 antibody, H2M12450N, reduced T47D viability to 21% with an IC_{50} value of 0.24 nM, whereas the isotype control reduced viability to only 64%. The antibodies had little to no impact on the viability of the NIH3T3 cell line.

Table 14. Anti-CD63 antibody internalization measured by an indirect cytotoxicity assay in T47D and NIH3T3 cells

Antibody	**T47D (nM) IC_{50}**	**T47D % Viability**	**NIH3T3 (nM) IC_{50}**	**NIH3T3 % Viability**
H2M12450N	0.24	21	ND	83
Isotype Control	ND	64	ND	91

ND = Not determined

[0257] *Biacore binding kinetics of anti-CD63 monoclonal antibodies binding to CD63 (EC2) loop reagents measured at 25°C and 37°C*

[0258] Equilibrium dissociation constants (K_D) for different CD63loop reagents binding to purified anti-CD63 monoclonal antibodies were determined using a real-time surface plasmon resonance based Biacore T200 biosensor or Biacore 2000 biosensor. All binding studies were performed in 10mM HEPES, 150mM NaCl, 3mM EDTA, and 0.05% v/v Surfactant Tween-20, pH 7.4 (HBS-ET) running buffer at 25°C and 37°C. The Biacore CM5 sensor chip surface was first derivatized by amine coupling with either a rabbit anti-mouse Fc specific polyclonal antibody (GE Healthcare,

Cat# BR100838) or an anti-human Fab kit (GE Healthcare, Cat# 28958325) to capture anti-CD63 monoclonal antibodies. Binding studies were performed on either recombinant human CD63 extracellular loop 2 expressed with a C-terminal Myc-Myc- hexahistidine (hCD63 EC loop 2-MMH; SEQ ID NO:80) or recombinant human CD63 extracellular loop 2 expressed with a C-terminal human Fc tag (hCD63 EC loop 2-hFc; SEQ ID NO:81). Different concentrations of hCD63 EC loop 2-MMH or hCD63 EC loop 2-hFc (either tested at 50nM – 12.5nM in a 4-fold dilution or at 90nM-0.37 nM in 3-fold serial dilutions) were first prepared in HBS-ET running buffer and were injected over the anti-mouse Fc captured anti-CD63 monoclonal antibody surface for 4 minutes at a flow rate of 35 µL/minute or 50 µL/minute, while the dissociation of monoclonal antibody bound CD63 reagent was monitored for 8 or 10 minutes in HBS-ET running buffer. The association rate (k_a) and dissociation rate (k_d) were determined by fitting the real-time binding sensorgrams to a 1:1 binding model with mass transport limitation using Scrubber 2.0c curve-fitting software. Binding dissociation equilibrium constant (K_D) and dissociative half-life ($t_{1/2}$) were calculated from the kinetic rates as:

$$K_D\ (M) = \frac{kd}{ka}, \quad \text{and} \quad t_{1/2}\ (\text{min}) = \frac{\ln(2)}{60 * kd}$$

[0259] Binding kinetics parameters for human CD63 EC loop 2 protein binding to different anti-CD63 monoclonal antibodies of the invention at 25°C and 37°C are shown in Tables 15 through 18.

[0260] At 25°C, all of the anti-CD63 monoclonal antibodies of the invention bound to hCD63 EC loop 2-MMH with K_D values ranging from 530pM to 11.0nM, as shown in Table 15. At 37°C, all of the anti-CD63 monoclonal antibodies of the invention bound to hCD63 EC loop 2-MMH with K_D values ranging from 1.15nM to 56.4nM, as shown in Table 16.

[0261] At 25°C, all of the anti-CD63 monoclonal antibodies of the invention bound to hCD63 EC loop 2-hFc with K_D values ranging from 150pM to 753pM, as shown in Table 17. At 37°C, all of the anti-CD63 monoclonal antibodies of the invention bound to hCD63 EC loop 2-hFc with K_D values ranging from 119pM to 3.38nM, as shown in Table 18.

