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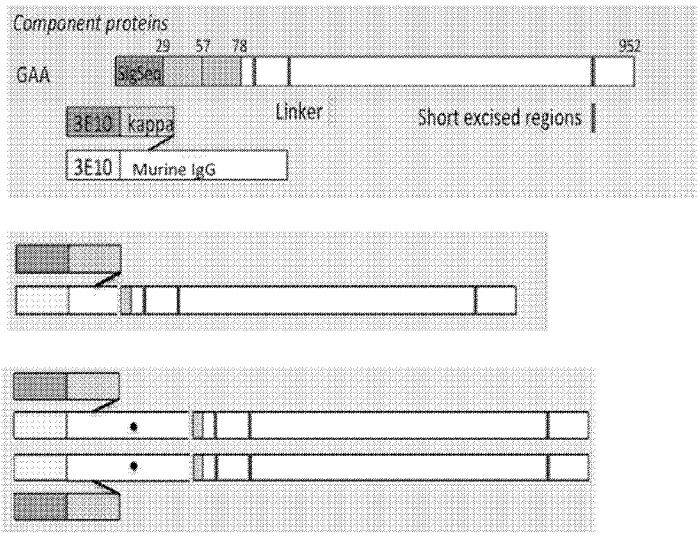
(54) **Title**: METHODS AND COMPOSITIONS FOR TREATMENT OF POMPE DISEASE

Figure 1



(57) **Abstract**: Provided herein are chimeric polypeptides comprising a mature acid-alpha glucosidase (GAA) polypeptide and an internalizing moiety. An exemplary internalizing moiety of the application is the 3E10 antibody which is capable of transiting a cellular membrane via an equilibrative nucleoside transporter 2 (ENT2) and binding DNA. Further provided are methods and uses of the said chimeric polypetides for treating Pompe disease, increasing acid alpha-glucosidase enzyme activity in a cell and decreasing glycogen accumulation in the cytoplasm, lysosomes and autophagic vacuoles of cells.

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— *with sequence listing part of description (Rule 5.2(a))*

METHODS AND COMPOSITIONS FOR TREATMENT OF POMPE DISEASE

RELATED APPLICATIONS

This application claims the benefit of priority to United States provisional application serial number 61/767,016, filed February 20, 2013 and United States provisional application serial number 61/926,874, filed January 13, 2014. The disclosures of each of the foregoing applications are hereby incorporated by reference in their entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 20, 2014, is named 106199-0007-WO1.txt and is 80,767 bytes in size.

BACKGROUND OF THE DISCLOSURE

Glycogen storage disease type II (GSDII or Pompe disease) is an autosomal recessive metabolic disorder characterized by a deficiency in the lysosomal enzyme acid α-glucosidase (GAA). Patients suffering from the disorder are unable to convert lysosomal stores of glycogen into glucose, which leads initially to accumulation of glycogen in the lysosome, and later to accumulation of glycogen in the cytoplasm and autophagic vesicles of cells. Eventually, the buildup of toxic levels of glycogen damages the cells and impairs proper function. In particular, muscle cell dysfunction is a hallmark of Pompe disease, with symptoms ranging from hypertrophic cardiomyopathy, weakness, skeletal muscle dysfunction and early infant death in infantile onset forms of the disease, to progressive degeneration of skeletal muscle function and respiratory muscle dysfunction in juvenile and adult onset forms of the disease.

Treatment of Pompe disease with enzyme replacement therapy (ERT) has provided partial restoration of muscle function and prolonged survival in some patients. However, prior therapies based on delivery of the 110 kDa precursor GAA protein have achieved delivery of protein only to the lysosome. Delivery of protein exclusively to the lysosome has proven ineffective to clear glycogen build-up in the cytoplasm or other extra-lysosomal spaces. Additionally, approaches based on delivering protein to the lysosome have relied

on uptake through mannose-6-phosphate receptors in the lysosome, and high dosages appear to be required.

SUMMARY OF THE DISCLOSURE

There is a need in the art for methods and compositions for clearing cytoplasmic glycogen build-up in patients with Pompe disease, as well as a need for alternative therapies for treating Pompe disease. Such methods and compositions would improve treatment of Pompe disease, particularly in patients whose disease is severe enough and/or advanced enough to have significant cytoplasmic glycogen accumulation. The present disclosure provides such methods and compositions. In certain embodiments, the methods and compositions provided herein decrease glycogen build-up in, at least, the cytoplasm. In certain embodiments, the methods and compositions also decrease glycogen build-up in lysosomes and autophagic vesicles. Similarly, the methods and compositions provided herein can be used to improve deleterious symptoms of Pompe Disease, for example, to decrease levels of one or more of alanine transaminase, aspartate transaminase, alkaline phosphatase, and creatine phosphokinase (e.g., to decrease abnormally elevated levels of one or more such enzymes, such as in serum).

In a first aspect, the disclosure provides a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety that promotes delivery into cells. In other words, the disclosure provides chimeric polypeptides having two portions: a portion comprising a mature GAA polypeptide (a GAA polypeptide comprising mature GAA; a GAA portion comprising a GAA polypeptide comprising a mature GAA) and a portion comprising an internalizing moiety that promotes delivery into cells. In certain embodiments, the internalizing moiety promotes transport into cytoplasm of cells. In certain embodiments, the chimeric polypeptide has acid alpha-glucosidase activity, and does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons, particularly does not comprise the precursor polypeptide of 110 kilodaltons generated endogenously by the cleavage of amino acids 1-56 of SEQ ID NO: 1 by signal peptidase and protease 1. In certain embodiments, the chimeric polypeptide does not comprise the signal sequence of a GAA precursor polypeptide (e.g., see the signal sequence depicted for SEQ ID NO: 1 or 2). In other words, the chimeric polypeptide comprises a mature GAA portion and an internalizing moiety portion but does not include amino acids 1-56 of SEQ ID NO: 1 (e.g., the GAA portion comprises a GAA polypeptide but does not

include amino acids 1-56 of SEQ ID NO: 1). The chimeric polypeptide may further comprise additional portions, such as linker moieties and/or tags but, in certain embodiments, does not include the GAA precursor polypeptide of approximately 110 kilodaltons (e.g., the GAA precursor as defined in Moreland et al, 2005, J. Biol. Chem. 280: 6780) and/or the full length GAA polypeptide set forth in SEQ ID NO: 1 or 2.

In certain embodiments, the mature GAA polypeptide has a molecular weight of approximately 70-76 kilodaltons. In certain embodiments, the mature GAA polypeptide has a molecular weight of about 70 kDa or about 76 kDa. In certain embodiments, the mature GAA polypeptide comprises an amino acid sequence selected from about: residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2; residues 204-782 of SEQ ID NOs: 1 or 2; residues 206-782 of SEQ ID NOs: 1 or 2; or residues 288-782 of SEQ ID NOs: 1 or 2. In certain embodiments, the mature GAA polypeptide consists of an amino acid sequence selected from about residues: residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2; residues 204-782 of SEQ ID NOs: 1 or 2; residues 206-782 of SEQ ID NOs: 1 or 2; or residues 288-782 of SEQ ID NOs: 1 or 2. In other embodiments, the C-terminal amino acid residues of the mature GAA polypeptide varies, such that the C-terminal amino acid residues is any of residues 816-881, as set forth in SEQ ID NOs: 1 or 2. In certain embodiments, the mature GAA polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4. In certain embodiments, the chimeric polypeptide comprises any of the foregoing. In certain embodiments, the GAA polypeptide portion of the chimeric polypeptide consists of any of the foregoing examples of mature GAA. Although the chimeric polypeptide comprises additional amino acid sequence, in certain embodiments, the chimeric polypeptide does include additional GAA amino acid sequence contiguous with the mature GAA polypeptide portion. In other embodiments, the chimeric polypeptide comprises mature GAA and also comprises additional N- and or C-termimal contiguous GAA polypeptide sequence, such as the longer active GAA polypeptides described herein (e.g., the GAA portion comprises a GAA polypeptide comprising mature GAA). As used herein, "GAA polypeptide" refers to a polypeptide that comprises a portion corresponding to mature GAA but may also include additional N- and/or C-terminal portions naturally present in a GAA polypeptide (e.g., a native GAA polypeptide). In certain embodiments, a GAA polypeptide for use in the chimeric polypeptides and methods of the disclosure does not include residues 1-56 of SEQ

ID NO: 1. In certain embodments, the GAA polypeptide does not correspond to the 110 kilodalton, GAA precursor polypeptide.

In some embodiments, the chimeric polypeptide has acid alpha-glucosidase activity.

In some embodiments, the chimeric polypeptide does not comprise the full length, GAA translation product set forth in SEQ ID NO: 1 (e.g., the GAA polypeptide portion does not include the full length, GAA translation product set forth in SEQ ID NO: 1). In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-56 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide lacks the portion corresponding to amino acids 1-56, preferably 1-57 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide comprises a GAA polypeptide that lacks at least a portion of the GAA full linker region, wherein the full linker region (SEQ ID NO: 31) corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2 (e.g., the chimeric polypeptide may include some of the full linker region but does not include all of the full linker region). In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-60 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide lacks the portion corresponding to amino acids 1-60 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 21. In some embodiments, the GAA polypeptide comprises amino acids 61-952 of SEQ ID NO: 1. In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-66 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide lacks the portion corresponding to amino acids 1-66 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22. In some embodiments, the GAA polypeptide comprises amino acids 67-952 of SEQ ID NO: 1. In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-69 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide lacks the portion corresponding to amino acids 1-69 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide or GAA polypeptide comprises the sequence of SEQ ID NO: 23. In some embodiments, the GAA

polypeptide comprises amino acis 70-952 of SEQ ID NO: 1. The disclosure contemplates combinations of any one or more of these features.

In certain embodiments, the chimeric polypeptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 11 or 12, or set forth in either of these sequence identifiers but in the absence of one or more epitope tags (e.g., in the absence of, for example, a His and/or Myc epitope tag).

In certain embodiments, the chimeric polypeptide and/or the mature GAA is glycosylated. In certain embodiments, the chimeric polypeptide and/or mature GAA is not glycosylated. In certain embodiments, the mature GAA has a glycosylation pattern that differs from that of naturally occurring human GAA.

In certain embodiments, the internalizing moiety promotes delivery of the chimeric polypeptide into cytoplasm of cells. In certain embodiments, the internalizing moiety promotes delivery of said chimeric polypeptide into muscle cells, such as skeletal or cardiac muscle cells (e.g., promotes delivery into cytoplasm of such cells). In certain embodiments, the internalizing moiety promotes delivery of said chimeric polypeptide into neurons or hepatocytes (e.g., promotes delivery into cytoplasm of such cells).

In certain embodiments, the chimeric polypeptide comprises *N*-linked oligosaccharide chains modified with M6P residues. In certain embodiments, the chimeric polypeptide comprises a KFERQ-like sequence (SEQ ID NO: 33).

In certain embodiments, the chimeric polypeptide further comprises one or more polypeptide portions that enhance one or more of in vivo stability, in vivo half life, uptake/administration, production, or purification. Exemplary polypeptide portions include epitope tags, such as HA and myc tags, as well as the Fc region of an immunoglobulin or all or a portion of HSA.

In certain embodiments, the internalizing moiety comprises an antibody or antigen binding fragment. In certain embodiments, the antibody or antigen binding fragment is a monoclonal antibody or fragment. In certain embodiments, the antibody or antigen binding fragment is human or humanized. In other embodiments, the antibody or antigen binding fragment is murine. Exemplary antigen binding fragments include, scFv, Fv, Fab, and the like. Further exemplary antigen binding fragments comprise a heavy chain variable region (VH) comprising CDR1, CDR2, and CDR3, and a light chain variable region (VL) comprising CDR1, CDR2, and CDR3. When referring to suitable internalizing moieties,

they preferably retain the antigen/target binding characteristics and cell penetrating characteristics when present in the context of the chimeric polypeptide.

In certain embodiments, the chimeric polypeptides comprise as an internalizing moiety, an antibody or antigen binding fragment thereof select from: monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing. In certain embodiments, the antibody or antigen binding fragment thereof is a monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or an antigen binding fragment of 3E10 or said 3E10 variant. In some embodiments, the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment. In some embodiments, the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof. In some embodiments, the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof. In some embodiments, the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof. For any description of an antibody or antigen binding fragment, the disclosure contemplates that the antibody or antigen binding fragment may comprise a heavy chain and a light chain, such as a heavy chain comprising a heavy chain variable domain and a light chain comprising a light chain variable domain. In some embodiments, the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 13;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 18, which CDRs are according to the Kabat system.

In some embodiments, the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 24;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 29, which CDRs are according to the IMGT system.

In other words, in certain embodiments, the antibody comprises a heavy chain comprising VH CDR1, VH CDR2, and VH CDR3 of the 3E10 antibody, as determined by Kabat and set forth above, and a light chain comprising a VL CDR1, a VL CD2, and a VL CD3 of the 3E10 antibody, as determined by Kabat and set forth above; or is an antigen binding fragment thereof. In certain other embodiments, the antibody comprises a heavy chain comprising VH CDR1, VH CDR2, and VH CDR3 of the 3E10 antibody, as determined by the IGMT system and set forth above, and a light chain comprising a VL CDR1, a VL CD2, and a VL CD3 of the 3E10 antibody, as determined by the IGMT system and set forth above; or is an antigen binding fragment thereof.

In certain embodiments, the 3E10 antibody, fragment, or variant comprises a heavy chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 9. In certain embodiments, the 3E10 antibody, fragment, or variant comprises a light chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 10. In certain embodiments, the 3E10 antibody, fragment, or variant comprises a heavy chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 9 and a light chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 10. It is understood, that these heavy and light chain regions may, in certain embodiments, be connected by a linker (e.g., such as in the context of an scFv – see SEQ ID NO: 11 and 12). Chimeric polypeptides having any combination of the foregoing or following internalizing moieties and mature GAA portions are contemplated. Moreover, chimeric polypeptides having any combination of the foregoing or following internalizing moieties and GAA portions (e.g., GAA polypeptides) described herein are contemplated. Any such chimeric polypeptides are suitable for use in any of the methods of the disclosure described herein.

In some embodiments, the internalizing moiety is an antibody or antigen-binding fragment (e.g., an antibody fragment). In some embodiments, the internalizing moiety is an antibody fragment, particularly an scFv. In other embodiments, the antibody fragment is a Fab. In some embodiments, the N-terminus of the GAA polypeptide portion is fused to the C-terminus of the heavy chain constant region portion of the Fab. In some embodiments, the N-terminus of the GAA polypeptide portion is fused to the C-terminus of the heavy chain constant region portion of the Fab by means of a linker. In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 30. In some embodiments, the antibody or antigen binding fragment is an antibody. In some embodiments, the N-terminus of the GAA polypeptide portion is fused to the C-terminus of the heavy chain Fc portion of the antibody. In some embodiments, the N-terminus of the GAA polypeptide portion is fused to the C-terminus of the heavy chain Fc portion of the antibody by means of a linker. In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 30.

In certain embodiments, the internalizing moiety comprises a homing peptide. In certain embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 1 (ENT1), ENT2, ENT3 or ENT4 transporter. In certain embodiments, the internalizing moiety transits cellular membranes via an ENT2 transporter. By way of example, 3E10 antibody or antigen binding fragment thereof transits cellular membranes via ENT2.

In certain embodiments, the internalizing moiety is capable of binding polynucleotides. In certain embodiments, the internalizing moiety is capable of binding DNA. For example, 3E10 and the particular 3E10 variant described herein are known to bind DNA (e.g., their target – or antigen is DNA). Although these and other DNA binding antibodies are typically not specifically reactive with a single antigen, they do bind DNA with relatively strong affinity. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 1 μM. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 100 nM, less than 75nM, less than 50nM, or even less than 30 nM. For the foregoing, K_D may be determined using, for example, SPR or QCM according to standard protocols.

In certain embodiments, the chimeric polypeptide is a chemical conjugate of mature GAA polypeptide to the internalizing moiety, such as a chemical conjugate comprising a GAA polypeptide portion and an internalizing moiety portion. In other words, the mature

GAA portion and the internalizing moiety portion are interconnected, directly or indirectly, via chemical conjugation. In certain embodiments, the chimeric polypeptide is a recombinant, co-translational fusion protein comprising the mature GAA polypeptide and the internalizing moiety. When the chimeric polypeptide is a fusion protein, the mature GAA portion and the internalizing moiety portion may be interconnected directly or indirectly. In certain embodiments, the chimeric polypeptide is produced recombinantly to recombinantly conjugate the mature GAA polypeptide to the internalizing moiety. In certain embodiments, the chimeric polypeptide is produced in a prokaryotic or eukaryotic cell, such as a yeast cell, an avian cell, an insect cell, or a mammalian cell. In certain embodiments, the chimeric polypeptide is produced in a prokaryotic cell, such as a bacterial cell. In certain embodiments, the internalizing moiety is an antibody or an antibody fragment. When the internalizing moiety is an antibody or antibody fragment, the GAA polypeptide may be produced as a fusion to any portion of the antibody or antibody fragment. For example, if the internalizing moiety is a full length antibody or an Fab, the GAA polypeptide may be fused recombinantly to (or chemically conjugated to), for example, the C-terminus of the heavy chain of the antibody or Fab.

Whether chemically or genetically conjugated, in certain embodiments, the conjugate comprises a linker that conjugates or joins, directly or indirectly, the mature GAA polypeptide to the internalizing moiety. In certain embodiments, the conjugate does not include a linker, and the mature GAA polypeptide is conjugated or joined directly to the internalizing moiety. Regardless of whether a linker joins the mature GAA and the internalizing moiety, portions of the internalizing moiety may be joined via a linker (e.g., an scFv has a linker joining VH and VL domains). In certain embodiments, the chimeric polypeptide has a total of 0, 1, or 2 linkers. In other embodiments, the chimeric polypeptide has more than two linkers. Any linkers may be cleavable. Chimeric polypeptides of the disclosure comprise a GAA portion (e.g., a GAA polypeptide) and an internalizing moiety portion. The disclosure contemplates that any of the GAA portions (e.g., a GAA polypeptide comprising mature GAA) and internalizing moiety portions can be joined, as described above,

In certain embodiments, the internalizing moiety is conjugated or joined, directly or indirectly, to the N-terminal or C-terminal amino acid of the mature GAA polypeptide or to a longer GAA polypeptide comprising a mature GAA polypeptide. In other words, regardless of whether the mature GAA portion and the internalizing portion are contiguous

or separated by one or more amino acid residues, the disclosure contemplates embodiments in which the mature GAA portion is located N-terminal to the internalizing moiety portion and embodiments in which the mature GAA portion is located C-terminal to the internalizing moiety portion. In certain embodiments, the internalizing moiety is conjugated or joined to an internal amino acid of the mature GAA polypeptide.

In a related aspect, the disclosure provides compositions comprising one or more chimeric polypeptides of the disclosure. Chimeric polypeptides for use in such compositions may have any combination of features, as set forth above. In one embodiment, the disclosure provides a composition comprising (a) a first chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide having a molecular weight of approximately 76 kDa and (ii) an internalizing moiety; and (b) a second chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide having a molecular weight of approximately 70 kDa and (ii) an internalizing moiety; wherein the first chimeric polypeptide and the second chimeric polypeptide each have acid alpha-glucosidase activity, and wherein neither the first chimeric polypeptide nor the second chimeric polypeptide comprise a GAA precursor polypeptide of approximately 110 kilodaltons. The foregoing is merely exemplary of suitable compositions. The internalizing moities for the first and second chimeric polypeptide may be the same or different.

In certain embodiments, the composition of two or more chimeric polypeptides further comprises a polypeptide comprising a precursor GAA polypeptide having a molecular weight of about 110 kDa. Also contemplated are compositions in which one or more chimeric polypeptides of the disclosure that do not include a GAA precursor polypeptide of approximately 110 kDa is combined with a polypeptide comprising a precursor GAA polypeptide having a molecular weight of about 110 kDa.

In a related embodiment, the disclosure provides a composition comprising (a) a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety that promotes transport into cytoplasm of cells; wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons; and (b) a polypeptide comprising a precursor GAA polypeptide having a molecular weight of about 110 kDa. The foregoing is merely exemplary of suitable compositions.

In another aspect, the disclosure provides a nucleic acid construct comprising a nucleotide sequence that encodes any of the chimeric polypeptides of the disclosure. For example, the disclosure provides a nucleic acid construct, comprising a nucleotide sequence that encodes a mature GAA polypeptide (or a GAA polypeptide portion), operably linked to a nucleotide sequence that encodes an internalizing moiety, wherein the nucleic acid construct encodes a chimeric polypeptide having acid alpha-glucosidase enzymatic activity and having the internalizing activity of the internalizing moiety, and wherein the nucleic acid construct does not encode a chimeric polypeptide comprising a GAA precursor polypeptide of approximately 110 kilodaltons or does not encode a polypeptide comprising a portion corresponding to residues 1-56 of SEQ ID NO: 1.

In some embodiments, the disclosure provides a vector comprising any of the nucleic acid constructs of the disclosure. In some embodiments, the disclosure provides a host cell comprising any of the vectors of the disclosure. In some embodiments, the host cell comprises and is capable of expressing the vector. In some embodiments, the disclosure provides method of producing a chimeric polypeptide comprising culturing any of the host cells of the disclosure under appropriate conditions to allow expression of the polypeptide to occur. The disclosure contemplates embodiments in which a chimeric polypeptide comprises a single polypeptide chain, as well as embodiments in which a chimeric polypeptide comprises more than one polypeptide chain. When a chimeric polypeptide comprises more than one polypeptide chain that associates in the active polypeptide, the disclosure contemplates methods and compositions in which both polypeptide chains are present in and expressed from the same vector, as well as methods and compositons in which each chain is present in and expressed from a different vector which may be co-expressed in the same host cell.

In another aspect, the disclosure provides a composition comprising any of the chimeric polypeptides of the disclosure, including any chimeric polypeptide having any combination of the foregoing aspects and embodiments, and a pharmaceutically acceptable carrier. In certain embodiments, the composition is a sterile composition. In certain embodiments, the composition is substantially pyrogen-free.

In another aspect, the disclosure provides a variety of in vitro and in vivo methods. In one aspect, the disclosure provides a method of treating Pompe disease in a subject in need thereof, comprising administering to the subject an effective amount of any one or more of the chimeric polypeptides or compositions of the disclosure.

In another aspect, the disclosure provides a method of treating Pompe disease in a subject in need thereof, comprising administering to the subject an effective amount of a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety; wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

In another aspect, the disclosure provides a method of increasing acid alpha-glucosidase enzyme activity in a cell, comprising contacting the cell with a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety; wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

In another aspect, the disclosure provides a method of decreasing glycogen accumulation in cytoplasm of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprises (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety that promotes delivery into cytoplasm of cells; wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

In another aspect, the disclosure provides a method of decreasing glycogen accumulation in cytoplasm and lysosomes of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprises (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety; wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

In another aspect, the disclosure provides a method of decreasing glycogen accumulation in cytoplasm, lysosomes, and autophagic vacuoles of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprises (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety; wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

The following exemplary embodiments are applicable to any of the foregoing methods of the disclosure. Moreover, the disclosure contemplates combinations of these features with each other, as well as with the aspects and embodiments of the disclosure detailed above and throughout the specification. For example, any of the chimeric polypeptides described herein, such as a chimeric polypeptide comprising a GAA polypeptide portion comprising a mature GAA polypeptide (e.g., a GAA polypeptide), as described herein, and an internalizing moiety portion, as described herein may be used in any of the in vivo or in vitro methods of the disclosure.

In certain embodiments, the method comprises use of a chimeric polypeptide, wherein the mature GAA polypeptide has a molecular weight of approximately 70-76 kilodaltons. In certain embodiments, the method comprises the use of a chimeric polypeptide, wherein the mature GAA polypeptide has a molecular weight of approximately 70 kilodaltons or approximately 76 kDa. In certain embodiments, the mature GAA polypeptide has a molecular weight of approximately 70-76 kilodaltons. In certain embodiments, the mature GAA polypeptide has a molecular weight of about 70 kDa or about 76 kDa. In certain embodiments, the mature GAA polypeptide comprises an amino acid sequence selected from: residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2; residues 204-782 of SEQ ID NOs: 1 or 2; residues 206-782 of SEQ ID NOs: 1 or 2; or residues 288-782 of SEQ ID NOs: 1 or 2. In certain embodiments, the mature GAA polypeptide consists of an amino acid sequence selected from residues: residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2; residues 204-782 of SEQ ID NOs: 1 or 2; residues 206-782 of SEQ ID NOs: 1 or 2; or residues 288-782 of SEQ ID NOs: 1 or 2. In other embodiments, the C-terminal amino acid residues of the mature GAA polypeptide varies, such that the C-terminal amino acid residues is any of residues 816-881, as set forth in SEQ ID NOs: 1 or 2. In certain embodiments, the mature GAA polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

In some embodiments, the method comprises use of a chimeric polypeptide that has acid alpha-glucosidase activity. The disclosure contemplates chimeric polypeptides comprising a GAA polypeptide and an internalizing moiety, as described herein, and methods of using any such chimeric polypeptides. In some embodiments, the method comprises use of a chimeric polypeptide that does not comprise the full length, GAA polypeptide set forth in SEQ ID NO: 1. In some embodiments, neither the GAA

polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-56 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide portion lacks the portion corresponding to amino acids 1-56, preferably 1-57 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide comprises a GAA polypeptide that lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2 (*i.e.*, SEQ ID NO: 31). In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-60 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide portion lacks the portion corresponding to amino acids 1-60 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 21. In some embodiments, the GAA polypeptide, for use in a chimeric polypeptide of the disclosure, comprises amino acids 61-952 of SEQ ID NO:1. In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-66 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide portion lacks the portion corresponding to amino acids 1-66, of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22. In some embodimetns, the GAA polypeptide, for use in a chimeric polypeptide of the disclosure, comprises amino acids 67-952 of SEQ ID NO: 1. In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-69 of SEQ ID NO: 1 or 2 (e.g., the GAA polypeptide portion lacks the portion corresponding to amino acids 1-69 of SEQ ID NO: 1 or 2, and this region is not present in the chimeric polypeptide). In some embodiments, the chimeric polypeptide or GAA polypeptide comprises the sequence of SEQ ID NO: 23. In some embodiments, the GAA polypeptide, for use in a chimeric polypeptide of the disclosure, comprises amino acids 70-952 of SEQ ID NO: 1.

In certain embodiments, the disclosure contemplates chimeric polypeptides comprising the amino acid sequence set forth in SEQ ID NO: 21, 22, or 23, and methods of using such chimeric polypeptides. In other words, the GAA polypeptide portion of such chimeric polypeptides comprises (or consists of) the amino acid sequence set forth in SEQ ID NO: 21, 22, or 23. Also contemplated are variants for use in a chimeric polypeptide of

the disclosure, such as variants at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 21, 22, or 23.

In certain embodiments, the method comprises use of a chimeric polypeptide comprising or consisting of the amino acid sequence set forth in SEQ ID NO: 11 or 12, or set forth in either of these sequence identifiers but in the absence of one or more epitope tags.

In certain embodiments, the chimeric polypeptide and/or the mature GAA is glycosylated. In certain embodiments, the chimeric polypeptide and/or mature GAA is not glycosylated. In certain embodiments, the mature GAA has a glycosylation pattern that differs from that of naturally occurring human GAA. In certain embodiments, the mature GAA comprises a KFERQ-like motif (SEQ ID NO: 33).

In certain embodiments of any of the foregoing, the internalizing moiety promotes delivery of the chimeric polypeptide into cytoplasm of cells. In certain embodiments, the internalizing moiety promotes delivery of said chimeric polypeptide into muscle cells, such as skeletal or cardiac muscle cells (e.g., promotes delivery into, at least, cytoplasm of muscle cells). In certain embodiments, the internalizing moiety promotes delivery of said chimeric polypeptide into hepatocytes or neurons (e.g., promotes delivery into, at least, cytoplasm of hepatocytes or neurons). It should be noted that when an internalizing moiety is described as promoting delivery into muscle cells, that does not imply that delivery is exclusive to muscle cells. All that is implied is that delivery is somewhat enriched to muscle cells versus one or more other cell types and that transit into cells is not ubiquitous across all cell types.

In certain embodiments, the chimeric polypeptide comprises *N*-linked oligosaccharide chains modified with M6P residues.

In certain embodiments, the method comprises use of chimeric polypeptides further comprising one or more polypeptide portions that enhance one or more of in vivo stability, in vivo half life, uptake/administration, production, or purification. Exemplary polypeptide portions include epitope tags, such as HA and myc tags, as well as the Fc region of an immunoglobulin or all or a portion of HSA.

In certain embodiments, the method comprises use of a chimeric polypeptide comprising an internalizing moiety comprising an antibody or antigen binding fragment. In certain embodiments, the antibody or antigen binding fragment is a monoclonal antibody or fragment. In certain embodiments, the antibody or antigen binding fragment is human or

humanized. In other embodiments, the antibody or antigen binding fragment is murine. Exemplary antigen binding fragments include, scFv, Fv, Fab, and the like. Further exemplary antigen binding fragments comprise a heavy chain variable region (VH) comprising CDR1, CDR2, and CDR3, and a light chain variable region (VL) comprising CDR1, CDR2, and CDR3. When referring to suitable internalizing moieties, they preferably retain the antigen binding characteristics and cell penetrating characteristics when present in the context of the chimeric polypeptide. In some embodiments, the antigen or antigen-binding fragment is a scFv. In other embodiments, the antibody or antigen binding fragment is a Fab. In some embodiments, the GAA polypeptide is fused to the C-terminus of the heavy chain segment of the Fab. In some embodiments, the GAA polypeptide is fused to the C-terminus of the heavy chain segment of the Fab by means of a linker. In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 30. In some embodiments, the antibody or antigen binding fragment is an antibody. In some embodiments, the GAA polypeptide portion is fused to the C-terminus of the heavy chain portion of the antibody. In some embodiments, the GAA polypeptide portion is fused to the C-terminus of the heavy chain portion of the antibody by means of a linker. In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 30.

In certain embodiments, the method comprises the use of a chimeric polypeptide comprising an antibody or antigen binding fragment thereof that is a monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing. In certain embodiments, the antibody or antigen binding fragment thereof is monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity , or an antigen binding fragment of 3E10 or said 3E10 variant. In some embodiments, the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment. In some embodiments, the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof. In some embodiment, the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof. In some embodiments, the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of

SEQ ID NO: 9 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof. For any description of an antibody or antigen binding fragment, the disclosure contemplates that the antibody or antigen binding fragment may comprise a heavy chain and a light chain, such as a heavy chain comprising a heavy chain variable domain and a light chain comprising a light chain variable domain. In some embodiments, the antibody or antigen binding fragment comprises

a VH CDR1 having the amino acid sequence of SEQ ID NO 13;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 18, which CDRs are according to Kabat. In some embodiments, the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 24;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 29, which CDRs are according to the IMGT system.

In certain embodiments, the 3E10 antibody, fragment, or variant comprises a heavy chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 9. In certain embodiments, the 3E10 antibody, fragment, or variant comprises a light chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 10. In certain embodiments, the 3E10 antibody, fragment, or variant comprises a heavy chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 9 and a light chain comprising an amino acid sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or even 100% identical to SEQ ID NO: 10. Is in understood, that these heavy and light chain regions may, in certain embodiments, be connected by a linker (e.g., such as in the context of an scFv – see SEQ ID NO: 11 and 12). Chimeric polypeptides having any combination

of the foregoing or following internalizing moieties and mature GAA portions are contemplated. In certain embodiments, the 3E10 antibody, fragment, or variant comprises:

a heavy chain CDR1 having the amino acid sequence of SEQ ID NO: 13,

a heavy chain CDR2 having the amino acid sequence of SEQ ID NO: 14,

a heavy chain CDR3 having the amino acid sequence of SEQ ID NO: 15,

a light chain CDR1 having the amino acid sequence of SEQ ID NO: 16,

a light chain CDR2 having the amino acid sequence of SEQ ID NO: 17, and/or

a light chain CDR3 having the amino acid sequence of SEQ ID NO: 18, which CDRs are according to Kabat. In some embodiments, the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 24;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 29, which CDRs are according to the IMGT system.

In certain embodiments, the internalizing moiety comprises a homing peptide. In certain embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 1 (ENT1), ENT2, ENT3 or ENT4 transporter. In certain embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter.

In certain embodiments, the internalizing moiety is capable of binding polynucleotides. In certain embodiments, the internalizing moiety is capable of binding DNA. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 1 μM. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 100 nM.

In certain embodiments, the subject in need of treatment is a subject whose Pompe disease has been refractory to one or more previous enzyme replacement therapies.

In certain embodiments, the subject in need of treatment is a human patient with infantile Pompe disease. In certain embodiments, the subject in need of treatment is a human patient with juvenile onset or adult onset Pompe disease. In certain embodiments, the subject in need of treatment is a human patient.

In certain embodiments, an administered chimeric polypeptide is transported to one or more of cytoplasm, lysosomes, and autophagic vesicles of a cell. In certain embodiments, administering the chimeric polypeptide reduces cytoplasmic glycogen accumulation. In certain embodiments, administering the chimeric polypeptide reduces lysosomal glycogen accumulation. In certain embodiments, administering the chimeric polypeptide reduces autophagic vacuole glycogen accumulation.

In certain embodiments, the subject in need thereof is a subject diagnosed with or expected of having Pompe disease. In certain embodiments, the subject in need thereof is a subject whose disease has been refractory to one or more previous enzyme replacement therapies. In certain embodiments, the subject in need thereof is a subject having pathologic cytoplasmic glycogen accumulation prior to initiation of treatment with said chimeric polypeptide. In certain embodiments, the subject in need thereof is a subject diagnosed with Pompe disease greater than six months prior to initiation of treatment with said chimeric polypeptide. In certain embodiments, the subject in need thereof is a subject diagnosed with Pompe disease at least one year prior to initiation of treatment with said chimeric polypeptide. In certain embodiments, the subject in need thereof is a subject in whom the onset of symptoms of Pompe disease occurred greater than six months prior to initiation of treatment with said chimeric polypeptide. In certain embodiments, the subject in need thereof is a subject in whom the onset of symptoms of Pompe disease occurred at least one year prior to initiation of treatment with said chimeric polypeptide. In certain embodiments, the subject in need thereof is a subject with Pompe disease in which pathologic cytoplasmic glycogen accumulation has not yet occurred. In such cases, administration of chimeric polypeptides of the disclosure (e.g., polypeptides that can be delivered into the cytoplasm) is used to prevent pathologic cytoplasmic glycogen accumulation from occurring.

In certain embodiments, the method comprises systemically administering the chimeric polypeptide. In certain embodiments, the method comprises intravenously administering the chimeric polypeptides.

The disclosure contemplates all combinations of any of the foregoing aspects and embodiments, as well as combinations with any of the embodiments set forth in the detailed description and examples. For example, any of the chimeric polypeptides and compositions, including chimeric polypeptides and compositions having any combination of mature GAA portions and an internalizing moiety portions, can be used in any of the

methods described herein. Moreover, the disclosure contemplates that, in certain embodiments, while comprising mature GAA, chimeric polypeptides of the disclosure may also include additional contiguous GAA sequence (e.g., the GAA portion comprises a GAA polypeptide that is larger than just mature GAA, but does not include the complete amino acid sequence set forth in SEQ ID NO: 1 and, preferably, does not include a portion corresponding to residues 1-56 or 1-57 of SEQ ID NO: 1 or 2).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagram schematically depicting two different fusion constructs generated. Figure 1A is a diagram schematically depicting the full-length GAA protein and its different regions, as well as the murine heavy and light chains. Amino acid residues 1-28 correspond to the signal sequence ("SigSeq") region, amino acids 29-56 correspond to the prepro region, and amino acids 57-78 corresponds to the full linker region. Residues 1-56 are highlighted in SEQ ID NO: 1 because, in accordance with Moreland et al., this is the portion of the GAA translation product that is cleaved by a signal peptidase and protease to produce the precursor GAA polypeptie of approximately 110 kilodaltons. Figure 1B is a diagram schematically depicting the murine 3E10 Fab-GAA fusion construct, while Figure 1C is a diagram schematically depicting the murine 3E10 mAb-GAA fusion construct.

DETAILED DESCRIPTION OF THE DISCLOSURE

Glycogen is a complex polysaccharide that provides a ready store of glucose to cells in the human body. Glycogen is found principally in the liver, where it is hydrolyzed and released into the bloodstream to provide glucose to other cells, and in muscle, where the glucose resulting from glycogen hydrolysis provides energy for muscle cells. The lysosomal enzyme acid α-glucosidase (GAA) is one of the enzymes that mediates glycogen hydrolysis.

Disruption of glycogen hydrolysis, typically resulting from genetic mutations in genes associated with the process, can lead to glycogen storage disorders. In many cases, the severity of the disease symptoms correlates directly with the extent of the mutation. A debilitating glycogen storage disorder is Glycogen storage disease type II (GSDII or Pompe disease), an autosomal recessive metabolic disorder characterized by a deficiency in the lysosomal enzyme acid α-glucosidase (GAA). Over 300 variants in GAA are known, and disease phenotype depends largely on the amount of residual enzyme activity (Schoser et

al., Therapeutic approaches in Glycogen Storage Disease type II (GSDII)/Pompe disease, Neurotherapeutics, 5(4): 569-578, 2008). The most severe form of Pompe disease is an infantile onset form, in which the initial diagnosis occurs between 0-7 months of age. Infantile onset Pompe disease is characterized by hypertrophic cardiomyopathy and profound generalized weakness, and death typically occurs by 1 year of age. Late onset Pompe disease (juvenile and adult onset) is less severe, as residual GAA activity is present. Symptoms appear after 1 year of age or in adulthood, and dysfunction occurs primarily in skeletal and respiratory muscles (Case and Kishnani, Physical therapy management of Pompe disease, Genet Med 8(5): 318-327, 2006). Although juvenile and adult onset Pompe disease are less severe, muscle dysfunction leads to increased and significant disability over time. In some cases, patients become wheelchair and/or ventilator dependent.

In all forms of Pompe disease, dysfunction of GAA impairs the hydrolysis of glycogen in the lysosomes and causes toxic levels of glycogen to accumulate (Geel et al., Pompe disease: Current state of treatment modalities and animal models, *Molecular Genetics and Metabolism*, 92:299-307, 2007). Initially, lysosomes in affected cells increase in size and number. Subsequently, the lysosomes rupture and leak glycogen into the cytoplasm. In muscle fibers, high levels of glycogen accompanied by a lack of glycogen hydrolysis may lead to local starvation and to an increased autophagic response. However, the autophagic vesicles cannot fuse properly with the lysosomes. In addition, cells uptake water to counteract the high concentrations of glycogen, which leads to cell swelling. Over a relatively brief period of time, glycogen accumulates not only in lysosomes but also in the cytoplasm.

Endogenous human GAA is a 952 amino acid protein, encoded by a gene of approximately 28 kb in length. In humans, 3 transcript variants are known (NM_000152.3 which encodes NP000143.2; NM_001079803.1 which encodes NP_001073271.1; and NM_001079804.1 which encodes NP_001073272.1). However, all three transcript variants encode a protein having substantially the same amino acid sequence. Endogenously, the GAA gene encodes a 952 or 957 amino acid polypeptide which includes a signal sequence. This polypeptide is glycosylated in the endoplasmic reticulum and the Golgi apparatus, resulting in a glycosylated precursor with an apparent molecular mass of 110 kDa. There are 7 potential glycosylation sites on the immature precursor, located at residues 140, 233, 390, 470, 652, 882, and 925 of SEQ ID NOs: 1 or 2. The immature precursor is targeted to the lysosomes through mannose-6-phophate receptors (MPRs) and a mannose-6-phosphate

(M6P)-independent pathway. The 110 kDa precursor protein is cleaved to give rise to an endosomal intermediate form of GAA having a molecular weight of about 95 kDa. Subsequent N-terminal and C-terminal proteolytic cleavages generate, in the lysosome, mature, active forms of GAA having molecular weights of about 76 kDa and about 70 kDa (Moreland et al., Lysosomal Acid α-Glucosidase Consists of Four Different Peptides Processed from a Single Chain Precursor, Journal of Biological Chemistry, 280(8): 6780-6791, 2005; which is incorporated by reference in its entirety). Owing to heterogeneity in the cleavage sites, alternative starting residues and/or ending residues may define the N and C terminal boundaries of mature GAA polypeptides, such as mature GAA polypeptides for use in the claimed disclosure. For example, the N-terminal residue of a mature GAA polypeptide of about 76 kDa may, in certain embodiments, correspond to residue 122 (Met) or 123 (Gly) of SEQ ID NOs: 1 or 2, while the N-terminal residue of a mature GAA polypeptide of about 70 kDa may, in certain embodiments, correspond to any of residues 204 (Ala), 206 (Ser), or 288 (Gly) of SEQ ID NOs: 1 or 2. Polypeptides having any of the foregoing N-terminal residues may have, for example, a C-terminal residue corresponding to any of residues 816 through 881 of SEQ ID NO:1 or 2, and may be residue 782 of SEQ ID NOs: 1 or 2. Additionally, the C-terminal residue may be any of residues 782 through 816, or residues 782 through 881, inclusive. The molecular weight of the mature GAA polypeptides may be about 76 kDa or about 70 kDa, or may vary according to the foregoing alternative starting and/or ending N and C terminal residues (e.g., corresponding to portions generated due to alternative cleavage).