Table 15 : Binding kinetics parameters of human CD63 EC loop2-MMH binding to anti-CD63 monoclonal antibodies at 25 °C

Antibody	mAb Capture Level (RU)	50nM or 90 nM Ag Bound (RU)	k_a ($M^{-1}s^{-1}$)	k_d (1/s)	K_D (M)	t½ (min)
H2M12450N	592	65	2.27E+05	2.79E-04	1.23E-09	41
H2M12395N	472	42	1.66E+05	1.82E-03	1.10E-08	6
H1M12451N	525	52	8.59E+04	4.30E-04	5.00E-09	27
H4H12450N	296	24	1.39E+05	7.37E-05	5.30E-10	156.7

Table 16: Binding kinetics parameters of human CD63 EC loop2-MMH binding to anti-CD63 monoclonal antibodies at 37 °C

Antibody	mAb Capture Level (RU)	50nM Ag Bound (RU)	k_a ($M^{-1}s^{-1}$)	k_d (1/s)	K_D (M)	t½ (min)
H2M12450N	617	58	1.75E+05	2.01E-04	1.15E-09	57
H2M12395N	535	31	1.65E+05	9.32E-03	5.64E-08	1
H1M12451N	597	44	8.89E+04	1.90E-03	2.14E-08	6

Table 17: Binding kinetics parameters of human CD63 EC loop2-hFC binding to anti-CD63 monoclonal antibodies at 25 °C

Antibody	mAb Capture Level (RU)	50nM Ag Bound (RU)	k_a ($M^{-1}s^{-1}$)	k_d (1/s)	K_D (M)	t½ (min)
H2M12450N	592	134	3.20E+05	4.66E-05	1.50E-10	248
H2M12395N	472	117	6.77E+05	5.10E-04	7.53E-10	23
H1M12451N	525	107	1.19E+05	8.40E-05	7.04E-10	138

Table 18 : Binding kinetics parameters of human CD63 EC loop2-hFC binding to anti-CD63 monoclonal antibodies at 37 °C

Antibody	mAb Capture Level (RU)	50nM Ag Bound (RU)	k_a ($M^{-1}s^{-1}$)	k_d (1/s)	K_D (M)	t½ (min)
H2M12450N	617	138	4.11E+05	4.89E-05	1.19E-10	236
H2M12395N	535	123	5.29E+05	1.79E-03	3.38E-09	6
H1M12451N	597	118	6.51E+05	3.31E-04	5.09E-10	35

[0262] *Generation of bispecific antibodies to determine internalization of bispecific complex having an anti-CD63 binding arm*

[0263] To assess the ability of anti-CD63 antibodies generated as described in this example to internalize as part of a bispecific antigen-binding molecule, the antibodies were reconstructed into bispecific formats where one binding arm was the anti-CD63 antibody VH/VL pair (see Table 11 parental antibodies) and the other was an irrelevant binding arm. Standard methods of making bispecific antibodies were used, and exemplary methods are described in, *e.g.,* US Application Publication No. 2010/0331527, and US Patent No. US5731168, each of which is incorporated herein by reference. The bispecific antibodies were tested for their ability to internalize using human CD63 expressing cells. For the assay, HEK293 cells, which endogenously express human CD63, were plated at a density of 10,000 cells/well in DMEM containing 10% FBS and penicillin-streptomycin/L-glutamine (Gibco, Cat# 10378016) in clear bottom black Poly-D-Lysine coated 96-well plates (Greiner, Cat# 655946). Two days later, the media was replaced with fresh media containing anti-CD63 bispecific antibodies and a negative control antibody in a 2-fold dilution series beginning at 10 µg/mL to 0.157 µg/mL, along with a media only control. Cells were then incubated at 37°C for 3 hours to allow for antibody internalization. Following the incubation, cells were washed with PBS, fixed in 4% paraformaldehyde (Thermo Scientific, Cat# 28908) for 20 minutes at room temperature, and subsequently permeablized with 0.2% Triton X-100 (Spectrum Chemical, Cat# TR135) in 5% normal goat serum (NGS) (Gibco, Cat# PCN5000) for 20 minutes at room temperature. Cells were then incubated with either 2 ug/mL of donkey anti-mouse IgG Alexa Fluor-647 Fab (Manufacture, Cat#115-606-006) or 2 **µg**/mL of goat anti-human IgG Alexa Fluor-647 Fab (Jackson ImmunoResearch, Cat#115-606-006) in 5% NGS for 1 hour at room temperature. The secondary antibody solution was removed, then cell were washed with PBS, and subsequently fresh PBS containing 2 drops/mL of NucBlue (Invitrogen, CAT# R37605) was added to stain live cell nuclei. Antibody internalization and nuclei were imaged at 40x on the ImageXpress High-Content Imaging System (Molecular Devices) and antibody internalization was quantified using the MetaXpress Software Transfluor Application Module (Molecular Devices). Antibody internalization is reported as pit integrated intensity per cell ± standard deviation (SD).

[0264] As shown in Table 15, all of the bispecific antibodies incorporating a single arm binding to human CD63 (derived from either H1M12451, H2M12450, or H2M12395) and an irrelevant non-

binding arm demonstrated efficient internalization into HEK293 cells. The bispecific antibody incorporating one binding arm of mAb12450 demonstrated a higher amount of internalization than the other bispecific antibodies tested.

Table 15: Internalization of anti-CD63 bispecific antibodies by HEK293 cells

	Internalization of antibody (pit integrated intensity) ± SD			
Concentration of antibody (µg/mL)	**H2M12395N bispecific**	**H2M12450N bispecific**	**H1M12451N bispecific**	**Negative control Ab**
10	1.05E+06 ± 4.56E+05	4.34E+06 ± 8.77E+05	8.26E+05 ± 2.67E+05	4.58E+03 ± 6.50E+03
5	1.07E+06 ± 4.06E+05	4.31E+06 ± 5.48E+05	5.45E+05 ± 5.20E+04	1.23E+03 ± 8.85E+02
2.5	2.73E+05 ± 6.01E+04	3.92E+06 ± 5.80E+05	3.27E+05 ± 1.06E+05	7.70E+02 ± 6.09E+02
1.25	1.89E+05 ± 6.61E+04	2.72E+06 ± 2.63E+05	1.20E+05 ± 3.91E+04	1.43E+03 ± 1.19E+03
0.625	2.03E+05 ± 9.37E+04	1.75E+06 ± 1.39E+05	7.87E+04 ± 1.07E+04	3.37E+03 ± 5.22E+03
0.3125	3.95E+04 ± 8.23E+03	8.57E+05 ± 1.60E+05	3.77E+04 ± 1.22E+04	1.99E+03 ± 2.19E+03
0.15625	2.81E+04 ± 1.30E+04	2.42E+05 ± 2.54E+04	7.23E+03 ± 6.09E+03	1.87E+03 ± 1.16E+03
0	3.72E+03 ± 1.66E+03	8.21E+03 ± 3.47E+03	1.66E+04 ± 1.80E+04	1.10E+03 ± 1.56E+03

CLAIMS

1. A polynucleotide encoding a multidomain therapeutic protein comprising a delivery domain and an enzyme domain,

wherein the delivery domain is an antigen-binding protein that binds to CD63,

wherein the delivery domain is a single-chain variable fragment (scFv),

wherein the enzyme domain comprises an alpha-glucosidase (GAA) or a biologically active portion thereof, and

wherein the polynucleotide further comprises a virus nucleic acid sequence, wherein the virus nucleic acid sequence is an adeno-associated virus (AAV) nucleic acid sequence.

2. The polynucleotide of claim 1, wherein:

(i) the delivery domain comprises:

(1) the amino acid sequence set forth in SEQ ID NO: 2;

(2) the HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR-3 amino acid sequences contained within an HCVR/LCVR amino acid sequence pair selected from the group consisting of SEQ ID NOs: 14/22, SEQ ID NOs: 30/38, SEQ ID NOs: 46/54, and SEQ ID NOs: 62/70;

(3) HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3 amino acid sequences selected from the group consisting of SEQ ID NOs: 16-18-20-24-26-28, SEQ ID NOs: 32-34-36-40-42-44, SEQ ID NOs: 48-50-52-56-58-60, and SEQ ID NOs: 64-66-68-72-74-76; or