The FDA approved a version of GAA referred to as alglucosidase alfa (Myozyme, Genzyme Corporation), a recombinant human GAA (rhGAA) analog of the 110 kDa precursor form of GAA, produced in CHO cells. Myozyme is believed to be targeted to the endocytic/lysosomal pathway, and is thought to exert its effects in the lysosome. Myozyme does not appear to treat glycogen accumulation in cytoplasm (Schoser et al., Therapeutic approaches in Glycogen Storage Disease type II (GSDII)/Pompe disease, Neurotherapeutics, 5(4): 569-578, 2008). As noted above, this therapy is believed to target the lysosome and is based on delivery of the immature precursor form of the protein. However, the precursor form of the protein is less active than the 76 kDa mature form of the GAA (Human Molecular Genetics, 7(11): 1815-1824, 1998). Thus, in certain aspects, it may be beneficial to either (i) deliver a mature form of GAA as a chimeric polypeptide, (ii) deliver a GAA polypeptide that, although longer than the mature form is shorter than the

110 kDa precursor form as a chimeric polypeptide, and/or (iii) to deliver a GAA polypeptide with activity of any size as a chimeric polypeptide connected to an internalizing moiety to facilitate delivery of polypeptide into cells, and even into the appropriate subcellular compartment. Without being bound by theory, even if a polypeptide of the disclosure has substantially the same activity as a precursor GAA polypeptide, delivery to the proper cellular location, optionally facilitated by an internalizing moiety that promotes delivery to the cytoplasm, would increase the effective GAA activity delivered to cells. In certain embodiments, the disclosure provides a chimeric polypeptide comprising a GAA polypeptide comprising mature GAA (a GAA portion comprising mature GAA) and an internalizing moiety portion that facilitate deliver into cells. In other words, the disclosure contemplates chimeric polypeptides comprising a GAA polypeptide and an internalizing moiety.

In one aspect, the disclosure provides compositions and methods for cytoplasmic delivery of a mature GAA molecule to affected cells, for example, skeletal muscle cells. Pompe patients exhibit a buildup of glycogen not only in lysosomes but also in cytoplasm and autophagic vesicles. GAA that is targeted to the lysosomes or to endocytic vesicles may not hydrolyze cytoplasmic glycogen. For those patients who begin treatment after the disease has progressed (in some cases, this may be patients who begin treatment after 6 months with GAA dysfunction), it may be too late for lysosome-targeted forms of GAA to clear glycogen effectively from the cells. In contrast, cytoplasm-targeted mature GAA can clear glycogen from the cytoplasm. Not only is mature GAA more active than immature GAA, but mature GAA also remains active at neutral pH, showing approximately 40% activity at neutral pH relative to the acidic environment of the lysosome (Human Molecular Genetics, 11(14), 2002). In fact, although the activity of mature GAA is reduced at neutral pH relative to its activity at acidic pH, even this reduced activity is greater than that of immature GAA – even when assessed under the endogenous acidic conditions of the lysosome. In addition, not only can cytoplasmically delivered mature GAA decrease glycogen accumulation in the cytoplasm, but such GAA may also be incorporated into autophagic vesicles and lysosomes. Without being bound by theory, autophagic vesicles that ultimately fuse with lysosomes may be one of the mechanisms to help promote delivery of cytoplasmic GAA to lysosomes as well. Accordingly, chimeric polypeptides of the disclosure delivered to the cytoplasm are, at least, useful for decreasing glycogen

accumulation in cytoplasm and may also help decrease glycogen accumulation in lysosomes and autophagic vesicles.

In certain embodiments, the disclosure provides a chimeric polypeptide comprising a GAA polypeptide and an internalizing moiety, as described herein. Any such chimeric polypeptide of the disclosure can comprise any of the GAA polypeptides described herein associated with any of the internalizing moiety portions described herein, and these chimeric polypeptides can be used in any of the methods of the disclosure.

In certain embodiments, chimeric polypeptides of the disclosure comprise a mature GAA polypeptide and may also contain some additional contiguous amino acid sequence from a GAA polypeptide (but not including the 110 kD precursor polypeptide or the signal sequence of the GAA precursor polypeptide). In other embodiments, the chimeric polypeptides of the disclosure comprise a mature GAA polypeptide but do not include additional contiguous amino acid sequence from a GAA polypeptide other than the mature GAA polypeptide. Thus, the disclosure contemplates chimeric polypeptides in which the GAA portion comprises or consists of a mature GAA polypeptide. Exemplary mature GAA polypeptides having a molecular weight of 70-76 kD are described herein. In certain embodiments, the chimeric polypeptide does not include the signal sequence of the precursor GAA polypeptide. In certain embodiments, the chimeric polypeptide does not include a portion corresponding to residues 1-56 of SEQ ID NO: 1 or 2.

In certain embodiments, the disclosure provides a chimeric polypeptide comprising a GAA polypeptide and an internalizing moiety, as described herein. Any such chimeric polypeptide of the disclosure can comprise any of the GAA polypeptides described herein associated with any of the internalizing moiety portions described herein, and these chimeric polypeptides can be used in any of the methods of the disclosure.

In certain embodiments, the GAA polypeptide portion comprises the amino acid sequence of SEQ ID NO: 21 (e.g., the GAA polypeptide comprises SEQ ID NO: 21), and thus, the chimeric polypeptide comprises a mature GAA having the amino acid sequence of SEQ ID NO: 3 or 4. In certain embodiments, the chimeric polypeptide does not include additional contiguous amino acid sequence from human GAA – other than SEQ ID NO: 21. In certain embodiments, the GAA polypeptide or chimeric polypeptide does not include residues 1-56 of SEQ ID NO: 1. In certain embodiments, the GAA polypeptide or chimeric polypeptide does not include residues 1-60 of SEQ D NO: 1. In certain embodiments, the GAA polypeptide portion comprises the amino acid sequence of SEQ ID NO: 22 (e.g., the

GAA polypeptide comprises SEQ ID NO: 22), and thus, the chimeric polypeptide comprises a mature GAA having the amino acid sequence of SEQ ID NO: 3 or 4. In certain embodiments, the chimeric polypeptide does not include additional contiguous amino acid sequence from human GAA – other than SEQ ID NO: 22. In certain embodiments, the GAA polypeptide or chimeric polypeptide does not include residues 1-66 of SEQ ID NO: 1. In certain embodiments, the GAA polypeptide portion comprises the amino acid sequence of SEQ ID NO: 23 (e.g., the GAA polypeptide comprises SEQ ID NO: 23), and thus, the chimeric polypeptide comprises a mature GAA having the amino acid sequence of SEQ ID NO: 3 or 4. In certain embodiments, the chimeric polypeptide does not include additional contiguous amino acid sequence from human GAA – other than SEQ ID NO: 23. In certain embodiments, the GAA polypeptide or chimeric polypeptide does not include residues 1-69 of SEQ ID NO: 1.

Thus, in certain aspects, the disclosure provides chimeric polypeptides comprising a mature acid alpha-glucosidase (GAA) polypeptide that may be used to treat symptoms associated with Pompe disease.

In certain embodiments, the disclosure provides a chimeric polypeptide comprising (i) a mature GAA polypeptide; and (ii) an internalizing moiety that promotes delivery into cells, such as into cytoplasm of cells. In a particular embodiment, the internalizing moiety helps target delivery of the chimeric polypeptide to muscle cells, such as skeletal muscle cells.

I. GAA polypeptides

It has been demonstrated that mature GAA polypeptides have enhanced glycogen clearance as compared to the full length, precursor GAA (Bijvoet, et al., 1998, Hum Mol Genet, 7(11): 1815-24), whether at low pH (*i.e.*, the pH of the lysosome or autophagic vacuole) or neutral pH (*i.e.*, the pH of the cytoplasm) conditions. In addition, while mature GAA is a lysosomal protein that has optimal activity at lower pHs, mature GAA retains approximately 40% activity at neutral pH (*i.e.*, the pH of the cytoplasm) (Martin-Touaux et al., 2002, Hum Mol Genet, 11(14): 1637-45). Accordingly, a GAA polypeptide comprising mature GAA is suitable for cytoplasmic delivery, and thus, suitable to address an unaddressed issue of Pompe disease: cytoplasmic glycogen accumulation. However, regardless of whether the GAA portion of a chimeric polypeptide comprises or consists of mature HAA, providing the GAA polypeptide in association with an internalizing moiety of

the disclosure facilitates delivery into cells and, in certain embodiments, delivery to cytoplasm.

As used herein, the mature GAA polypeptides include variants, and in particular the mature, active forms of the protein (the active about 76 kDa or about 70 kDa forms or similar forms having an alternative starting and/or ending residue, collectively termed "mature GAA"). The term "mature GAA" refers to a polypeptide having an amino acid sequence corresponding to that portion of the immature GAA protein that, when processed endogenously, has an apparent molecular weight by SDS-PAGE of about 70 kDa to about 76 kDa, as well as similar polypeptides having alternative starting and/or ending residues, as described above. In some embodiments, the GAA polypeptide lacks the signal sequence (amino acids 1-27 of SEQ ID NOs: 1 or 2 or the sequence designated by amino acids 1-56 of SEQ ID NO: 1-56). Exemplary mature GAA polypeptides include polypeptides having residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2; or residues 204-782 of SEQ ID NOs: 1 or 2. The term "mature GAA" includes polypeptides that are glycosylated in the same or substantially the same way as the endogenous, mature proteins, and thus have a molecular weight that is the same or similar to the predicted molecular weight. The term also includes polypeptides that are not glycosylated or are hyper-glycosylated, such that their apparent molecular weight differ despite including the same primary amino acid sequence. Any such variants or isoforms, functional fragments or variants, fusion proteins, and modified forms of the mature GAA polypeptides have at least a portion of the amino acid sequence of substantial sequence identity to the native mature GAA protein, and retain enzymatic activity. In certain embodiments, a functional fragment, variant, or fusion protein of a mature GAA polypeptide comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to mature GAA polypeptides set forth in one or both of SEQ ID NOs: 3 and 4, or is at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to mature GAA polypeptides corresponding to one or more of: residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2; or residues 204-782 of SEQ ID NOs: 1 or 2. In some embodiments, the GAA polypeptide is a GAA polypeptide from a non-human species, *e.g.*, mouse, rat, dog, zebrafish, pig, goat, cow, horse, monkey or ape. In some embodiments, the GAA protein comprises the mature form, but not the full-length form, of a bovine GAA protein having the amino acid sequence of SEQ ID NO: 32.

Here and elsewhere in the specification, sequence identity refers to the percentage of residues in the candidate sequence that are identical with the residue of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology.

Methods and computer programs for the alignment of sequences and the calculation of percent identity are well known in the art and readily available. Sequence identity may be measured using sequence analysis software. For example, alignment and analysis tools available through the ExPasy bioinformatics resource portal, such as ClustalW algorithm, set to default parameters. Suitable sequence alignments and comparisons based on pair-wise or global alignment can be readily selected. One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J Mol Biol 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). In certain embodiments, the now current default settings for a particular program are used for aligning sequences and calculating percent identity.

In certain specific embodiments, the chimeric polypeptide comprises a mature GAA polypeptide, such as a GAA polypeptide comprising mature GAA. The mature GAA has an activity that is similar to or substantially equivalent to the activity of endogenous forms of human GAA that are about 76 kDa or about 70 kDa. For example, the mature GAA may be 7-10 fold more active for glycogen hydrolysis than the 110 kDa precursor form, with the comparison being made under the same or similar conditions (e.g. the mature GAA-chimeric polypeptides disclosed herein as compared with endogenous human immature precursor GAA under acidic or neutral pH conditions) The mature GAA polypeptide may be the 76 kDa or the 70 kDa form of GAA, or similar forms that use alternative starting and/or ending residues. As noted in Moreland et al. (Lysosomal Acid α-Glucosidase Consists of Four Different Peptides Processsed from a Single Chain Precursor, Journal of Biological Chemistry, 280(8): 6780-6791, 2005), the nomenclature used for the processed forms of GAA is based on an apparent molecular mass as determined by SDS-PAGE. In some embodiments, mature GAA may lack the N-terminal sites that are normally glycosylated in the endoplasmic reticulum. An exemplary mature GAA polypeptide

comprises SEQ ID NO: 3 or SEQ ID NO:4. Further exemplary mature GAA polypeptide may comprise or consist of an amino acid sequence corresponding to about: residues 122-782 of SEQ ID NOs: 1 or 2; residues 123-782 of SEQ ID NOs: 1 or 2, such as shown in SEQ ID NO: 3; residues 204-782 of SEQ ID NOs: 1 or 2; residues 206-782 of SEQ ID NOs: 1 or 2; residues 288-782 of SEQ ID NOs: 1 or 2, as shown in SEQ ID NO: 4. Mature GAA polypeptides may also have the N-terminal and or C-terminal residues described above.

In certain embodiments, the chimeric polypeptide does not comprise a full-length GAA polypeptide, but comprises a mature GAA polypeptide and at least a portion of the full-length GAA polypeptide. In other words, in certain embodiments, the chimeric polypeptide comprises a GAA polypeptide and an internalizing moiety. In some embodiments, the chimeric polypeptide does not comprise a full-length GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or 2, but comprises a mature GAA polypeptide sequence comprising the amino acid sequences of SEQ ID NOs: 3 or 4 and at least a portion of the amino acids corresponding to amino acids 1-121 of SEQ ID NOs: 1-2 and/or at least a portion of the amino acids corresponding to amino acids 783-952 of SEQ ID NO: 1. In some embodiments, the chimeric polypeptide does not comprise a full-length GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or 2, but comprises a mature GAA polypeptide sequence comprising the amino acid sequences of SEQ ID NOs: 3 or 4 and at least a portion of the amino acids corresponding to amino acids 783-952 of SEQ ID NO: 1. In some embodiments, the chimeric polypeptide does not comprise a full-length GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or 2, but comprises a mature GAA polypeptide sequence comprising the amino acid sequences of SEQ ID NOs: 3 or 4 and at least a portion of the amino acids corresponding to amino acids 783-957 of SEQ ID NO: 2. These are exemplary of GAA polypeptides.

In certain embodiments, the GAA polypeptide portion (e.g., the portion comprising a GAA polypeptide comprising mature GAA; e.g., a GAA polypeptide) of the chimeric proteins described herein comprise a mature form of GAA but does not comprise a GAA translation product set forth in SEQ ID NO: 1. In some embodiments, neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-27 or 1-56 of SEQ ID NO: 1 or 2. In some embodiments, the GAA polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to amino acids 57-78 of SEQ ID NOs: 1 or 2

(*i.e.*, SEQ ID NO: 31). In some embodiments, the GAA polypeptide does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-60, 1-61, 1-62, 1-63, 1-64, 1-65, 1-66, 1-67, 1-68, 1-69, 1-70, 1-71, 1-72, 1-73, 1-74, 1-75, 1-80, 1-85, 1-90, 1-95, 1-100, 1-105, 1-110, 1-115, 1-120 or 1-121 of SEQ ID NOs: 1 or 2. In particular embodiments, the GAA polypeptide does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-60 of SEQ ID NOs: 1 or 2 (*e.g.*, the chimeric polypeptide comprises a GAA polypeptide portion comprising a GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 21). In other embodiments, the GAA portion does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-66 of SEQ ID NO: 1 or 2 (*e.g.*, the chimeric polypeptide comprises a GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 22). In some embodiments, the GAA portion does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-69 of SEQ ID NO: 1 or 2 (*e.g.*, the chimeric polypeptide comprises a GAA polypeptide portion comprising a GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 23).

In other embodiments, the mature GAA polypeptides may be glycosylated, or may be not glycosylated. For those mature GAA polypeptides that are glycosylated, the glycosylation pattern may be the same as that of naturally-occurring human GAA or may be different. One or more of the glycosylation sites on the precursor GAA protein may be removed in the final mature GAA construct.

Mature GAA has been isolated from tissues such as bovine testes, rat liver, pig liver, human liver, rabbit muscle, human heart, human urine, and human placenta. Mature GAA may also be produced using recombinant techniques, for example by transfecting Chinese hamster ovary (CHO) cells with a vector that expresses full-length human GAA or a vector that expresses mature GAA. Recombinant human GAA (rhGAA) or mature GAA is then purified from CHO-conditioned medium, using a series of ultrafiltration, diafiltration, washing, and eluting steps, as described by Moreland et al. (Lysosomal Acid α-Glucosidase Consists of Four Different Peptides Processsed from a Single Chain Precursor, Journal of Biological Chemistry, 280(8): 6780-6791, 2005). Mature GAA fragments may be separated according to methods known in the art, such as affinity chromatography and SDS page.

In certain embodiments, mature GAA, or fragments or variants are human mature GAA.

In certain embodiments, fragments or variants of the mature GAA polypeptides can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding a mature GAA polypeptide. In addition, fragments or variants can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments or variants can be produced (recombinantly or by chemical synthesis) and tested to identify those fragments or variants that can function as a native GAA protein, for example, by testing their ability hydrolyze glycogen and/or treat symptoms of Pompe disease.

In certain embodiments, the present disclosure contemplates modifying the structure of a mature GAA polypeptide for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified mature GAA polypeptides are considered functional equivalents of the naturally-occurring GAA polypeptide. Modified polypeptides can be produced, for instance, by amino acid substitution, deletion, or addition. For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the GAA biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains.

This disclosure further contemplates generating sets of combinatorial mutants of an mature GAA polypeptide, as well as truncation mutants, and is especially useful for identifying functional variant sequences. Combinatorially-derived variants can be generated which have a selective potency relative to a naturally occurring GAA polypeptide. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding wild-type GAA polypeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of GAA function. Such variants can be utilized to alter the mature GAA polypeptide level by modulating their half-life. There are many ways by which the library of potential mature GAA variants sequences can be generated, for example, from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated

into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential polypeptide sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al., (1981) Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acid Res. 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, mature GAA polypeptide variants can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) Biochemistry 33:1565-1572; Wang et al., (1994) J. Biol. Chem. 269:3095-3099; Balint et al., (1993) Gene 137:109-118; Grodberg et al., (1993) Eur. J. Biochem. 218:597-601; Nagashima et al., (1993) J. Biol. Chem. 268:2888-2892; Lowman et al., (1991) Biochemistry 30:10832-10838; and Cunningham et al., (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by saturation mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of mature GAA.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of the mature GAA polypeptides. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library

into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In certain embodiments, a mature GAA polypeptide may include a peptide and a peptidomimetic. As used herein, the term "peptidomimetic" includes chemically modified peptides and peptide-like molecules that contain non-naturally occurring amino acids, peptoids, and the like. Peptidomimetics provide various advantages over a peptide, including enhanced stability when administered to a subject. Methods for identifying a peptidomimetic are well known in the art and include the screening of databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known crystal structures (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). Where no crystal structure of a target molecule is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems; San Leandro Calif.), contains about 100,000 compounds that are commercially available and also can be searched to identify potential peptidomimetics of the mature GAA polypeptides.

In certain embodiments, a mature GAA polypeptide may further comprise post-translational modifications. Exemplary post-translational protein modification include phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination, glycosylation, carbonylation, sumoylation, biotinylation or addition of a polypeptide side chain or of a hydrophobic group. As a result, the modified mature GAA polypeptides may contain non-amino acid elements, such as lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of a mature GAA polypeptide may be tested for its biological activity, for example, its ability to treat Pompe disease. In certain embodiments, the mature GAA polypeptide may further comprise one or more polypeptide portions that enhance one or more of in vivo stability, in vivo half life, uptake/administration, and/or purification. In other embodiments, the internalizing moiety comprises an antibody or an antigen-binding fragment thereof.

In one specific embodiment of the present disclosure, a mature GAA polypeptide may be modified with nonproteinaceous polymers. In one specific embodiment, the polymer is polyethylene glycol ("PEG"), polypropylene glycol, or polyoxyalkylenes, in the manner as set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. PEG is a well-known, water soluble polymer that is commercially available or can be prepared by ring-opening polymerization of ethylene glycol according to methods well known in the art (Sandler and Karo, Polymer Synthesis, Academic Press, New York, Vol. 3, pages 138-161).

By the terms "biological activity", "bioactivity" or "functional" is meant the ability of the mature GAA protein to carry out the functions associated with wildtype GAA proteins, for example, the hydrolysis of α-1,4- and α-1,6-glycosidic linkages of glycogen, for example lysosomal glycogen. The terms "biological activity", "bioactivity", and "functional" are used interchangeably herein. In certain embodiments, and as described herein, a mature GAA protein or chimeric polypeptide having biological activity has the ability to hydrolyze glycogen. In other embodiments, a mature GAA protein or chimeric polypeptide having biological activity has the ability to lower the concentration of lysosomal and/or cytoplasmic glycogen. In still other embodiments, a mature GAA protein or chimeric polypeptide has the ability to treat symptoms associated with Pompe disease. As used herein, "fragments" are understood to include bioactive fragments (also referred to as functional fragments) or bioactive variants that exhibit "bioactivity" as described herein. That is, bioactive fragments or variants of mature GAA exhibit bioactivity that can be measured and tested. For example, bioactive fragments/functional fragments or variants exhibit the same or substantially the same bioactivity as native (i.e., wild-type, or normal) GAA protein, and such bioactivity can be assessed by the ability of the fragment or variant to, e.g., hydrolyze glycogen in vitro or in vivo. As used herein, "substantially the same" refers to any parameter (e.g., activity) that is at least 70% of a control against which the parameter is measured. In certain embodiments, "substantially the same" also refers to any parameter (e.g., activity) that is at least 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, 99%, 100%, 102%, 105%, or 110% of a control against which the parameter is measured, when assessed under the same or substantially the same conditions. In certain embodiments, fragments or variants of the mature GAA polypeptide will preferably retain at least 50%, 60%, 70%, 80%, 85%, 90%, 95% or 100% of the GAA biological activity associated with the native GAA polypeptide, when assessed under the same or substantially the same

conditions. In certain embodiments, fragments or variants of the mature GAA polypeptide have a half-life ($t_{1/2}$) which is enhanced relative to the half-life of the native protein. Preferably, the half-life of mature GAA fragments or variants is enhanced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 400% or 500%, or even by 1000% relative to the half-life of the native GAA protein, when assessed under the same or substantially the same conditions. In some embodiments, the protein half-life is determined in vitro, such as in a buffered saline solution or in serum. In other embodiments, the protein half-life is an *in vivo* half life, such as the half-life of the protein in the serum or other bodily fluid of an animal. In addition, fragments or variants can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments or variants can be produced (recombinantly or by chemical synthesis) and tested to identify those fragments or variants that can function as well as or substantially similarly to a native GAA protein.