(4) an HCVR/LCVR amino acid pair set forth in SEQ ID NOs: 14/22, SEQ ID NOs: 30/38, SEQ ID NOs: 46/54, or SEQ ID NOs: 62/70,

optionally wherein the HCVR/LCVR amino acid pair comprises from N-terminal to C-terminal: the HCVR, an optional linker, and the LCVR and/or optionally wherein the enzyme domain is attached to the C-terminus of the LCVR, optionally via a linker, and any combination thereof; and/or

(ii) the enzyme domain comprises the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 13, or SEQ ID NO: 78, or a biologically active fragment thereof,

optionally wherein the multidomain therapeutic protein comprises the amino acid sequence set forth in SEQ ID NO: 79.

3. The polynucleotide of claim 1 or 2, wherein:

(i) the polynucleotide further comprises a locus-targeting sequence;

(ii) the AAV nucleic acid sequence comprises an internal terminal repeat sequence, optionally wherein the internal terminal repeat sequence that comprises the sequence set forth in SEQ ID NO: 6, SEQ ID NO: 7, or both;

(iii) the polynucleotide further comprises a tissue-specific regulatory element, optionally wherein the tissue-specific regulatory element comprises a liver-specific promoter;

(iv) the polynucleotide further comprises a tissue-specific regulatory element comprising the sequence set forth in SEQ ID NO: 8, SEQ ID NO: 9, or both; and/or

(v) the polynucleotide comprises the nucleic acid sequence set forth in SEQ ID NO: 11 and/or encodes the amino acid sequence set forth in SEQ ID NO: 10 or SEQ ID NO: 78.

4. The polynucleotide of any one of claims 1-3, wherein the delivery domain comprises HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3 amino acid sequences contained within the HCVR/LCVR pair set forth in SEQ ID NOs: 46/54.

5. The polynucleotide of any one of claims 1-4, wherein the delivery domain comprises the HCVR/LCVR pair set forth in SEQ ID NOs: 46/54.

6. The polynucleotide of any one of claims 1-5, wherein the multidomain therapeutic protein comprises the amino acid sequence set forth in SEQ ID NO: 79.

7. A gene therapy vector comprising the polynucleotide of any one of claims 1-6, optionally wherein the gene therapy vector is selected from the group consisting of:

(i) a viral vector, optionally wherein the viral vector is a natural virus, an engineered virus, or a chimeric virus;

(ii) a naked polynucleotide; and

(iii) a polynucleotide complex, optionally wherein the polynucleotide complex is a lipid nanoparticle comprising the polynucleotide and lipids.

8. The gene therapy vector of claim 7, wherein the gene therapy vector is:

(i) a viral vector selected from the group consisting of a retrovirus, an adenovirus, a herpes simplex virus, a pox virus, a vaccinia virus, a lentivirus, and an adeno-associated virus; and/or

(ii) an AAV2/8 chimera or an AAV pseudotyped to a specific tissue, optionally wherein the AAV is pseudotyped to the liver.

9. A recombinant multidomain therapeutic protein comprising a delivery domain and an enzyme domain, wherein the recombinant multidomain therapeutic protein is encoded by the polynucleotide of any one of claims 1-6.

10. A method of expressing in a cell a recombinant multidomain therapeutic protein comprising a delivery domain and an enzyme domain, wherein the cell is *ex vivo*, the method comprising:

(a) contacting the cell with the gene therapy vector of claim 7 or 8 containing the polynucleotide encoding the recombinant multidomain therapeutic protein;

(b) allowing the polynucleotide to integrate into a genomic locus of the cell; and

(c) allowing the cell to produce the recombinant multidomain therapeutic protein.

11. A gene therapy vector for use in a method of expressing in a cell a recombinant multidomain therapeutic protein comprising a delivery domain and an enzyme domain, wherein the cell is *in vivo*, the method comprising:

(a) contacting the cell with the gene therapy vector of claim 7 or 8 containing the polynucleotide encoding the recombinant multidomain therapeutic protein;

(b) allowing the polynucleotide to integrate into a genomic locus of the cell; and

(c) allowing the cell to produce the recombinant multidomain therapeutic protein.