With respect to methods of increasing GAA bioactivity in cells, the disclosure contemplates all combinations of any of the foregoing aspects and embodiments, as well as combinations with any of the embodiments set forth in the detailed description and examples. The described methods based on administering chimeric polypeptides or contacting cells with chimeric polypeptides can be performed in vitro (e.g., in cells or culture) or in vivo (e.g., in a patient or animal model). In certain embodiments, the method is an in vitro method. In certain embodiments, the method is an in vivo method.

In some aspects, the present disclosure also provides a method of producing any of the foregoing chimeric polypeptides as described herein. Further, the present disclosure contemplates any number of combinations of the foregoing methods and compositions.

In certain aspects, a mature GAA polypeptide may be a fusion protein which further comprises one or more fusion domains. Well-known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, and an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), which are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt-conjugated resins are used. Fusion domains also include "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG,

influenza virus haemagglutinin (HA), His, and c-myc tags. An exemplary His tag has the sequence HHHHHH (SEQ ID NO: 7), and an exemplary c-myc tag has the sequence EQKLISEEDL (SEQ ID NO: 8). It is recognized that any such tags or fusions may be appended to the mature GAA portion of the chimeric polypeptide or may be appended to the internalizing moiety portion of the chimeric polypeptide, or both. In certain embodiments, the chimeric polypeptides comprise a "AGIH" portion (SEQ ID NO: 19) on the N-terminus (or within 10 amino acid residues of the N-terminus) of the chimeric polypeptide, and such chimeric polypeptides may be provided in the presence or absence of one or more epitope tags. In further embodiments, the chimeric polypeptide comprises a serine at the N-terminal most position of the polypeptide. In some embodiments, the chimeric polypeptides comprise an "SAGIH" (SEQ ID NO: 20) portion at the N-terminus (or within 10 amino acid residues of the N-terminus) of the polypeptide, and such chimeric polypeptides may be provided in the presence or absence of one or more epitope tags.

In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain embodiments, the mature GAA polypeptides may contain one or more modifications that are capable of stabilizing the polypeptides. For example, such modifications enhance the in vitro half life of the polypeptides, enhance circulatory half life of the polypeptides or reducing proteolytic degradation of the polypeptides.

In some embodiments, a mature GAA polypeptide may be a fusion protein with an Fc region of an immunoglobulin. As is known, each immunoglobulin heavy chain constant region comprises four or five domains. The domains are named sequentially as follows: CH1-hinge-CH2-CH3(-CH4). The DNA sequences of the heavy chain domains have cross-homology among the immunoglobulin classes, e.g., the CH2 domain of IgG is homologous to the CH2 domain of IgA and IgD, and to the CH3 domain of IgM and IgE. As used herein, the term, "immunoglobulin Fc region" is understood to mean the carboxyl-terminal portion of an immunoglobulin chain constant region, preferably an immunoglobulin heavy chain constant region, or a portion thereof. For example, an immunoglobulin Fc region may comprise 1) a CH1 domain, a CH2 domain, and a CH3 domain, 2) a CH1 domain and a CH2 domain, 3) a CH1 domain and a CH3 domain, 4) a CH2 domain and a CH3 domain, or 5) a combination of two or more domains and an immunoglobulin hinge region. In a

preferred embodiment the immunoglobulin Fc region comprises at least an immunoglobulin hinge region a CH2 domain and a CH3 domain, and preferably lacks the CH1 domain. In one embodiment, the class of immunoglobulin from which the heavy chain constant region is derived is IgG (Igγ) (γ subclasses 1, 2, 3, or 4). Other classes of immunoglobulin, IgA (Igα), IgD (Igδ), IgE (Igε) and IgM (Igμ), may be used. The choice of appropriate immunoglobulin heavy chain constant regions is discussed in detail in U.S. Pat. Nos. 5,541,087, and 5,726,044. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of skill in the art. The portion of the DNA construct encoding the immunoglobulin Fc region preferably comprises at least a portion of a hinge domain, and preferably at least a portion of a CH_3 domain of Fc γ or the homologous domains in any of IgA, IgD, IgE, or IgM. Furthermore, it is contemplated that substitution or deletion of amino acids within the immunoglobulin heavy chain constant regions may be useful in the practice of the disclosure. One example would be to introduce amino acid substitutions in the upper CH2 region to create a Fc variant with reduced affinity for Fc receptors (Cole et al. (1997) J. IMMUNOL. 159:3613). One of ordinary skill in the art can prepare such constructs using well known molecular biology techniques.

In certain embodiments of any of the foregoing, the GAA portion of the chimeric protein comprises one of the mature forms of GAA, e.g., the 76 kDa fragment, the 70 kDa fragment, similar forms that use an alternative start and/or stop site, or a functional fragment thereof. In certain embodiments, such mature GAA polypeptide or functional fragment thereof retains the ability of to hydrolyze glycogen, as evaluated in vitro or in vivo. Further, in certain embodiments, the chimeric polypeptide that comprises such a mature GAA polypeptide or functional fragment thereof can hydrolyze glycogen. Exemplary bioactive fragments comprise at least 50, at least 60, at least 75, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 230, at least 250, at least 260, at least 275, or at least 300 consecutive amino acid residues of a full length mature GAA polypeptide.

In certain embodiments, the GAA polypeptide portion of the chimeric proteins described herein comprise a mature form of GAA but does not comprise a GAA polypeptide set forth in SEQ ID NO: 1. In some embodiments, the GAA polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to amino acids 57-78 of SEQ ID NOs: 1 or 2 (*i.e.*, SEQ ID NO: 31). In some embodiments,

the GAA polypeptide does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-60, 1-61, 1-62, 1-63, 1-64, 1-65, 1-66, 1-67, 1-68, 1-69, 1-70, 1-71, 1-72, 1-73, 1-74, 1-75, 1-80, 1-85, 1-90, 1-95, 1-100, 1-105, 1-110, 1-115, 1-120 or 1-121 of SEQ ID NOs: 1 or 2. In particular embodiments, the GAA polypeptide does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-60 of SEQ ID NOs: 1 or 2 (*e.g.*, the chimeric polypeptide comprises a GAA polypeptide portion comprising a GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 21). In other embodiments, the GAA portion does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-66 of SEQ ID NO: 1 or 2 (*e.g.*, the chimeric polypeptide comprises a GAA polypeptide portion comprising a GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 22). In some embodiments, the GAA portion does not comprise a contiguous amino acid sequence corresponding to the amino acids 1-69 of SEQ ID NO: 1 or 2 (*e.g.*, the chimeric polypeptide comprises a GAA polypeptide portion comprising a GAA polypeptide comprising the amino acid sequence of SEQ ID NO: 23).

In certain embodiments, the disclosure contemplates chimeric proteins where the mature GAA portion is a variant of any of the foregoing mature GAA polypeptides or functional fragments. Exemplary variants have an amino acid sequence at least 90%, 92%, 95%, 96%, 97%, 98%, or at least 99% identical to the amino acid sequence of a native GAA polypeptide or bioactive fragment thereof, and such variants retain the ability of native GAA to hydrolyze glycogen, as evaluated in vitro or in vivo. The disclosure contemplates chimeric proteins and the use of such proteins wherein the GAA portion comprises any of the mature GAA polypeptides, forms, or variants described herein in combination with any internalizing moiety described herein. Exemplary mature GAA polypeptides are set forth in SEQ ID NOs: 3 and 4. Moreover, in certain embodiments, the mature GAA portion of any of the foregoing chimeric polypeptides may, in certain embodiments, by a fusion protein. Any such chimeric polypeptides comprising any combination of GAA portions and internalizing moiety portions, and optionally including one or more linkers, one or more tags, etc., may be used in any of the methods of the disclosure.

II. InternalizingMoieties

As used herein, the term "internalizing moiety" refers to a moiety capable of interacting with a target tissue or a cell type to effect delivery of the attached molecule into the cell (i.e., penetrate desired cell; transport across a cellular membrane; deliver across cellular membranes to, at least, the cytoplasm). Preferably, this disclosure relates to an internalizing moiety which promotes delivery to, for example, muscle cells and liver cells. Internalizing moieties having limited cross-reactivity are generally preferred. In certain embodiments, this disclosure relates to an internalizing moiety which selectively, although not necessarily exclusively, targets and penetrates muscle cells. In certain embodiments, the internalizing moiety has limited cross-reactivity, and thus preferentially targets a particular cell or tissue type. However, it should be understood that internalizing moieties of the subject disclosure do not exclusively target specific cell types. Rather, the internalizing moieties promote delivery to one or more particular cell types, preferentially over other cell types, and thus provide for delivery that is not ubiquitous. In certain embodiments, suitable internalizing moieties include, for example, antibodies, monoclonal antibodies, or derivatives or analogs thereof. Other internalizing moieties include for example, homing peptides, fusion proteins, receptors, ligands, aptamers, peptidomimetics, and any member of a specific binding pair. In certain embodiments, the internalizing moiety mediates transit across cellular membranes via an ENT2 transporter. In some embodiments, the internalizing moiety helps the chimeric polypeptide effectively and efficiently transit cellular membranes. In some embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside (ENT) transporter. In some embodiments, the internalizing moiety transits cellular membranes via an ENT1, ENT2, ENT3 or ENT4 transporter. In some embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter. In some embodiments, the internalizing moiety promotes delivery into muscle cells (*e.g.*, skeletal or cardiac muscle). In other embodiments, the internalizing moiety promotes delivery into cells other than muscle cells, e.g., neurons, epithelial cells, liver cells, kidney cells or Leydig cells. For any of the foregoing, in certain embodiments, the internalizing moiety promotes delivery of a chimeric polypeptide into the cytoplasm.

In certain embodiments, the internalizing moiety promotes delivery of a chimeric polypeptide into the cytoplasm. Without being bound by theory, regardless of whether the GAA polypeptide portion of the chimeric polypeptide comprises or consists of mature

GAA, this facilitates delivery to the cytoplasm and, optionally, to the lysosome and/or autophagic vesicles.

In certain embodiments, the internalizing moiety is capable of binding polynucleotides. In certain embodiments, the internalizing moiety is capable of binding DNA. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 1 μM. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 100 nM, less than 75nM, less than 50nM, or even less than 30nM. K_D can be measured using Surface Plasmon Resonance (SPR) or Quartz Crystal Microbalance (QCM), in accordance with currently standard methods. By way of example, a 3E10 antibody or antibody fragment, including an antibody or antibody fragment comprising a VH having the amino acid sequence set forth in SEQ ID NO: 9 and a VL having an amino acid sequence set forth in SEQ ID NO: 10) is know to bind DNA with a K_D of less than 100nM.

In some embodiments, the internalizing moiety targets a mature GAA polypeptide to muscle cells, and mediates transit of the polypeptide across the cellular membrane into the cytoplasm of the muscle cells.

As used herein, the term "internalizing moiety" refers to a moiety capable of interacting with a target tissue or a cell type. Preferably, this disclosure relates to an internalizing moiety which promotes delivery to, for example, muscle cells and liver cells. Internalizing moieties having limited cross-reactivity are generally preferred. However, it should be understood that internalizing moieties of the subject disclosure do not exclusively target specific cell types. Rather, the internalizing moieties promote delivery to one or more particular cell types, preferentially over other cell types, and thus provide for delivery that is not ubiquitous. In certain embodiments, suitable internalizing moieties include, for example, antibodies, monoclonal antibodies, or derivatives or analogs thereof; and other internalizing moieties include for example, homing peptides, fusion proteins, receptors, ligands, aptamers, peptidomimetics, and any member of a specific binding pair. In some embodiments, the internalizing moiety helps the chimeric polypeptide effectively and efficiently transit cellular membranes. In some embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside (ENT) transporter. In some embodiments, the internalizing moiety transits cellular membranes via an ENT1, ENT2, ENT3 or ENT4 transporter. In some embodiments, the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter. In some

embodiments, the internalizing moiety promotes delivery into muscle cells (*e.g.*, skeletal or cardiac muscle). In other embodiments, the internalizing moiety promotes delivery into cells other than muscle cells, e.g., neurons, epithelial cells, liver cells, kidney cells or Leydig cells.

(a) Antibodies

In certain aspects, an internalizing moiety may comprise an antibody, including a monoclonal antibody, a polyclonal antibody, and a humanized antibody. Without being bound by theory, such antibody may bind to an antigen of a target tissue and thus mediate the delivery of the subject chimeric polypeptide to the target tissue (e.g., muscle). In some embodiments, internalizing moieties may comprise antibody fragments, derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, human antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent internalizing moieties including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, human antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent internalizing moieties including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ($(scFv)_2$ fragments), diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized (*i.e.*, leucine zipper or helix stabilized) scFv fragments; receptor molecules which naturally interact with a desired target molecule. In some embodiments, the antibodies or variants thereof may be chimeric, *e.g.*, they may include variable heavy or light regions from the murine 3E10 antibody, but may include constant regions from an antibody of another species (e.g,, a human). In some embodiments, the antibodies or variants thereof may comprise a constant region that is a hybrid of several different antibody subclass constant domains (*e.g.*, any combination of IgG1, IgG2a, IgG2b, IgG3 and IgG4).

In certain embodiments, the antibodies or variants thereof, may be modified to make them less immunogenic when administered to a subject. For example, if the subject is human, the antibody may be "humanized"; where the complementarity determining region(s) of the hybridoma-derived antibody has been transplanted into a human monoclonal antibody, for example as described in Jones, P. et al. (1986), *Nature,* 321, 522-

525 or Tempest et al. (1991), *Biotechnology,* 9, 266-273. The term humanization and humanized is well understood in the art when referring to antibodies. In some embodiments, the internalizing moiety is any peptide or antibody-like protein having the complementarity determining regions (CDRs) of the 3E10 antibody sequence, or of an antibody that binds the same epitope (e.g., the same target, such as DNA) as 3E10. Also, transgenic mice, or other mammals, may be used to express humanized or human antibodies. Such humanization may be partial or complete.

In certain embodiments, the internalizing moiety comprises the monoclonal antibody 3E10 or an antigen binding fragment thereof. For example, the antibody or antigen binding fragment thereof may be monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or an antigen binding fragment of 3E10 or said 3E10 variant. Additionally, the antibody or antigen binding fragment thereof may be an antibody that binds to the same epitope (e.g., target, such as DNA) as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10, or an antigen binding fragment thereof. These are exemplary of agents that target ENT2. In certain embodiments, the internalizing moiety is capable of binding polynucleotides. In certain embodiments, the internalizing moiety is capable of binding DNA. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 1 μM. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 100 nM, less than 75 nM, less than 50 nM, or even less than 30 nM. K_D is determined using SPR or QCM, according to manufacturer's instructions and current practice.

In certain embodiments, the antigen binding fragment is an Fv or scFv fragment thereof. Monoclonal antibody 3E10 can be produced by a hybridoma 3E10 placed permanently on deposit with the American Type Culture Collection (ATCC) under ATCC accession number PTA-2439 and is disclosed in US Patent No. 7,189,396. Additionally or alternatively, the 3E10 antibody can be produced by expressing in a host cell nucleotide sequences encoding the heavy and light chains of the 3E10 antibody. The term "3E10 antibody" or "monoclonal antibody 3E10" are used to refer to the antibody, regardless of the method used to produce the antibody. Similarly, when referring to variants or antigen-binding fragments of 3E10, such terms are used without reference to the manner in which the antibody was produced. At this point, 3E10 is generally not produced by the hybridoma but is produced recombinantly. Thus, in the context of the present application, 3E10 antibody will refer to an antibody having the sequence of the hybridoma or comprising a

variable heavy chain domain comprising the amino acid sequence set forth in SEQ ID NO: 9 (which has a one amino acid substitution relative to that of the 3E10 antibody deposited with the ATCC, as described herein) and the variable light chain domain comprising the amino acid sequence set forth in SEQ ID NO: 10.

The internalizing moiety may also comprise variants of mAb 3E10, such as variants of 3E10 which retain the same cell penetration characteristics as mAb 3E10, as well as variants modified by mutation to improve the utility thereof (e.g., improved ability to target specific cell types, improved ability to penetrate the cell membrane, improved ability to localize to the cellular DNA, convenient site for conjugation, and the like). Such variants include variants wherein one or more conservative substitutions are introduced into the heavy chain, the light chain and/or the constant region(s) of the antibody. Such variants include humanized versions of 3E10 or a 3E10 variant. In some embodiments, the light chain or heavy chain may be modified at the N-terminus or C-terminus. Similarly, the foregoing description of variants applies to antigen binding fragments. Any of these antibodies, variants, or fragments may be made recombinantly by expression of the nucleotide sequence(s) in a host cell.

Monoclonal antibody 3E10 has been shown to penetrate cells to deliver proteins and nucleic acids into the cytoplasmic or nuclear spaces of target tissues (Weisbart RH et al., J Autoimmun. 1998 Oct;11(5):539-46; Weisbart RH, et al. Mol Immunol. 2003 Mar;39(13):783-9; Zack DJ et al., J Immunol. 1996 Sep 1;157(5):2082-8.). Further, the VH and Vk sequences of 3E10 are highly homologous to human antibodies, with respective humanness z-scores of 0.943 and -0.880. Thus, Fv3E10 is expected to induce less of an anti-antibody response than many other approved humanized antibodies (Abhinandan KR et al., Mol. Biol. 2007 369, 852–862). A single chain Fv fragment of 3E10 possesses all the cell penetrating capabilities of the original monoclonal antibody, and proteins such as catalase, dystrophin, HSP70 and p53 retain their activity following conjugation to Fv3E10 (Hansen JE et al., Brain Res. 2006 May 9;1088(1):187-96; Weisbart RH et al., Cancer Lett. 2003 Jun 10;195(2):211-9; Weisbart RH et al., J Drug Target. 2005 Feb;13(2):81-7; Weisbart RH et al., J Immunol. 2000 Jun 1;164(11):6020-6; Hansen JE et al., J Biol Chem. 2007 Jul 20;282(29):20790-3). The 3E10 is built on the antibody scaffold present in all mammals; a mouse variable heavy chain and variable kappa light chain. 3E10 gains entry to cells via the ENT2 nucleotide transporter that is particularly enriched in skeletal muscle and cancer cells, and in vitro studies have shown that 3E10 is nontoxic. (Weisbart RH et al.,

Mol Immunol. 2003 Mar;39(13):783-9; Pennycooke M et al., Biochem Biophys Res Commun. 2001 Jan 26;280(3):951-9).

The internalizing moiety may also include mutants of mAb 3E10, such as variants of 3E10 which retain the same or substantially the same cell penetration characteristics as mAb 3E10, as well as variants modified by mutation to improve the utility thereof (e.g., improved ability to target specific cell types, improved ability to penetrate the cell membrane, improved ability to localize to the cellular DNA, improved binding affinity, and the like). Such mutants include variants wherein one or more conservative substitutions are introduced into the heavy chain, the light chain and/or the constant region(s) of the antibody. Numerous variants of mAb 3E10 have been characterized in, e.g., US Patent 7,189,396 and WO 2008/091911, the teachings of which are incorporated by reference herein in their entirety.

In certain embodiments, the internalizing moiety comprises an antibody or antigen binding fragment comprising an VH domain comprising an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 99%, or 100% identical to SEQ ID NO: 9 and/or a VL domain comprising an amino acid sequence at least 85%, 90%, 95%, 96%, 97%, 99%, or 100% identical to SEQ ID NO: 10, or a humanized variant thereof. Of course, such internalizing moieties transit cells via ENT2 and/or bind the same epitope (e.g., target, such as DNA) as 3E10.

In certain embodiments, the internalizing moiety is capable of binding polynucleotides. In certain embodiments, the internalizing moiety is capable of binding DNA. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 1 μM. In certain embodiments, the internalizing moiety is capable of binding DNA with a K_D of less than 100 nM.

In certain embodiments, the internalizing moiety is an antigen binding fragment, such as a single chain Fv of 3E10 (scFv) comprising SEQ ID NOs: 9 and 10. In certain embodiments, the internalizing moiety comprises a single chain Fv of 3E10 (or another antigen binding fragment), and the amino acid sequence of the V_H domain is at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 9, and amino acid sequence of the V_L domain is at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 10. The variant 3E10 or fragment thereof retains the function of an internalizing moiety. When the internalizing moiety is an scFv, the VH and VL domains are typically

connected via a linker, such as a gly/ser linker. The VH domain may be N-terminal to the VL domain or vice versa.

In some embodiments, the internalizing moiety comprises one or more of the CDRs of the 3E10 antibody. In certain embodiments, the internalizing moiety comprises one or more of the CDRs of a 3E10 antibody comprising the amino acid sequence of a V_H domain that is identical to SEQ ID NO: 9 and the amino acid sequence of a V_L domain that is identical to SEQ ID NO: 10. The CDRs of the 3E10 antibody may be determined using any of the CDR identification schemes available in the art. For example, in some embodiments, the CDRs of the 3E10 antibody are defined according to the Kabat definition as set forth in Kabat et al. Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). In other embodiments, the CDRs of the 3E10 antibody are defined according to Chothia et al., 1987, J Mol Biol. 196: 901–917 and Chothia et al., 1989, Nature. 342:877–883. In other embodiments, the CDRs of the 3E10 antibody are defined according to the international ImMunoGeneTics database (IMGT) as set forth in LeFranc et al., 2003, Development and Comparative Immunology, 27: 55-77. In other embodiments, the CDRs of the 3E10 antibody are defined according to Honegger A, Pluckthun A., 2001, J Mol Biol., 309:657–670. In some embodiments, the CDRs of the 3E10 antibody are defined according to any of the CDR identification schemes discussed in Kunik et al., 2012, PLoS Comput Biol. 8(2): e1002388. In order to number residues of a 3E10 antibody for the purpose of identifying CDRs according to any of the CDR identification schemes known in the art, one may align the 3E10 antibody at regions of homology of the sequence of the antibody with a "standard" numbered sequence known in the art for the elected CDR identification scheme. Maximal alignment of framework residues frequently requires the insertion of "spacer" residues in the numbering system, to be used for the Fv region. In addition, the identity of certain individual residues at any given site number may vary from antibody chain to antibody chain due to interspecies or allelic divergence.

In certain embodiments, the internalizing moiety comprises at least 1, 2, 3, 4, or 5 of the CDRs of 3E10 as determined using the Kabat CDR identification scheme (e.g., the CDRs set forth in SEQ ID NOs: 13-18). In other embodiments, the internalizing moiety comprises at least 1, 2, 3, 4 or 5 of the CDRs of 3E10 as determined using the IMGT identification scheme (e.g., the CDRs set forth in SEQ ID NOs: 24-29). In certain embodiments, the internalizing moiety comprises all six CDRs of 3E10 as determined using

the Kabat CDR identification scheme (e.g., comprises SEQ ID NOs 13-18). In other embodiments, the internalizing moiety comprises all six CDRS of 3E10 as determined using the IMGT identification scheme (e.g., which are set forth as SEQ ID NOs: 24-29). For any of the foregoing, in certain embodiments, the internalizing moiety is an antibody that binds the same epitope (e.g., the same target, such as DNA) as 3E10 and/or the internalizing moiety competes with 3E10 for binding to antigen. Exemplary internalizing moieties target and transit cells via ENT2.

The present disclosure utilizes the cell penetrating ability of 3E10 or 3E10 fragments or variants to promote delivery of mature GAA and GAA polypeptides comprising mature GAA *in vivo* or into cells in vitro, such as into cytoplasm of cells. 3E10 and 3E10 variants and fragments are particularly well suited for this because of their demonstrated ability to effectively promote delivery to muscle cells, including skeletal and cardiac muscle, as well as diaphragm. Thus, in certain embodiments, 3E10 and 3E10 variants and fragments (or antibodies or antibody fragments that bind the same epitope and/or transit cells via ENT2) are useful for promoting effective delivery into cells in subjects, such as human patients or model organisms, having Pompe Disease or symptoms that recapitulate Pompe Disease. In certain embodiments, chimeric polypeptides in which the internalizing moiety is related to 3E10 are suitable to facilitate delivery of a GAA polypeptide comprising mature GAA to the cytoplasm of cells.

As described further below, a recombinant 3E10 or 3E10-like variant or fragment can be conjugated, linked or otherwise joined to a mature GAA polypeptide, such as to a GAA polypeptide comprising a mature GAA polypeptide. In the context of making chimeric polypeptides to mature GAA, chemical conjugation, as well as making the chimeric polypeptide as a fusion protein is available and known in the art.

Preparation of antibodies or fragments thereof (e.g., a single chain Fv fragment encoded by V_H-linker-V_L or V_L-linker-V_H or a Fab) is well known in the art. In particular, methods of recombinant production of mAb 3E10 antibody fragments have been described in WO 2008/091911. Further, methods of generating scFv fragments of antibodies or Fabs are well known in the art. When recombinantly producing an antibody or antibody fragment, a linker may be used. For example, typical surface amino acids in flexible protein regions include Gly, Asn and Ser. One exemplary linker is provided in SEQ ID NO: 5 or 6. Permutations of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the criteria (e.g., flexible with minimal hydrophobic or charged

character) for a linker sequence. Another exemplary linker is of the formula $(G_4S)n$, wherein n is an integer from 1-10, such as 2, 3, or 4 (SEQ ID NO: 34). Other near neutral amino acids, such as Thr and Ala, can also be used in the linker sequence.

In addition to linkers interconnecting portions of, for example, an scFv, the disclosure contemplates the use of additional linkers to, for example, interconnect the mature GAA portion to the antibody portion of the chimeric polypeptide.

Preparation of antibodies may be accomplished by any number of well-known methods for generating monoclonal antibodies. These methods typically include the step of immunization of animals, typically mice, with a desired immunogen (*e.g.*, a desired target molecule or fragment thereof). Once the mice have been immunized, and preferably boosted one or more times with the desired immunogen(s), monoclonal antibody-producing hybridomas may be prepared and screened according to well known methods *(see*, for example, Kuby, Janis, *Immunology*, Third Edition, pp. 131-139, W.H. Freeman & Co. (1997), for a general overview of monoclonal antibody production, that portion of which is incorporated herein by reference). Over the past several decades, antibody production has become extremely robust. *In vitro* methods that combine antibody recognition and phage display techniques allow one to amplify and select antibodies with very specific binding capabilities. See, for example, Holt, L. J. et al., "The Use of Recombinant Antibodies in Proteomics," *Current Opinion in Biotechnology*, *2000*, 11:445-449, incorporated herein by reference. These methods typically are much less cumbersome than preparation of hybridomas by traditional monoclonal antibody preparation methods. In one embodiment, phage display technology may be used to generate an internalizing moiety specific for a desired target molecule. An immune response to a selected immunogen is elicited in an animal (such as a mouse, rabbit, goat or other animal) and the response is boosted to expand the immunogen-specific B-cell population. Messenger RNA is isolated from those B-cells, or optionally a monoclonal or polyclonal hybridoma population. The mRNA is reverse-transcribed by known methods using either a poly-A primer or murine immunoglobulin-specific primer(s), typically specific to sequences adjacent to the desired V_H and V_L chains, to yield cDNA. The desired V_H and V_L chains are amplified by polymerase chain reaction (PCR) typically using V_H and V_L specific primer sets, and are ligated together, separated by a linker. V_H and V_L specific primer sets are commercially available, for instance from Stratagene, Inc. of La Jolla, California. Assembled V_H-linker-V_L product (encoding an scFv fragment) is selected for and amplified by PCR. Restriction sites are introduced into

the ends of the V_H-linker-V_L product by PCR with primers including restriction sites and the scFv fragment is inserted into a suitable expression vector (typically a plasmid) for phage display. Other fragments, such as an Fab' fragment, may be cloned into phage display vectors for surface expression on phage particles. The phage may be any phage, such as lambda, but typically is a filamentous phage, such as fd and M13, typically M13.

In certain embodiments, an antibody or antibody fragment is made recombinantly in a host cell. In other words, once the sequence of the antibody is known (for example, using the methods described above), the antibody can be made recombinantly using standard techniques.

In certain embodiments, the internalizing moieties may be modified to make them more resistant to cleavage by proteases. For example, the stability of an internalizing moiety comprising a polypeptide may be increased by substituting one or more of the naturally occurring amino acids in the (L) configuration with D-amino acids. In various embodiments, at least 1%, 5%, 10%, 20%, 50%, 80%, 90% or 100% of the amino acid residues of internalizing moiety may be of the D configuration. The switch from L to D amino acids neutralizes the digestion capabilities of many of the ubiquitous peptidases found in the digestive tract. Alternatively, enhanced stability of an internalizing moiety comprising an peptide bond may be achieved by the introduction of modifications of the traditional peptide linkages. For example, the introduction of a cyclic ring within the polypeptide backbone may confer enhanced stability in order to circumvent the effect of many proteolytic enzymes known to digest polypeptides in the stomach or other digestive organs and in serum. In still other embodiments, enhanced stability of an internalizing moiety may be achieved by intercalating one or more dextrorotatory amino acids (such as, dextrorotatory phenylalanine or dextrorotatory tryptophan) between the amino acids of internalizing moiety. In exemplary embodiments, such modifications increase the protease resistance of an internalizing moiety without affecting the activity or specificity of the interaction with a desired target molecule.

(b) Homing peptides

In certain aspects, an internalizing moiety may comprise a homing peptide which selectively directs the subject chimeric mature GAA polypeptide to a target tissue (e.g., muscle). For example, delivering a chimeric polypeptide to the muscle can be mediated by a homing peptide comprising an amino acid sequence of ASSLNIA (SEQ ID NO: 35). Further exemplary homing peptides are disclosed in WO 98/53804. Homing peptides for a

target tissue (or organ) can be identified using various methods well known in the art. Additional examples of homing peptides include the HIV transactivator of transcription (TAT) which comprises the nuclear localization sequence Tat48-60; Drosophila antennapedia transcription factor homeodomain (e.g., Penetratin which comprises Antp43-58 homeodomain 3rd helix); Homo-arginine peptides (e.g., Arg7 peptide-PKC-ε agonist protection of ischemic rat heart ("Arg7" is disclosed as SEQ ID NO: 36)); alpha-helical peptides; cationic peptides ("superpositively" charged proteins). In some embodiments, the homing peptide transits cellular membranes via an equilibrative nucleoside (ENT) transporter. In some embodiments, the homing peptide transits cellular membranes via an ENT1, ENT2, ENT3 or ENT4 transporter. In some embodiments, the homing peptide targets ENT2. In other embodiments, the homing peptide targets muscle cells. The muscle cells targeted by the homing peptide may include skeletal, cardiac or smooth muscle cells. In other embodiments, the homing peptide targets neurons, epithelial cells, liver cells, kidney cells or Leydig cells.

In certain embodiments, the homing peptide is capable of binding polynucleotides. In certain embodiments, the homing peptide is capable of binding DNA. In certain embodiments, the homing peptide is capable of binding DNA with a K_D of less than 1 μM. In certain embodiments, the homing peptide is capable of binding DNA with a K_D of less than 100 nM.

Additionally, homing peptides for a target tissue (or organ) can be identified using various methods well known in the art. Once identified, a homing peptide that is selective for a particular target tissue can be used, in certain embodiments.

An exemplary method is the *in vivo* phage display method. Specifically, random peptide sequences are expressed as fusion peptides with the surface proteins of phage, and this library of random peptides are infused into the systemic circulation. After infusion into host mice, target tissues or organs are harvested, the phage is then isolated and expanded, and the injection procedure repeated two more times. Each round of injection includes, by default, a negative selection component, as the injected virus has the opportunity to either randomly bind to tissues, or to specifically bind to non-target tissues. Virus sequences that specifically bind to non-target tissues will be quickly eliminated by the selection process, while the number of non-specific binding phage diminishes with each round of selection. Many laboratories have identified the homing peptides that are selective for vasculature of brain, kidney, lung, skin, pancreas, intestine, uterus, adrenal gland, retina, muscle, prostate,

or tumors. See, for example, Samoylova et al., 1999, *Muscle Nerve*, 22:460; Pasqualini et al., 1996, *Nature*, 380:364; Koivunen et al., 1995, *Biotechnology*, 13:265; Pasqualini et al., 1995, *J. Cell Biol.*, 130:1189; Pasqualini et al., 1996, *Mole. Psych.*, 1:421, 423; Rajotte et al., 1998, *J. Clin. Invest.*, 102:430; Rajotte et al., 1999, *J. Biol. Chem.*, 274:11593. See, also, U.S. Patent Nos. 5,622,699; 6,068,829; 6,174,687; 6,180,084; 6,232,287; 6,296,832; 6,303,573; 6,306,365. Homing peptides that target any of the above tissues may be used for targeting a mature GAA protein to that tissue.

(c) Additional Targeting to lysosomes and autophagic vesicles

A traditional method of targeting a protein to lysosomes is modification of the protein with M6P residues, which directs their transport to lysosomes through interaction of M6P residues and M6PR molecules on the inner surface of structures such as the Golgi apparatus or late endosome. Transport of endogenous GAA to the lysosome depends on M6P and M6PR interaction. There are also forms of M6P independent transport of GAA, as evidenced by normal activity of GAA even in patients with I-cell disease, which manifests with severe deficiencies in other lysosomal enzymes (Wisselar et al., J. Biological Chemistry, 268(3): 2223-2231, 1993). Further evidence of M6P independent transport of GAA is evidenced by a study showing no disruption in lysosomal GAA in muscle-specific M6PR-knockout mice targeting (Wylie et al., 2003, Am J Pathol, 162(1): 321-28). In certain embodiments, chimeric polypeptides of the present disclosure (e.g., polypeptides comprising mature GAA and an internalizing moiety) may further include modification to facilitate additional targeting to the lysosome through M6PRs or in pathways independent of M6PRs. Such targeting moieties may be added, for example, at the N-terminus or C-terminus of a chimeric polypeptide, and via conjugation to 3E10 or mature GAA. In other embodiments, the GAA portion of a chimeric polypeptide comprises all or some of the endogenous sequences to facilitate M6P transport.

In some embodiments, the chimeric polypeptides of the present disclosure are transported to lysosomes via the cellular process of autophagy. Autophagy is a catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery. During this process, targeted cytoplasmic constituents are isolated from the rest of the cell within vesicles called autophagosomes, which are then fused with lysosomes and degraded or recycled. Uptake of proteins into autophagic vesicles is mediated by the formation of a membrane around the targeted region

of a cell and subsequent fusion of the vesicle with a lysosome. Several mechanisms for autophagy are known, including macroautophagy in which organelles and proteins are sequestered within the cell in a vesicle called an autophagic vacuole. Upon fusion with the lysosome, the contents of the autophagic vacuole are degraded by acidic lysosomal hydrolases. In microautophagy, lysosomes engulf cytoplasm directly, and in chaperone-mediated autophagy, proteins with a consensus peptide sequence are bound by a hsc70-containing chaperone-cochaperone complex, which is recognized by a lysosomal protein and translocated across the lysosomal membrane. Autophagic vacuoles have a lysosomal environment (low pH), which is conducive for activity of enzymes such as mature GAA.

Autophagy naturally occurs in muscle cells of mammals (Masiero et al, 2009, Cell Metabolism, 10(6): 507-15). It also has been demonstrated that autophagic degradation is enhanced in Pompe Disease (Malicdan et al., 2008, Neuromuscular Disorders, 18: 521-29; Fukuda et al, 2006, Mol Ther, 14(6): 831-39; Takikita et al, 2009, Autophagy, 5(5): 729-31; Raben et al., 2008, 17(24): 3897-3908). Moreover, the autophagic vacuoles present in Pompe Disease contain glycogen (Malicdan et al., 2008, Neuromuscular Disorders, 18: 521-29). As the autophagic vacuoles take up proteins from the cytoplasm, the chimeric polypeptides of the present disclosure are expected to be taken up by glycogen-containing autophagic vesicles, where the chimeric polypeptides would be free to degrade the glycogen present within those vacuoles. As such, in some embodiments, the chimeric polypeptides are capable of taken up by autophagic vacuoles without addition of any autophagic vacuole-specific targeting motif.

In certain embodiments, the chimeric polypeptides of the present disclosure may further include modification to facilitate additional targeting to autophagic vesicles. One known chaperone-targeting motif is KFERQ-like motif (SEQ ID NO: 33). Accordingly, this motif can be added to chimeric polypeptides as described herein, in order to target the polypeptides for autophagy. Such targeting moieties may be added, for example, at the N-terminus or C-terminus of a chimeric polypeptide, and via conjugation to 3E10 or mature GAA.

M6P residues or chaperone-targeting motifs may be added to the mature GAA polypeptides. Mature GAA polypeptides of the present disclosure may comprise, for example, the 76 kDa form of GAA or the 70 kDa form of GAA or similar forms that use an alternative starting and/or ending site, administered either separately or in combination. For combinations of 70 kDa and 76 kDa forms of GAA, or similar forms of GAA as described

herein, the internalizing motifs may be added to either or both of the mature GAA polypeptides.

III. Chimeric Polypeptides

Chimeric polypeptides of the present disclosure can be made in various manners. The chimeric polypeptides may comprise any of the internalizing moiety portions and the mature GAA polypeptide portions disclosed herein (e.g., a GAA polypeptide comprising mature GAA, as described herein) . As used herein, chimeric polypeptides of the disclosure comprising (i) a GAA polypeptide portion and (ii) an internalizing moiety portion, such as a GAA polypeptide portion comprising a GAA polypeptide comprising a mature GAA (e.g., the GAA polypeptide portion comprises a GAA polypeptide which includes mature GAA but is longer than the mature GAA generated in vivo by endogenous processing of a GAA precursor. In addition, any of the chimeric polypeptides disclosed herein may be utilized in any of the methods or compositions disclosed herein. In some embodiments, an internalizing moiety (*e.g.* an antibody or a homing peptide) is linked, directly or indirectly, to any one of the mature GAA polypeptides, fragments or variants disclosed herein. In some embodiments, the chimeric polypeptide does not comprise an: i) immature GAA polypeptide of approximately 110kDa and/or, ii) immature GAA possessing the signal sequence, *i.e.*, amino acid residues 1-27 of SEQ ID NO: 1 or 2. In other words, the disclosure contemplates chimeric polypeptides in which the chimeric polypeptide comprises a mature GAA polypeptide, but may also include additional polypeptide sequence from a GAA polypeptide, including sequence contiguous with the mature GAA polypeptide (e.g., the GAA polypeptide portion comprises a GAA polypeptide comprising a mature GAA polypeptide sequence). For example, in some embodiments, the chimeric polypeptides comprise a GAA polypeptide comprising the amino acid sequence of any of SEQ ID NOs: 21-23 (e.g., SEQ ID NOs 21-23 are exemplary of GAA polypeptides comprising mature GAA but which also include additional contiguous amino acids of a GAA polypeptide). The disclosure also contemplates embodiments in which the chimeric polypeptide comprises a mature GAA polypeptide but does not include additional GAA polypeptide sequence contiguous with the mature GAA polypeptide portion. Finally, the disclosure contemplates embodiments in which the chimeric polypeptide does not include additional GAA polypeptide portions in addition to the mature GAA polypeptide.

In certain embodiments, it may be desirable to conjugate any of the internalizing moieties described herein with a mature GAA polypeptide (*e.g.*, a GAA polypeptide having the amino acid sequence of SEQ ID NO: 3 or 4) in order to reduce the likelihood that a chimeric polypeptide comprising a larger GAA polypeptide (*e.g.*, a GAA polypeptide having the amino acid sequence of any of SEQ ID NOs: 21-24) is inadvertently cleaved at any of the cleavage sites present in the full-length GAA polypeptide (*e.g.*, cleaving between any of the amino acids corresponding to amino acids 56-57, 77-78, 113-114, 121-122, 200-201, 203-204, 781-782, or 791-792 of SEQ ID NO: 1) by a subject's proteases prior to uptake of the chimeric polypeptide by a targeted cell in the subject.

In certain embodiments, the C-terminus of a mature GAA polypeptide can be linked, directly or indirectly, to the N-terminus of an internalizing moiety (e.g., an antibody, an antibody fragment, or a homing peptide). Alternatively, the C-terminus of an internalizing moiety (e.g., an antibody, an antibody fragment, or a homing peptide) can be linked, directly or indirectly, to the N-terminus of a mature GAA polypeptide. For example, chimeric polypeptides can be designed to place the mature GAA polypeptide at the amino or carboxy terminus of either the antibody heavy or light chain of mAb 3E10. In some embodiments, the GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22 or 23 fused to the C-terminus of an internalizing moiety. In some embodiments, the GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22 or 23 fused to the C-terminus of the heavy chain segment of a Fab internalizing moiety. In some embodiments, the GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22 or 23 fused to the C-terminus of the heavy chain segment of a full-length antibody internalizing moiety.

In certain embodiments, potential configurations include the use of truncated portions of an antibody's heavy and light chain sequences (e.g., mAB 3E10) as needed to maintain the functional integrity of the attached mature GAA polypeptide. Further still, the internalizing moiety can be linked to an exposed internal (non-terminus) residue of mature GAA or a variant thereof. In further embodiments, any combination of the mature GAA-internalizing moiety configurations can be employed, thereby resulting in a mature GAA: internalizing moiety ratio that is greater than 1:1 (e.g., two mature GAA molecules to one internalizing moiety).

The mature GAA polypeptide and the internalizing moiety may be linked directly to each other. Alternatively, they may be linked to each other via a linker sequence, which

separates the mature GAA polypeptide and the internalizing moiety by a distance sufficient to ensure that each domain properly folds into its secondary and tertiary structures. Preferred linker sequences (1) should adopt a flexible extended conformation, (2) should not exhibit a propensity for developing an ordered secondary structure which could interact with the functional domains of the mature GAA polypeptide or the internalizing moiety, and (3) should have minimal hydrophobic or charged character, which could promote interaction with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Permutations of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, can also be used in the linker sequence. In a specific embodiment, a linker sequence length of about 20 amino acids can be used to provide a suitable separation of functional protein domains, although longer or shorter linker sequences may also be used. The length of the linker sequence separating the mature GAA polypeptide and the internalizing moiety can be from 5 to 500 amino acids in length, or more preferably from 5 to 100 amino acids in length. Preferably, the linker sequence is from about 5-30 amino acids in length. In preferred embodiments, the linker sequence is from about 5 to about 20 amino acids, and is advantageously from about 10 to about 20 amino acids. In other embodiments, the linker joining the mature GAA polypeptide to an internalizing moiety can be a constant domain of an antibody (e.g., constant domain of mAb 3E10 or all or a portion of an Fc region of another antibody). In certain embodiments, the linker is a cleavable linker. In certain embodiments, the linker sequence comprises the linker sequence of SEQ ID NO: 30.