12. The method of claim 10 or the gene therapy vector for use of claim 11, wherein:

(i) the gene therapy vector is an adeno-associated virus (AAV) vector;

(ii) the genomic locus of the cell is a safe harbor locus;

(iii) the genomic locus is at or proximal to a locus selected from the group consisting of an EESYR locus, a SARS locus, position 188,083,272 of human chromosome 1 or its non-human mammalian orthologue, position 3,046,320 of human chromosome 10 or its non-human mammalian orthologue, position 67,328,980 of human chromosome 17 or its non-human mammalian orthologue, an adeno-associated virus site 1 (AAVS1) on human chromosome 19 or its non-human mammalian orthologue, a chemokine receptor 5 (CCR5) gene, a chemokine receptor gene encoding an HIV-1 coreceptor, a mouse Rosa26 locus or its non-murine mammalian orthologue, and a human albumin (*alb*) locus;

(iv) the cell is a human cell or a mouse cell; and/or

(v) the cell is a liver cell.

13. The gene therapy vector of claim 7 or 8, for use in a method of reducing glycogen accumulation in a tissue in a patient in need thereof, comprising administering to the patient the gene therapy vector.

14. The gene therapy vector for use of claim 13, wherein:

(i) the tissue is selected from the group consisting of heart, liver, and skeletal muscle;

(ii) high serum levels of GAA are maintained for at least 12 weeks, or serum levels of GAA at 12 weeks are about 2.5-3-fold higher than serum levels of GAA in a subject administered a gene therapy vector comprising a polynucleotide encoding a GAA without the anti-CD63 scFv delivery domain;

(iii) glycogen levels in heart, skeletal muscle, and liver tissue are reduced to wild-type levels; and/or

(iv) glycogen levels in the tissue are maintained at wild-type levels 3 months after administration of the gene therapy vector.

15. The gene therapy vector of claim 7 or 8 for use in a method of treating enzyme deficiency in a patient in need thereof and/or tolerizing the patient to the enzyme for which it is deficient, comprising administering to the patient the gene therapy vector.

16. The gene therapy vector for use of claim 15, wherein:

(i) the enzyme deficiency is a deficiency in alpha-glucosidase;

(ii) the anti-CD63 scFv-GAA comprises the amino acid sequence set forth in SEQ ID NO: 10 or SEQ ID NO: 79;

(iii) high serum levels of GAA are maintained in the patient for at least 12 weeks after administration of the gene therapy vector;

(iv) serum levels of GAA in the patient at 12 weeks after administration of the gene therapy vector are about 2.5-3-fold higher than serum levels of GAA in a subject administered a gene therapy vector comprising a polynucleotide encoding a GAA without the anti-CD63 scFv delivery domain;

(v) glycogen levels in heart, skeletal muscle, and liver tissue in the patient are reduced to wild-type levels;

(vi) glycogen levels in heart, skeletal muscle, and liver tissue in the patient are maintained at wild-type levels 3 months after administration of the gene therapy vector;

(vii) muscle strength of the patient after treatment is restored to wild-type levels;

(viii) the patient has Pompe disease;

(ix) the patient has reduced antibodies to the enzyme compared to a control patient who received the enzyme domain in absence of the delivery domain;

(x) the method further comprises immunosuppressing the patient with one or more immunosuppressant agents;

(xi) the method further comprises as a last step administering to the patient the enzyme in which it is deficient, optionally wherein the patient is administered the enzyme in

which it is deficient via a multidomain therapeutic protein, optionally wherein the multidomain therapeutic protein comprises an anti-CD63 antibody or antigen-binding portion thereof; and/or

(xii) the patient is administered a therapeutically effective amount of the polynucleotide that makes unnecessary any further administration of GAA, a biologically active portion thereof, or a multidomain therapeutic polypeptide comprising GAA or a biologically active portion thereof to the patient.

17. A pharmaceutical composition comprising the gene therapy vector of claim 7 or 8 and a pharmaceutically acceptable carrier.