In other embodiments, the mature GAA polypeptide or functional fragment thereof may be conjugated or joined directly to the internalizing moiety. For example, a recombinantly conjugated chimeric polypeptide can be produced as an in-frame fusion of the mature GAA portion and the internalizing moiety portion. In certain embodiments, the linker may be a cleavable linker. In any of the foregoing embodiments, the internalizing moiety may be conjugated (directly or via a linker) to the N-terminal or C-terminal amino acid of the mature GAA polypeptide, such as to the N-terminal or C-terminal amino acid of a GAA polypeptide comprising a mature GAA. In other embodiments, the internalizing moiety may be conjugated (directly or indirectly) to an internal amino acid of the mature GAA polypeptide. Note that the two portions of the construct are conjugated/joined to each other. Unless otherwise specified, describing the chimeric polypeptide as a conjugation of

the mature GAA portion to the internalizing moiety is used equivalently as a conjugation of the internalizing moiety to the mature GAA portion. Further, unless otherwise specified, conjugation and/or joining refers to either chemical or genetic conjugation.

In certain embodiments, the chimeric polypeptides of the present disclosure can be generated using well-known cross-linking reagents and protocols. For example, there are a large number of chemical cross-linking agents that are known to those skilled in the art and useful for cross-linking the mature GAA polypeptide with an internalizing moiety (e.g., an antibody). For example, the cross-linking agents are heterobifunctional cross-linkers, which can be used to link molecules in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art, including succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidyloxycarbonyl-a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage in vivo. In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers. Disuccinimidyl subcrate (DSS), bismaleimidohexane (BMH) and dimethylpimelimidate.2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[B-(4 -azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in this disclosure. For a recent review of protein coupling techniques, see Means et al. (1990) Bioconjugate Chemistry. 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon

groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product. Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with --SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds. The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules.

In some embodiments, the chimeric polypeptide comprises multiple linkers. For example, if the chimeric polypeptide comprises an scFv internalizing moiety, the chimeric polypeptide may comprise a first linker conjugating the GAA to the internalizing moiety, and a second linker in the scFv conjugating the V_H domain (*e.g.*, SEQ ID NO: 9) to the V_L domain (*e.g.*, SEQ ID NO: 10).

Preparing protein-conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263, incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

In certain specific embodiments, chimeric polypeptides of the disclosure can be produced by using a universal carrier system. For example, a mature GAA polypeptide can be conjugated to a common carrier such as protein A, poly-L-lysine, hex-histidine, and the like. The conjugated carrier will then form a complex with an antibody which acts as an

internalizing moiety. A small portion of the carrier molecule that is responsible for binding immunoglobulin could be used as the carrier.

In certain embodiments, chimeric polypeptides of the disclosure can be produced by using standard protein chemistry techniques such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant G. A. (ed.), Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992). In addition, automated peptide synthesizers are commercially available (e.g., Advanced ChemTech Model 396; Milligen/Biosearch 9600). In any of the foregoing methods of cross-linking for chemical conjugation of mature GAA to an internalizing moiety, a cleavable domain or cleavable linker can be used. Cleavage will allow separation of the internalizing moiety and the mature GAA polypeptide. For example, following penetration of a cell by a chimeric polypeptide, cleavage of the cleavable linker would allow separation of mature GAA from the internalizing moiety.

In certain embodiments, the chimeric polypeptides comprising a GAA polypeptide portion (e.g., a GAA polypeptide comprising a mature GA polypeptide sequence) and an internalizing moiety portion can be generated as a fusion protein containing the GAA polypeptide and the internalizing moiety. In certain embodiments, the chimeric polypeptides of the present disclosure can be generated as a fusion protein containing a mature GAA polypeptide and an internalizing moiety (e.g., an antibody or a homing peptide), expressed as one contiguous polypeptide chain. In certain embodiments, the chimeric polypeptide is generated as a fusion protein that comprises a GAA polypeptide portion and internalizing moiety portion, wherein the GAA polypeptide portion comprises a mature GAA polypeptide and also includes additional polypeptide sequence from a GAA polypeptide, including sequence contiguous with the mature GAA polypeptide. In preparing such fusion protein, a fusion gene is constructed comprising nucleic acids which encode a mature GAA polypeptide and an internalizing moiety, and optionally, a peptide linker sequence to span the mature GAA polypeptide and the internalizing moiety. The use of recombinant DNA techniques to create a fusion gene, with the translational product being the desired fusion protein, is well known in the art. Both the coding sequence of a gene and its regulatory regions can be redesigned to change the functional properties of the protein product, the amount of protein made, or the cell type in which the protein is produced. The coding sequence of a gene can be extensively altered--for example, by fusing part of it to the coding sequence of a different gene to produce a novel hybrid gene

that encodes a fusion protein. Examples of methods for producing fusion proteins are described in PCT applications PCT/US87/02968, PCT/US89/03587 and PCT/US90/07335, as well as Traunecker et al. (1989) Nature 339:68, incorporated by reference herein. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. Alternatively, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. In another method, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992). The chimeric polypeptides encoded by the fusion gene may be recombinantly produced using various expression systems as is well known in the art (also see below).

Recombinantly conjugated chimeric polypeptides include embodiments in which the mature GAA polypeptide is conjugated to the N-terminus or C-terminus of the internalizing moiety. Exemplary chimeric polypeptides in which mature GAA polypeptides are conjugated to variant light and heavy chains of Fv3E10 are indicated in SEQ ID NOS: 11 and 12. In certain embodiments, a chimeric polypeptide of the disclosure further comprises, at the N-terminus (at or within 10 amino acid residues of the N-terminus), an amino acid sequence set forth in SEQ ID NO: 19 or 20.

Recombinantly conjugated chimeric polypeptides include embodiments in which the internalizing moiety is N-terminal to the GAA polypeptide and embodiments in which the internalizing moiety is C-terminal to the GAA polypeptide portion.

We note that methods of making fusion proteins recombinantly are well known in the art. Any of the chimeric proteins described herein can readily be made recombinantly. This includes proteins having one or more tags and/or one or more linkers. For example, if the chimeric polypeptide comprises an scFv internalizing moiety, the chimeric polypeptide may comprise a first linker interconnection the internalizing moiety to the mature GAA polypeptide portion, and a second linker in the scFv conjugating the V_H domain. Moreover, in certain embodiments, the chimeric polypeptides comprise a "AGIH" portion (SEQ ID

NO: 19) on the N-terminus of the chimeric polypeptide (or within 10 amino acid residues of the N-terminus), and such chimeric polypeptides may be provided in the presence or absence of one or more epitope tags. In further embodiments, the chimeric polyepeptide comprises a serine at the N-terminal most position of the polypeptide. In some embodiments, the chimeric polypeptides comprise an "SAGIH" (SEQ ID NO: 20) portion at the N-terminus of the polypeptide (or within 10 amino acid residues of the N-terminus), and such chimeric polypeptides may be provided in the presence or absence of one or more epitope tags.

In some embodiments, the immunogenicity of the chimeric polypeptide may be reduced by identifying a candidate T-cell epitope within a junction region spanning the chimeric polypeptide and changing an amino acid within the junction region as described in U.S. Patent Publication No. 2003/0166877.

Chimeric polypeptides according to the disclosure can be used for numerous purposes. We note that any of the chimeric polypeptides described herein can be used in any of the methods described herein, and such suitable combinations are specifically contemplated.

Chimeric polypeptides described herein can be used to deliver mature GAA polypeptide to cells, particular to a muscle cell. In certain embodiments, chimeric polypeptides deliver mature GAA to liver cells. Thus, the chimeric polypeptides can be used to facilitate transport of mature GAA to cells in vitro or in vivo. By facilitating transport to cells, the chimeric polypeptides improve delivery efficiency, thus facilitating working with mature GAA polypeptide in vitro or in vivo. Further, by increasing the efficiency of transport, the chimeric polypeptides may help decrease the amount of mature GAA needed for in vitro or in vivo experimentation. Moreover, by facilitating delivery to the cytoplasm, the chimeric polypeptides and methods of the disclosure can address the problems associated with cytoplasmic accumulation of glycogen in, for example, Pompe disease.

The chimeric polypeptides can be used to study the function of mature GAA in cells in culture, as well as to study transport of mature GAA. The chimeric polypeptides can be used to identify binding partners for mature GAA in cells, such as transport between cytoplasm and lysosome. The chimeric polypeptides can be used in screens to identify modifiers (*e.g.*, small organic molecules or polypeptide modifiers) of mature GAA activity in a cell. The chimeric polypeptides can be used to help treat or alleviate the symptoms of

Pompe disease in humans or in an animal model. The foregoing are merely exemplary of the uses for the subject chimeric polypeptides.

Any of the chimeric polypeptides described herein, including chimeric polypeptides combining any of the features of the GAA polypeptides, internalizing moieties, and linkers, may be used in any of the methods of the disclosure.

IV. GAA-Related Nucleic Acids And Expression

In certain embodiments, the present disclosure makes use of nucleic acids for producing a mature GAA polypeptide (including functional fragments, variants, and fusions thereof), such as for producing GAA polypeptides comprising a mature GAA polypeptide. In certain specific embodiments, the nucleic acids may further comprise DNA which encodes an internalizing moiety (e.g., an antibody or a homing peptide) for making a recombinant chimeric protein of the disclosure. In certain embodiments, the nucleic acid construct does not encode a chimeric polypeptide comprising a GAA precursor polypeptide of approximately 110 kDa. In certain embodiments, the nucleic acid construct encodes a GAA polypeptide comprising mature GAA but does not encode a GAA polypeptide comprising (i) the amino acid sequence set forth in SEQ ID NO: 1 or 2 or (ii) a portion corresponding to residues 1-27 and/or 1-56 of SEQ ID NO: 1 or 2. All these nucleic acids are collectively referred to as mature GAA nucleic acids because they encode a polypeptide comprising a mature GAA polypeptide and, optionally, additional contiguous portions of a GAA polypeptide.

The nucleic acids may be single-stranded or double-stranded, DNA or RNA molecules. In certain embodiments, the disclosure relates to isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to a region of a GAA nucleotide sequence (e.g., GenBank Accession No.: NM_000152.3 which encodes NP000143.2; NM_001079803.1 which encodes NP_001073271.1; and NM_001079804.1 which encodes NP_001073272.1) that encodes mature GAA (e.g., mature GAA nucleotide sequence). The nucleotide sequences for GAA are hereby incorporated by reference in their entirety. In further embodiments, the GAA nucleic acid sequences can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

In certain embodiments, mature GAA nucleic acids also include nucleotide sequences that hybridize under highly stringent conditions to any of the above-mentioned

native GAA nucleotide sequences (e.g., GenBank Accession No.: NM_ 000152.3; NM_001079803.1; and NM_001079804.1), or complement sequences thereof. One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the disclosure provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the native mature GAA nucleic acids due to degeneracy in the genetic code are also within the scope of the disclosure. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this disclosure.

In certain embodiments, the recombinant mature GAA nucleic acids may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate for a host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the

disclosure. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used. In certain aspects, this disclosure relates to an expression vector comprising a nucleotide sequence encoding a mature GAA polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the encoded polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology*: *Methods in Enzymology*, Academic Press, San Diego, CA (1990). It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell (e.g., Chines Hamster Ovary cells) to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

In some embodiments, a nucleic acid construct, comprising a nucleotide sequence that encodes a mature GAA polypeptide or a bioactive fragment thereof, is operably linked to a nucleotide sequence that encodes an internalizing moiety, wherein the nucleic acid construct encodes a chimeric polypeptide having mature GAA biological activity. In certain embodiments, the nucleic acid constructs may further comprise a nucleotide sequence that encodes a linker.

This disclosure also pertains to a host cell transfected with a recombinant gene which encodes a mature GAA polypeptide or a chimeric polypeptide of the disclosure. The host cell may be any prokaryotic or eukaryotic cell. For example, a mature GAA polypeptide or a chimeric polypeptide may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

The present disclosure further pertains to methods of producing a mature GAA polypeptide or a chimeric polypeptide of the disclosure. For example, a host cell transfected with an expression vector encoding a mature GAA polypeptide or a chimeric

polypeptide can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The polypeptide may be secreted and isolated from a mixture of cells and medium containing the polypeptides. Alternatively, the polypeptides may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The polypeptides can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the polypeptides (e.g., a GAA polypeptide). In a preferred embodiment, the polypeptide is a fusion protein containing a domain which facilitates its purification.

A recombinant mature GAA nucleic acid can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant polypeptide include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*. The preferred mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant polypeptide by the use of a

baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

The disclosure contemplates methods of producing chimeric proteins recombinantly, such as described above. Suitable vectors and host cells may be readily selected for expression of proteins in, for example, yeast or mammalian cells. Host cells may express a vector encoding a chimeric polypeptide stably or transiently. Such host cells may be cultured under suitable conditions to express chimeric polypeptide which can be readily isolated from the cell culture medium.

Chimeric polypeptides of the disclosure (e.g., polypeptides comprising a GAA portion comprising mature GAA and an internalizing moiety portion) may be expressed as a single polypeptide chain or as more than one polypeptide chains. An example of a single polypeptide chain is when a GAA portion is fused inframe to an internalizing moiety, which internalizing moiety is an scFv. In certain embodiments, this single polypeptide chain is expressed from a single vectors as a fusion protein.

An example of more than one polypeptide chains is when the internalizing moiety is an antibody or Fab. In certain embodiments, the heavy and light chains of the antibody or Fab may be expressed in a host cell expressing a single vector or two vectors (one expressing the heavy chain and one expressing the light chain). In either case, the GAA polypeptide may be expressed as an inframe fusion to, for example, the C-terminus of the

heavy chain such that the GAA polypeptide is appended to the internalizing moiety but at a distance to the antigen binding region of the internalizing moiety.

As noted above, methods for recombinantly expressing polypeptides, including chimeric polypeptides, are well known in the art. Nucleotide sequences expressing a GAA polypeptide, such as a human GAA polypeptide, having a particular amino acid sequence are available and can be used. Moreover, nucleotide sequences expressing an internalizing moiety portion, such as expressing a 3E10 antibody, scFv, or Fab comprising the VH and VL set forth in SEQ ID NO: 9 and 10) are publicly available and can be combined with nucleotide sequence encoding suitable heavy and light chain constant regions. The disclosure contemplates nucleotide sequences encoding any of the chimeric polypeptides of the disclosure, vectors (single vector or set of vectors) comprising such nucleotide sequences, host cells comprising such vectors, and methods of culturing such host cells to express chimeric polypeptides of the disclosure.

V. Methods of Treatment and other Methods of Use

For any of the methods described herein, the disclosure contemplates the use of any of the chimeric polypeptides and/or compositions described throughout the application. In addition, for any of the methods described herein, the disclosure contemplates the combination of any step or steps of one method with any step or steps from another method.

For example, a chimeric polypeptide of the disclosure comprising a GAA polypeptide portion and an internalizing moiety portion can be used in any of the methods of the disclosure.

In certain embodiments, GAA polypeptides may comprise one of the mature, active forms of the GAA protein, such as the 70 kDa form or the mature 76 kDa form, or a combination of the two. Mature GAA polypeptides may also be administered in combination with the immature 110 kDa form of GAA, in order to target as many organelles and cellular regions/compartments as possible. In addition, mature GAA polypeptides may be administered in combination with and/or following administration of immunotolerizing fragments of GAA, such as small fragments of GAA, and/or immunosuppressive compounds. In some embodiments, the GAA polypeptides comprise a mature GAA polypeptide as well as additional polypeptide sequence from a GAA polypeptide, such as sequence contiguous with the mature GAA polypeptide.

In certain embodiments, the present disclosure provides methods of delivering chimeric polypeptides to cells, including cells in culture (in vitro or ex vivo) and cells in a subject. Delivery to cells in culture, such as healthy cells or cells from a model of disease, have numerous uses. These uses include to identify GAA substrates or binding partners, to evaluate localization and/or trafficking (e.g., to cytoplasm, lysosome, and/or autophagic vesicles), to evaluate enzymatic activity under a variety of conditions (e.g., pH), to assess glycogen accumulation, and the like. In certain embodiments, chimeric polypeptides of the disclosure can be used as reagents to understand GAA activity, localization, and trafficiking in healthy or diease contexts.

Delivery to subjects, such as to cells in a subject, have numerous uses. Exemplary therapeutic uses are described below. Moreover, the chimeric polypeptides may be used for diagnostic or research purposes. For example, a chimeric polypeptide of the disclosure may be detectably labeled and administerd to a subject, such as an animal model of disease or a patient, and used to image the chimeric polypeptide in the subject's tissues (e.g., localization to muscle and/or liver). Additionally exemplary uses include delivery to cells in a subject, such as to an animal model of disease (e.g., Pompe disease). By way of example, chimeric polypeptides of the disclosure may be used as reagents and delivered to animals to understand GAA bioactivity, localization and trafficking, protein-protein interactions, enzymatic activity, and impacts on animal physiology in healthy or diseased animals.

In certain embodiments, the present disclosure provides methods of treating conditions associated with dysfunction of GAA and Pompe disease. Such conditions include, but are not limited to, aberrant accumulation of glycogen in the lysosomes, cytoplasm, and/or autophagic vesicles of affected cells, for example heart and skeletal muscle cells; cell starvation; disorganization of microtubule structure; increase in number and/or size of lysosomes, rupture of lysosomes; accumulation of cellular debris including autophagic components; disruption of mitochondrial structure; cell swelling; motorneuron disease; muscle weakness; progressive muscle decline; damage to skeletal, respiratory, and/or cardiac muscles; and premature death. Some symptoms of Pompe disease do not manifest until the patients have lived without functional GAA or with diminished levels of GAA for extended periods of time, such as 6 months or longer. In these cases, there has been additional time for glycogen to accumulate not only in the lysosomes but also in cytoplasm and autophagic vacuoles of the patient's cells, triggering disruption of cell and

organelle function as described above. When the disease progresses to this stage, traditional enzyme replacement therapies that target GAA to the lysosome may no longer be effective or may be inadequate to treat the condition. Thus, in some embodiments, administration of the mature GAA polypeptides of the present disclosure targets polypetides comprising the mature GAA to the cytoplasm of affected cells and treats symptoms associated with accumulation of glycogen in the cytoplasm and/or autophagic vacuoles.

These methods involve, in certain embodiments, administering to the individual a therapeutically effective amount of a chimeric polypeptide as described above (e.g., a chimeric polypeptide comprising (i) a GAA portion comprising a GAA polypeptide and (ii) an internalizing moiety portion). These methods are particularly aimed at therapeutic and prophylactic treatments of animals, and more particularly, humans. With respect to methods for Pompe disease, the disclosure contemplates all combinations of any of the foregoing aspects and embodiments, as well as combinations with any of the embodiments set forth in the detailed description and examples.

The present disclosure provides a method of delivering a chimeric polypeptide or nucleic acid construct into a cell via an equilibrative nucleoside transporter (ENT2) pathway, comprising contacting a cell with a chimeric polypeptide or nucleic acid construct. In certain embodiments, the method comprises contacting a cell with a chimeric polypeptide, which chimeric polypeptide comprises a mature GAA polypeptide or bioactive fragment thereof and an internalizing moiety which mediates transport across a cellular membrane via an ENT2 pathway, thereby delivering the chimeric polypeptide into the cell. In certain embodiments, the cell is a muscle cell. The muscle cells targeted using the claimed method may include skeletal, cardiac or smooth muscle cells. In other embodiments, the chimeric polypeptides are delivered to liver.

The present disclosure also provides a method of delivering a chimeric polypeptide or nucleic acid construct into a cell via a pathway that allows access to cells other than muscle cells. Other cell types that could be targeted using the claimed method include, for example, liver cells, neurons, epithelial cells, uterine cells, and kidney cells.

Conditions associated with GAA dysfunction and Pompe disease are manifold. Pompe disease is characterized by massive accumulation of glycogen in many tissues, but predominantly skeletal and cardiac muscles, leading to severe dysfunction of the affected tissues. The disease symptoms are progressive. Early onset form of the disease manifests

clinically in infants as a combination of hypotonia and generalized muscle weakness, such as a head lag or a "floppy baby" appearance. Cardiomegaly appears in an estimated 92-95% of all infant patients, and heart failure often occurs. Respiratory failure due to weakness of the diaphragm is common, and infants may present with difficulties feeding. Without treatment, infants usually die within the first two years of life.

In juvenile and adult onset forms of the disease, symptoms include musculoskeletal dysfunction, such as muscle weakness, gait abnormalities, muscle pain, frequent falls, difficulty chewing; respiratory complications due to weakening of the diaphragm and other respiratory muscles; cardiac abnormalities such as arrhythmias; and gastrointestinal problems such as difficulty swallowing or feeding. Patients may become dependent on ventilators as the respiratory complications progress, or on wheelchairs as motor function declines.

The terms "treatment", "treating", and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. "Treating" a condition or disease refers to curing as well as ameliorating at least one symptom of the condition or disease, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject in need relative to a subject which does not receive the composition. "Treatment" as used herein covers any treatment of a disease or condition of a mammal, particularly a human, and includes: (a) preventing symptoms of the disease or condition from occurring in a subject which may be predisposed to the disease or condition but has not yet begun experiencing symptoms; (b) inhibiting the disease or condition (e.g., arresting its development); or (c) relieving the disease or condition (e.g., causing regression of the disease or condition, providing improvement in one or more symptoms). For example, "treatment" of Pompe disease encompasses a complete reversal or cure of the disease, or any range of improvement in symptoms and/or adverse effects attributable to Pompe disease. Merely to illustrate, "treatment" of Pompe disease includes an improvement in any of the following effects associated with dysfunction of GAA (or combination thereof): decreased GAA activity (e.g., treatment increases GAA activity), glycogen accumulation in cells (e.g., treatment decreases glycogen accumulation), increased creatine kinase levels, elevation of urinary glucose tetrasaccharide, reduction in heart size, hypertrophic cardiomyopathy, respiratory complications, dependence on a ventilator, muscle dysfunction and/or weakening, loss of motor function, dependence on a wheelchair or other form of mobility assistance,

dependence on neck or abdominal support for sitting upright, ultrastructural damage of muscle fibers, loss of muscle tone and function. Improvements in any of these symptoms can be readily assessed according to standard methods and techniques known in the art. Other symptoms not listed above may also be monitored in order to determine the effectiveness of treating Pompe disease. The population of subjects treated by the method of the disclosure includes subjects suffering from the undesirable condition or disease, as well as subjects at risk for development of the condition or disease. In certain embodiments, administering a mature GAA chimeric polypeptide may have any one or more of the following affects: decrease accumulation of glycogen in cytoplasm of cells, decrease accumulation of glycogen in cytoplasm of muscle cells, decrease accumulation of glycogen in cytoplasm of liver cells, decrease accumulation of glycogen in cytoplasm of neurons, decrease accumulation of glycogen in lysosomes of cells, decrease accumulation of glycogen in lysosomes of muscle cells, decrease accumulation of glycogen in lysosomes of liver cells, decrease accumulation of glycogen in lysosomes of neurons, decrease accumulation of glycogen in autophagic vacuoles of cells, decrease accumulation of glycogen in autophagic vacuoles of muscle cells, decrease accumulation of autophagic vacuoles in cytoplasm of liver cells, decrease accumulation of glycogen in autophagic vacuoles of neurons, decrease elevated levels of alanine transaminase (such as elevated levels in serum), decrease elevated levels of aspartate transaminase (such as elevated levels in serum), decrease elevated levels of alkaline phosphatase (such as elevated levels in serum), and/or decrease elevated levels of creatine phosphokinase (such as elevated levels in serum). It should be noted that any of the GAA chimeric polypeptides described above or herein may be used in any of the methods described herein.

In certain embodiments, the subjects in need of treatment are subjects having infantile form of Pompe disease. In other embodiments, the subjects in need of treatment are subjects having juvenile onset

By the term "therapeutically effective dose" is meant a dose that produces the desired effect for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (see, e.g., Lloyd (1999) The Art, Science and Technology of Pharmaceutical Compounding).

In certain embodiments, one or more chimeric polypeptides of the present disclosure can be administered, together (simultaneously) or at different times (sequentially). In

addition, chimeric polypeptides of the present disclosure can be administered alone or in combination with one or more additional compounds or therapies for treating Pompe disease. For example, one or more chimeric polypeptides can be co-administered in conjunction with one or more other therapeutic compounds. In some embodiments, the one or more chimeric polypeptides can be co-administered in conjunction with alglucosidase alfa (Myozyme, Genzyme Corporation). When co-administration is indicated, the combination therapy may encompass simultaneous or alternating administration. In addition, the combination may encompass acute or chronic administration. Optionally, the chimeric polypeptide of the present disclosure and additional compounds act in an additive or synergistic manner for treating Pompe disease. Additional compounds to be used in combination therapies include, but are not limited to, small molecules, polypeptides, antibodies, antisense oligonucleotides, and siRNA molecules. Depending on the nature of the combinatory therapy, administration of the chimeric polypeptides of the disclosure may be continued while the other therapy is being administered and/or thereafter. Administration of the chimeric polypeptides may be made in a single dose, or in multiple doses. In some instances, administration of the chimeric polypeptides is commenced at least several days prior to the other therapy, while in other instances, administration is begun either immediately before or at the time of the administration of the other therapy.

One type of combination therapy makes use of molecules that promote muscle synthesis and/or fat reduction. Molecules such as IGF-1, growth hormones, steroids, β-2 agonists (for example Clenbuterol), and myostatin inhibitors may be administered to patients in order to build muscle tissue and reduce fat infiltration. These molecules may also increase ENT2 levels. Accordingly, the molecules may be administered before treatment with mature GAA polypeptides begins, in between treatments with mature GAA polypeptides, or after treatment with mature GAA polypeptides.

In another example of combination therapy, one or more chimeric polypeptides of the disclosure can be used as part of a therapeutic regimen combined with one or more additional treatment modalities. By way of example, such other treatment modalities include, but are not limited to, dietary therapy, occupational therapy, physical therapy, ventilator supportive therapy, massage, acupuncture, acupressure, mobility aids, assistance animals, and the like.

Note that although the chimeric polypeptides described herein can be used in combination with other therapies, in certain embodiments, a chimeric polypeptide is

provided as the sole form of therapy. Regardless of whether administrated alone or in combination with other medications or therapeutic regiments, the dosage, frequency, route of administration, and timing of administration of the chimeric polypeptides is determined by a physician based on the condition and needs of the patient.

Chimeric polypeptides of the disclosure have numerous uses, including in vitro and in vivo uses. In vivo uses include not only therapeutic uses but also diagnostic and research uses in, for example, any of the foregoing animal models. By way of example, chimeric polypeptides of the disclosure may be used as research reagents and delivered to animals to understand GAA bioactivity, localization and trafficking, protein-protein interactions, enzymatic activity, and impacts on animal physiology in healthy or diseases animals.

Chimeric polypeptides may also be used in vitro to evaluate, for example, GAA bioactivity, localization and trafficking, protein-protein interactions, and enzymatic activity in cells in culture, including healthy and GAA deficient cells in culture. The disclosure contemplates that chimeric polypeptides of the disclosure may be used to deliver GAA to cytoplasm, lysosome, and/or autophagic vesicles of cells, including cells in culture.

VI. Gene Therapy

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding polypeptides of mature GAA and or chimeric polypeptides comprising mature GAA in mammalian cells or target tissues. In certain embodiments, the chimeric polypeptides for use in the methods described herein comprise a mature GAA polypeptide, but also include additional polypeptide sequence from a GAA polypeptide, including sequence contiguous with the mature GAA polypeptide. Such methods can be used to administer nucleic acids encoding polypeptides of the disclosure (e.g., mature GAA, including variants thereof, and include chimeric polypeptides) to cells in vitro. The disclosure contemplates that gene transfer methods may be used to deliver nucleic acid encoding any of the chimeric polypeptides of the disclosure or GAA polypeptides. In some embodiments, the nucleic acids encoding mature GAA are administered for in vivo or ex vivo gene therapy uses. In other embodiments, gene delivery techniques are used to study the activity of chiermic polypeptides or GAA polypeptide or to study Pompe disease in cell based or animal models, such as to evaluate cell trafficking, enzyme activity, and protein-protein interactions following delivery to healthy or diseased cells and tissues. Non-viral vector delivery systems include DNA plasmids, naked nucleic

acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. Such methods are well known in the art.

Methods of non-viral delivery of nucleic acids encoding engineered polypeptides of the disclosure include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection methods and lipofection reagents are well known in the art (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (ex vivo administration) or target tissues (in vivo administration). The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art.

The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding mature GAA or its variants take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro and the modified cells are administered to patients (ex vivo). Conventional viral based systems for the delivery of polypeptides of the disclosure could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Viral vectors are currently the most efficient and versatile method of gene transfer in target cells and tissues. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene

expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SW), human immuno deficiency virus (HIV), and combinations thereof, all of which are well known in the art.

In applications where transient expression of the polypeptides of the disclosure is preferred, adenoviral based systems are typically used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures. Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, et al.; *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski et al., *J. Virol.* 63:03822-3828 (1989).

Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system.

Replication-deficient recombinant adenoviral vectors (Ad) can be engineered such that a transgene replaces the Ad E1a, E1b, and E3 genes; subsequently the replication defector vector is propagated in human 293 cells that supply deleted gene function in trans. Ad vectors can transduce multiple types of tissues in vivo, including nondividing, differentiated cells such as those found in the liver, kidney and muscle system tissues. Conventional Ad vectors have a large carrying capacity.

Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and 42 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for

the protein to be expressed. The missing viral functions are supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. A viral vector is typically modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the viruses outer surface. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. This principle can be extended to other pairs of virus expressing a ligand fusion protein and target cell expressing a receptor. For example, filamentous phage can be engineered to display antibody fragments (e.g., FAB or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences thought to favor uptake by specific target cells, such as muscle cells.

Gene therapy vectors can be delivered in vivo by administration to an individual patient, by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application. Alternatively, vectors can be delivered to cells ex vivo, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

Ex vivo cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. For example, cells are isolated from the subject organism, transfected with a nucleic acid (gene or cDNA) encoding, e.g., mature GAA or its variants, and re-infused back into

the subject organism (e.g., patient). Various cell types suitable for ex vivo transfection are well known to those of skill in the art.

In certain embodiments, stem cells are used in ex vivo procedures for cell transfection and gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types in vitro, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Stem cells are isolated for transduction and differentiation using known methods.

Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing therapeutic nucleic acids can be also administered directly to the organism for transduction of cells in vivo. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present disclosure, as described herein.

VII. Methods of Administration

Various delivery systems are known and can be used to administer the chimeric polypeptides of the disclosure. Any such methods may be used to administer any of the chimeric polypeptides described herein. Methods of introduction can be enteral or parenteral, including but not limited to, intradermal, intramuscular, intraperitoneal, intramyocardial, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, and oral routes. The chimeric polypeptides may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

In certain embodiments, the chimeric polypeptide is administered intravenously.

In certain embodiments, it may be desirable to administer the chimeric polypeptides of the disclosure locally to the area in need of treatment (e.g., muscle); this may be achieved, for example, and not by way of limitation, by local infusion during surgery, by means of a catheter, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes.

In another embodiment, such local administration can be to all or a portion of the heart. For example, administration can be by intrapericardial or intramyocardial administration. Similarly, administration to cardiac tissue can be achieved using a catheter, wire, and the like intended for delivery of agents to various regions of the heart.

In another embodiment, local administration is directed to the liver. Glycogen storage and glycogenolysis in the liver affect the availability of glycogen for many other tissues in the body. For example, a venous catheter may be placed in the hepatic portal vein to deliver chimeric polypeptides directly to the liver. In addition, in some embodiments where the internalizing moieties of the chimeric polypeptides show a lower affinity for liver cells than for other cell types, delivery through the hepatic portal vein ensures that adequate concentrations of mature GAA reach the liver cells.

Note that the disclosure contemplates methods in which chimeric polypeptides are administered, at the same or different times, via one than one route of administration. For example, the disclosure contemplates a regimen in which chimeric polypeptides are administered systemically, such as by intravenous infusion, in combination with local administration via the hepatic portal vein.

In other embodiments, the chimeric polypeptides of the disclosure can be delivered in a vesicle, in particular, a liposome (see Langer, 1990, Science 249:1527-1533). In yet another embodiment, the chimeric polypeptides of the disclosure can be delivered in a controlled release system. In another embodiment, a pump may be used (see Langer, 1990, supra). In another embodiment, polymeric materials can be used (see Howard et al., 1989, J. Neurosurg. 71:105). In certain specific embodiments, the chimeric polypeptides of the disclosure can be delivered intravenously.

In certain embodiments, the chimeric polypeptides are administered by intravenous infusion. In certain embodiments, the chimeric polypeptides are infused over a period of at least 10, at least 15, at least 20, or at least 30 minutes. In other embodiments, the chimeric polypeptides are infused over a period of at least 60, 90, or 120 minutes. Regardless of the

infusion period, the disclosure contemplates that each infusion is part of an overall treatment plan where chimeric polypeptide is administered according to a regular schedule (e.g., weekly, monthly, etc.).

The foregoing applies to any of the chimeric polypeptides, compositions, and methods described herein. The disclosure specifically contemplates any combination of the features of such chimeric polypeptides, compositions, and methods (alone or in combination) with the features described for the various pharmaceutical compositions and route of administration described in this section.

VIII. Pharmaceutical Compositions

In certain embodiments, the subject chimeric polypeptides of the present disclosure are formulated with a pharmaceutically acceptable carrier. One or more chimeric polypeptides can be administered alone or as a component of a pharmaceutical formulation (composition). Any of the chimeric polypeptides described herein may be formulated, as described herein. In certain embodiments, the composition includes two or more chimeric polypeptides of the disclosure, such as a chimeric polypeptide comprising a mature GAA of approximately 70 kDa and a chimeric polypeptide comprising a mature GAA of approximately 76 kDa. The chimeric polypeptides may be formulated for administration in any convenient way for use in human or veterinary medicine. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Formulations of the subject chimeric polypeptides include, for example, those suitable for oral, nasal, topical, parenteral, rectal, and/or intravaginal administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

In certain embodiments, methods of preparing these formulations or compositions include combining another type of therapeutic agents and a carrier and, optionally, one or

more accessory ingredients. In general, the formulations can be prepared with a liquid carrier, or a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Formulations for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a subject chimeric polypeptide therapeutic agent as an active ingredient. Suspensions, in addition to the active compounds, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more chimeric polypeptide therapeutic agents of the present disclosure may be mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like. Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as 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, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

In certain embodiments, methods of the disclosure include topical administration, either to skin or to mucosal membranes such as those on the cervix and vagina. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl alcohol, dimethyl sulfoxide, and azone. Additional agents may further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples are salicylic acid and sulfur. Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, and inhalants. The subject polypeptide therapeutic agents may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required. The ointments, pastes, creams and gels may contain, in addition to a subject chimeric polypeptide agent, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof. Powders and sprays can contain, in addition to a subject chimeric polypeptides, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Pharmaceutical compositions suitable for parenteral administration may comprise one or more chimeric polypeptides in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes

which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

Injectable depot forms are made by forming microencapsule matrices of one or more polypeptide therapeutic agents in biodegradable polymers such as polylactide-polyglycolide. Depending on 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 also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

In a preferred embodiment, the chimeric polypeptides of the present disclosure are formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

In another embodiment, the chimeric polypeptides of the present disclosure are formulated for subcutaneous administration to human beings.

In certain embodiments, the chimeric polypeptides of the present disclosure are formulated for deliver to the heart, such as for intramyocardial or intrapericaridal delivery.

In certain embodiments, the composition is intended for local administration to the liver via the hepatic portal vein, and the chimeric polypeptides are formulated accordingly.

Note that, in certain embodiments, a particular formulation is suitable for use in the context of deliver via more than one route. Thus, for example, a formulation suitable for intravenous infusion may also be suitable for delivery via the hepatic portal vein. However, in other embodiments, a formulation is suitable for use in the context of one route of delivery, but is not suitable for use in the context of a second route of delivery.

The amount of the chimeric polypeptides of the disclosure which will be effective in the treatment of a tissue-related condition or disease (e.g., Pompe disease) can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each subject's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-5000 micrograms of the active chimeric polypeptide per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

In certain embodiments, compositions of the disclosure, including pharmaceutical preparations, are non-pyrogenic. In other words, in certain embodiments, the compositions are substantially pyrogen free. In one embodiment the formulations of the disclosure are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside a microorganism and are released only when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, even low amounts of endotoxins must be removed from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper

limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in relatively large dosages and/or over an extended period of time (e.g., such as for the patient's entire life), even small amounts of harmful and dangerous endotoxin could be dangerous. In certain specific embodiments, the endotoxin and pyrogen levels in the composition are less then 10 EU/mg, or less then 5 EU/mg, or less then 1 EU/mg, or less then 0.1 EU/mg, or less then 0.01 EU/mg, or less then 0.001 EU/mg.

The foregoing applies to any of the chimeric polypeptides, compositions, and methods described herein. The disclosure specifically contemplates any combination of the features of such chimeric polypeptides, compositions, and methods (alone or in combination) with the features described for the various pharmaceutical compositions and route of administration described in this section.

IX. Animal Models

Pompe disease has been modeled in animals such as Brahman and Shorthorn cattle, Lapland dog, cats, sheep, and a strain of Japanese quail (Kikuchi et al., Clinical and Metabolic Correction of Pompe Disease by Enzyme Therapy in Acid Maltase-deficient Quail, J. Clin. Invest., 101(4): 827-833, 1998). In addition, mouse models have been developed by targeted disruption of the GAA gene (summarized in Geel et al., Pompe disease: Current state of treatment modalities and animal models, *Molecular Genetics and Metabolism*, 92:299-307, 2007). Briefly, mice possessing a knockout in exon 13 of the GAA gene exhibit glycogen accumulation in lysosomes of liver, heart, and skeletal muscle cells, but remain phenotypically normal (Bijvoet et al., Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease, Human Molecular Genetics, 7(1): 53-62, 1998). Mice in which exon 6 of the GAA gene was replaced by a neomycin resistance gene flanked by LoxP sites was developed, and lacked GAA function in several tissues. This mouse has also been crossed with Cre-producing mice, and the resultant progeny have abnormal lysosomal glycogen storage in heart and skeletal muscle (Raben et al., Targeted Disruption of the Acid α-Glucosidase Gene in Mice Causes an Illness with Critical Features of Both Infantile and Adult Human Glycogen Storage Disease Type II, J. Biological Chemistry, 272(30): 19086-19092, 1998). A similar mouse model has targeted replacement of exon 14 with a neomycin cassette and is comparable to the neomycin-exon

6 mouse (Raben et al., Modulation of disease severity in mice with targeted disruption of the acid alpha-glucosidase gene, Neuromuscl. Disord. 10: 283-291, 2000). Two additional mouse models have been developed to address issues of immune response: one mouse model in which the exon 6 deletion was targeted to maintain GAA function in the liver while keeping the disease phenotype in other tissues, and one GAA knockout mouse model in SCID mice, which do not produce anti-hGAA antibodies upon administration of hGAA (Raben et al., Induction of tolerance to a recombinant human enzyme, acid alpha-glucosidase, in enzyme deficient knockout mice, Transgenic Research, 12:171-178, 2003; Xu et al., Improved efficacy of gene therapy approaches for Pompe disease using a new, immune-deficient GSD-II mouse model, Gene Therapy, 11:15890-1598, 2004). More recently, a double KO mouse has been developed that pairs deletion of GAA and deletion of glycogen synthase 1 to help determine the effects of decreased glycogen production (Xu et al., Impaired organization and function of myofilaments in single muscle fibers from a mouse model of Pompe disease, *J Appl Physiol* 108: 1383-1388, 2010).

Accordingly, in certain embodiments, the present disclosure contemplates methods of surveying improvements in disease phenotypes using the mature GAA constructs (e.g., the chimeric polypeptides comprising mature GAA) disclosed herein in any one or more animal models, such as the mouse models described herein. By way of example, various parameters can be examined in experimental animals treated with a subject chimeric polypeptide, and such animals can be compared to controls. Exemplary parameters that can be assessed to evaluate potential efficacy include, but are not limited to: increase in lifespan; increase in glycogen clearance, decrease in glycogen accumulation, decrease in alanine transaminase serum levels, decrease in aspartate transaminase serum levels, decrease in alkaline phosphatase serum levels, decrease in creatine phosphokinase serum levels, improved muscle strength, for example in open field and open wire hang paradigms, restoration of function of GAA in lysosomes in liver, skeletal muscle, smooth muscle and/or cardiac muscle. Increase in glycogen clearance and decrease in glycogen accumulation may be assessed, for example, by periodic acid Schiff staining in a biopsy (*e.g.*, muscle, liver or neuronal) from a treated or untreated animal model.

Moreover, once it is established that, for example, 3E10*mature GAA results in an improvement in any one or more of these phenotypes, a complete pharmacokinetic study to determine the effective dose, clearance rate, volume of distribution, and half-life of 3E10-

mature GAA can be determined. The PK/PD/TK of the final product can be examined in larger animals such as rats, dogs, and primates.

The above models are exemplary of suitable animal model systems for assessing the activity and effectiveness of the subject chimeric polypeptides and/or formulations. These models have correlations with symptoms of GAA deficiency, and thus provide appropriate models for studying Pompe disease. Activity of the subject chimeric polypeptides and/or formulations can be assessed in any one or more of these models, and the results compared to that observed in wildtype control animals and animals not treated with the chimeric polypeptides. Similarly, the subject chimeric polypeptides can be evaluated using cells in culture, for example, cells prepared from any of the foregoing mutant mice or other animals, as well as wild type cells, such as fibroblasts Additionally, cell free systems may be used to assess, for example, enzymatic activity of the subject chimeric polypeptides. An example of an in vitro assay for testing activity of the chimeric polypeptides disclosed herein would be to treat Pompe cells with or without the chimeric polypeptides and then, after a period of incubation, stain the cells for the presence of glycogen, *e.g.*, by using a periodic acid Schiff (PAS) stain. Another example of an in vitro assay for testing activity of the chimeric polypeptides disclosed herein would be a cell or cell-free assay in which whether the ability of the chimeric polypeptides to hydrolyze 4-methylumbelliferyl-α-D-glucoside as a substrate is assessed.

Chimeric polypeptides of the disclosure have numerous uses, including in vitro and in vivo uses. In vivo uses include not only therapeutic uses but also diagnostic and research uses in, for example, any of the foregoing animal models. By way of example, chimeric polypeptides of the disclosure may be used as research reagents and delivered to animals to understand GAA bioactivity, localization and trafficking, protein-protein interactions, enzymatic activity, and impacts on animal physiology in healthy or diseases animals.

Chimeric polypeptides may also be used in vitro to evaluate, for example, GAA bioactivity, localization and trafficking, protein-protein interactions, and enzymatic activity in cells in culture, including healthy and GAA deficient cells in culture. The disclosure contemplates that chimeric polypeptides of the disclosure may be used to deliver GAA to cytoplasm, lysosome, and/or autophagic vesicles of cells, including cells in culture.

X. Kits

In certain embodiments, the disclosure also provides a pharmaceutical package or kit comprising one or more containers filled with at least one chimeric polypeptide of the disclosure. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

In certain embodiments, the kit includes additional materials to facilitate delivery of the subject chimeric polypeptides. For example, the kit may include one or more of a catheter, tubing, infusion bag, syringe, and the like. In certain embodiments, the chimeric polypeptide is packaged in a lyophilized form, and the kit includes at least two containers: a container comprising the lyophilized chimeric polypeptide and a container comprising a suitable amount of water, buffer, or other liquid suitable for reconstituting the lyophilized material.

The foregoing applies to any of the chimeric polypeptides, compositions, and methods described herein. The disclosure specifically contemplates any combination of the features of such chimeric polypeptides, compositions, and methods (alone or in combination) with the features described for the various kits described in this section.

EXEMPLIFICATION

The disclosure now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present disclosure, and are not intended to limit the disclosure. For example, the particular constructs and experimental design disclosed herein represent exemplary tools and methods for validating proper function. As such, it will be readily apparent that any of the disclosed specific constructs and experimental plan can be substituted within the scope of the present disclosure.

Example 1: Chemical conjugation of 3E10 and human mature GAA (mAb3E10*mature GAA)

Chemical conjugation

In this example, ten milligrams (10 mg) of an exemplary 3E10 antigen binding fragment comprising a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9 and a light chain variable domain comprising the amino acid sequence of

SEQ ID NO: 10 (e.g., such as an scFv in which the VH and VL domains are interconnected via a linker) are conjugated covalently, directly or indirectly, to 76 kDa or 70 kDa mature human GAA, or to a GAA polypeptide comprising a mature human GAA, in a 1/1 molar ratio with the use of two different heterobifunctional reagents, succinimidyl 3-(2-pyridyldithio) propionate and succinimidyl trans-4-(maleimidylmethyl) cyclo-hexane-1-carboxylate. This reaction modifies the lysine residues of 3E10 into thiols and adds thiolreactive maleimide groups to mature GAA (Weisbart RH, et al., J Immunol. 2000 Jun 1; 164(11): 6020-6). After deprotection, each modified protein is reacted to each other to create a stable thioether bond. Chemical conjugation is performed, and the products are fractionated by gel filtration chromatography. The composition of the fractions is assessed by native and SDS-PAGE in reducing and nonreducing environments. Fractions containing the greatest ratio of 3E10-mature GAA conjugate to free 3E10 and free mature GAA are pooled and selected for use in later studies.

Similarly, conjugates are made in which an antigen binding portion of 3E10 (such as a single chain Fv fragment) or a full length 3E10 or a 3E10 Fab is conjugated to a mature GAA polypeptide, such as mature GAA polypeptide having a molecular weight of approximately 70-76 kDa (e.g., mature, active forms of GAA), or similar forms that use an alternative starting and/or ending residue, or to a GAA polypeptide comprising a mature GAA polypeptide. Other exemplary conjugates include conjugates in which the internalizing moiety is either a full length 3E10 mAb, or variant thereof, or an antigen binding fragment of the foregoing. The foregoing methods can be used to make chemical conjugates that include any combination of GAA portions and internalizing moiety portions, and the foregoing are merely exemplary. Both N-terminal and C-terminal conjugates are made (e.g., conjugates in which the 3E10 portion is N-terminal to the mature GAA portion and conjugates in which the 3E10 portion is C-terminal to the mature GAA portion). Moreover, the experimental approaches detailed herein can be used to evaluate any such chimeric polypeptide or to compare activity amongst chimeric polypeptides.

In vitro assessment of chemically conjugated 3E10 and mature GAA

Preparations of conjugated Fv3E10-mature GAA chimeric polypeptides and suitable control polypeptide are summarized in Table 1. The listed chimeric polypeptides are solely for illustrative purposes, and any chimeric polypeptides of the disclosure comprising a GAA polypeptide portion comprising a mature GAA and an internalizing moiety portion are similarly contemplated. Subject chimeric polypeptides are added, for example, to cell

cultures and the extent of protein uptake, protein localization and/or GAA enzymatic activity are determined and compared to controls. Similarly, GAA enzymatic activity can be assessed in cell free systems. We note that although, in this example, the internalizing moiety portion and GAA portion are chemically conjugated, each individual portion may be made recombinantly (e.g., by expressing nucleotide sequence encoding the polypeptide in a cell in culture and purifying the expressed polypeptide).

Table 1. Exemplary Chemically Conjugated Fv3E10 to human mature GAA

Note: Fv3E10 refers to an scFv antigen binding fragment of 3E10, as described above

Group	Polypeptides	
1	Fv3E10*mature GAA (76 kDa) Chemically conjugated	Fv3E10*mature GAA (70kDa) Chemically conjugated
2	Fv3E10 & mature GAA (76 kDa) Mixed unconjugated	Fv3E10 & mature GAA (70 kDa) Mixed unconjugated
3	Fv3E10 alone	Fv3E10 alone
4	mature GAA(76 kDa) alone	mature GAA(70 kDa) alone

i) Enzymatic activity of 3E10-mature GAA

GAA enzymatic activity is measured by determining the rate of 3E10-mature GAA catalyzed hydrolysis of a synthetic substrate, p-nitrophenyl-D-α-glucopyranoside, in 50 mM sodium acetate, 0.1% BSA, pH 4.3, as described in McVie et al. (Biochemical and Pharmacological Characterization of Different Recombinant Acid α-Glucosidase Preparations Evaluated for the Treatment of Pompe Disease, *Mol Genet Metab.*, 94(4): 448–455, 2008). The released chromophore, p-nitrophenol, is quantified spectrophotometrically at an alkaline pH (> 10.2) at 400 nm. One unit of mature GAA is defined as that amount of activity which resulted in the hydrolysis of 1 μmol of substrate per minute at 37°C under the assay conditions. Duplicate experiments are performed for Fv3E10 and mature GAA, Fv3E10 alone, or mature GAA alone.

ii) Uptake of 3E10-mature GAA

Uptake of 3E10-mature GAA is first assessed in COS-7 cells. Previous studies indicate that ENT2 is involved in 3E10 transport across the membrane of COS-7 cells

(Hansen et al., J. Biol. Chem., 282: 20790-20793, 2007), and a similar strategy can be used to determine transport of the chimeric 3E10-mature GAA across the membrane. Briefly, purified chimeric polypeptides are prepared in PBS with 10% fetal calf serum; control buffer is PBS with 10% fetal calf serum. 50 μL of control buffer or 3E10-mature GAA is added to COS-7 cells and incubated for 1 hour. The buffer is aspirated, cells are washed, fixed in chilled 100% ethanol, and stained with either an antibody to 3E10 or to GAA.

To demonstrate that muscle cells also uptake Fv3E10-mature GAA polypeptides, the same experiment is conducted in muscle cells. The murine cardiomyocte HL-1 cell line expresses ENT2 (Naydenova et al., Inosine and equilibrative nucleoside transporter 2 contribute to hypoxic preconditioning in the murine cardiomyocyte HL-1 cell line, Am J Physiol. Heart Circ. Physiol., 294(6):H2687-2692, 2008), and this cell line can be used in place of COS-7 cells in the above experiment.

Human Pompe fibroblasts (TR4192) are grown to confluence in a T-75 flask using MEM/FBS media. The cells are washed with PBS, trypsinized and plated at 1×10^6 cells/mL in a 96-well plate (100μL/well). Plates are incubated overnight at 37 °C. Following incubation, samples of Fv3E10 and mature GAA, Fv3E10 alone, or mature GAA alone (each assayed in triplicate) are diluted in reduced serum media (MEM/1% FBS) and added to the cells. Following a 24 hour incubation at 37 °C, cells are washed and lysed with the addition of PBS/1% Triton X-100 and frozen at -80 °C. A protein determination assay using bicinchoninic acid (BCA) and an activity analysis (using 4-methylumbelliferyl-α-D-glucoside as the substrate for GAA) are performed on the cell lysates in order to determine the extent of mature GAA uptake by cells McVie et al. (Biochemical and Pharmacological Characterization of Different Recombinant Acid α-Glucosidease Preparations Evaluated for the Treatment of Pompe Disease, *Mol Genet Metab.*, 94(4): 448–455, 2008).

iii) Immunoblot detection of cell-penetrating 3E10-mature GAA

Additional tests are performed to determine the uptake of 3E10-mature GAA in muscle fibers isolated from either wildtype or GAA KO mice (Bijvoet et al., Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease, Human Molecular Genetics, 7(1): 53-62, 1998). White gastrocnemius (G), tibialis anterior (TA) and extensor digitorum longus (EDL) muscles are removed immediately after sacrifice from WT and GAA-KO mice and pinned to Sylgaard coated dishes for fixation with 2% paraformaldehyde in 0.1M phosphate buffer for 1 h, followed by fixation in methanol (-

20 °C) for 6 min (Fukuda et al., Autophagy and Mis-targeting of Therapeutic Enzyme in Skeletal Muscle in Pompe Disease, Mol Ther., 14(6): 831-839, 2006). Single fibers are obtained by manual teasing.

Ten to 100 μM of chemically conjugated 3E10-mature GAA, an unconjugated mixture of Fv3E10 and mature GAA, Fv3E10 alone, or mature GAA alone are cultured with the isolated myofibers. The specificity of 3E10-mature GAA for the ENT2 transporter is validated by addition of nitrobenzylmercaptopurine riboside (NBMPR), an ENT2 specific inhibitor (Hansen et al., 2007, J.Biol.Chem., 282(29): 20790-3) to ENT2 transfected cells just prior to addition of 3E10-mature GAA. Eight to 24 hours later the myofibers are collected for immunoblot and Periodic acid-Schiff (PAS) stain for glycogen.

Myofibers are collected and resuspended in 500 ul PBS, lysed, and the supernatants are collected for immunoblot analysis of 3E10 and mature GAA. Epitope tagging will not, in certain embodiments, be employed, therefore the presence of a coincident anti-3E10 and anti-GAA immunoreactive band of ~150-156 kDa (for the full length 3E10 + 76 kDa GAA or full length 3E10 + 70 kDa GAA) in 3E10*mature GAA treated cells versus 3E10-alone and GAA-alone controls will constitute successful penetration of chemically conjugated 3E10*mature GAA. Tubulin detection is used as a loading control.

In addition, coverslips of treated cells are washed, fixed in 100% ethanol, rehydrated, and 3E10 and mature GAA are detected with previously described antibodies (Fukuda et al., Autophagy and Mis-targeting of Therapeutic Enzyme in Skeletal Muscle in Pompe Disease, Mol Ther., 14(6): 831-839, 2006).

Respective tissues are frozen, homogenized, and centrifuged to remove insoluble proteins, and protein content of the supernatants are measured by the Bradford assay. Equivalent amounts of protein are electrophoretically separated in a 10% polyacrylamide-SDS gel, are transferred to a nylon membrane, and are probed with a rabbit anti-human GAA polyclonal antibody. Detection of the bound anti-GAA antibody is visualized via the ECL detection system (Amersham Pharmacia).

iv) Tissue glycogen content

Myofibers from treated wildtype mice and GAA KO mice are fixed with 5% formaldehyde in 95% ethanol for 5 minutes at room temperature. Glycogen content is evaluated using high resolution light microscopy and computer assisted histomorphometry as described in McVie et al. (Biochemical and Pharmacological Characterization of Different Recombinant Acid α-Glucosidease Preparations Evaluated for the Treatment of

Pompe Disease, *Mol Genet Metab.*, 94(4): 448–455, 2008). Briefly, representative samples of muscle tissue are fixed in 3% gluteraldehyde in 0.2mol/L sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA) and embedded in epon-araldite. One micron sections are stained with Periodic acid-Schiff (PAS) reaction and counterstained with Richardson's solution. This results in high quality tissue preservation in which glycogen is fully retained and appears purple against a blue counterstain of myocyte cytoplasm. One representative field from each slide will be photographed with a Nikon DXM1200 digital camera (Nikon Inc, Instrument Group, Meville, NY) and analyzed using Metamorph Imaging Processing and Analysis software (version 4.6; Universal Imaging Corporation). For each image, glycogen load will be expressed as a percentage of total tissue area.

Example 2 Genetic construct of fv 3E10 and human mature GAA (Fv3E10-GS3-mature GAA)

Mammalian expression vectors encoding a genetic fusion of Fv3E10 and one of the two mature forms of human GAA (e.g., such as a GAA polypeptide comprising a mature GAA) (fv3E10-GS3-mature GAA, comprising the scFv of mAb 3E10 fused to mature GAA by, for example, the GS3 linker) will be generated. Note that in the examples, we have used "Fv3E10" to refer to an scFv of 3E10. Note that these genetic fusions are also referred to as recombinant conjugates or recombinantly produced conjugates. Other linkers may similarly be used. Further, linkerless fusions where the 3E10 moiety and the mature GAA moiety are directly fused may also be used. Similarly fusions to a portion of a full length antibody or Fab may be made. As with the chemical conjugates, recombinant fusions comprising any of the chimeric polypeptides of the disclosure are contemplated. Recombinantly produced chimeric polypetides may comprises a GAA polypeptide portion, according to the disclosure (e.g., a GAA polypeptide comprising a mature GAA) and an internalizing moiety portion, according to the disclosure.

Additional recombinantly produced conjugates will similarly be made for later testing. By way of non-limiting example: (a) mature GAA-GS3-3E10, (b) 3E10-GS3-mature GAA, (c) mature GAA-GS3-Fv3E10, (d) mature GAA-3E10, (e) 3E10-mature GAA, (f) mature GAA-Fv3E10. Note that throughout the examples, the abbreviation Fv is used to refer to a single chain Fv of 3E10. Similarly, mAb 3E10 and 3E10 are used interchangeably. Similarly, mature GAA refers to a mature GAA protein having a

molecular weight of from about 70-76 kDa, such as a mature GAA protein having a molecular weight of about 76 kDa or about 70 kDa. These and other chimeric polypeptides can be tested using, for example, the assays detailed herein. Further polypeptides in which the chimeric polypeptides comprise a mature GAA polypeptide, but which polypeptides also include additional contiguous GAA polypeptide sequence (but not the entire 110 kD precursor polypeptide and/or not the signal sequence of the set forth in residues 1-56 or SEQ ID NO: 1 or 2) are also contemplated and can similarly be made and tested.

Create the cDNA for human GAA and confirm activity in vitro

i) Synthesis of the cDNA for GAA

The full-length, 3.6 kb human GAA cDNA that encodes a full length, precursor form of human GAA (hGAA cDNA) may be found at http://www.ncbi.nlm.nih.gov/sites/entrez, for example, under GenBank Accession No. NM_000152.3. This cDNA sequences and other transcript variants are hereby incorporated in their entirety. A portion of such a human cDNA sequence corresponding approximately to the region that encodes mature GAA is used herein to generate a recombinant construct. However, it is also contemplated that the full length cDNA can be used.

The mature GAA cDNA along with flanking restriction sites that facilitate cloning into appropriate expression vectors will be synthesized and sequenced by Genscript or other qualified manufacturer of gene sequences. To maximize expression, the mature GAA cDNA will be codon optimized for mammalian and pichia expression. In the event that mammals or pichia prefer a different codon for a given amino acid, we will use the next best candidate to unify the preference. The resulting cDNA is cloned into a CMV-based mammalian expression cassette and large scale preps of the plasmid pCMV-mature GAA will be made using the Qiagen Mega Endo-free plasmid purification kit. To avoid complicating immune responses to the 3E10-GAA protein, epitopes or purification tags are not, in certain embodiments, included. However, conjugates that do include such tags may also be made and tested.

ii) Transfection of cells in vitro

A strategy to assess the function of GAA in transfected cells is described above. Ten micrograms of the plasmid pCMV (mock) or pCMV-mature GAA is transfected into 1) COS-7 cells, 2) HL-1 cells, 3) myofibers from wildtype mice, and 4) myofibers from GAA KO mice using commercially available transfection reagents (Table 2). To track the

efficiency of transfection, duplicate transfections with plasmids encoding a suitable reporter such as beta-galactosidase or GFP is performed. Forty-eight hours later transfected cells are pelleted by centrifugation resuspended in 500 µl PBS for protein and immunoblot analysis.

Table 2. Transfection strategy for pCMV and pCMV-mature GAA

Group	Cells	Transfected plasmid
1	COS-7	pCMV (mock)
2	COS-7	pCMV-mature GAA (76 kDa or 70 kDa)
3	HL-1	pCMV (mock)
4	HL-1	pCMV-mature GAA (76 kDa or 70 kDa)
5	Myofibers from GAA KO mice $6^{neo}/6^{neo}$	pCMV (mock)
6	Myofibers from GAA KO mice $6^{neo}/6^{neo}$	pCMV-mature GAA (76 kDa or 70 kDa)
7	Myofibers from WT mice	pCMV (mock)
8	Myofibers from WT mice	pCMV-mature GAA (76 kDa or 70 kDa)

iii) Viral infection with AAV cDNA construct

Constructs described above are cloned into an adenovirus vector plasmid, according to methods described in Sun et al., (Enhanced Efficacy of an AAV Vector Encoding Chimeric, Highly-Secreted Acid α-glucosidase in Glycogen Storage Disease Type II, Mol Ther., 14(6): 822–830, 2006). These constructs provide a means to test the cDNA constructs in cells, and/or use constructs in vivo for gene therapy.

Briefly, 293 cells are transfected with an AAV vector plasmid, the AAV packaging plasmid p5E18-VD 2/8, and pAdHelper (Stratagene, La Jolla, CA). Cell lysate is harvested

48 hours following infection, freeze-thawed 3 times, and isolated by sucrose cushion pelleting followed by 2 cesium chloride gradient centrifugation steps. AAV stocks are dialyzed against 3 changes of Hanks buffer, and aliquots are stored at -80 °C. The number of vector DNA containing-particles is determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks are handled according to Biohazard Safety Level 2 guidelines published by the NIH.

The uptake of chimeric mature GAA is analyzed in (1) COS-7 cells, (2) HL-1 cells, and (3) Pompe disease patient cells as described in Example 1 above. COS-7 cells, HL-1 cells, or fibroblasts from a GSD-II patient are grown in medium containing 10% FBS and incubated for 40 hours with the medium of transfected 293 cells producing chimeric hGAA with activity of 300 nmol.hr.ml. GAA activity and glycogen in cultured patient fibroblasts is analyzed as described above.

iii) Immunoblot detection of transfected human GAA, and assay of GAA mediated hydrolysis of glycogen.

The same procedures described in Example 1 are utilized.

Create and validate cDNA Fv3E10 genetically conjugated to 76 kDa or 70 kDa forms of GAA

i) Synthesis of the cDNA for Fv3E10

The cDNA encoding the mouse Fv3E10 variable light chain linked to the 3E10 heavy chain (SEQ ID NOs: 9-10) contains a mutation in the VH CDR1 that enhances the cell penetrating capacity of the Fv fragment (Zack et al., 1996, J Immunol, 157(5): 2082-8). The 3E10 cDNA is flanked by restriction sites that facilitate cloning in frame with the cDNA coding sequence that corresponds to the amino acid sequences in the mature forms of GAA (SEQ ID NOS: 3-4) or with a GAA polypeptide comprising mature GAA. The constructs are synthesized and sequenced by Genscript or other qualified manufacturer of gene sequences. To maximize expression the 3E10 cDNA will be codon optimized for mammalian and pichia expression. In the event that mammals or pichia prefer a different codon for a given amino acid, the next best candidate to unify the preference will be used. The resulting cDNA will be cloned into a mammalian expression cassette and large scale preps of the plasmid pCMV-3E10-mature GAA will be made using the Qiagen Mega Endo-

free plasmid purification kit. The constructs will be tested in 1) COS-7 cells, 2) HL-1 cells, 3) myofibers from wildtype mice, and 4) myofibers from GAA KO mice. A transfection strategy is outlined in Table 3.

Table 3. Transfection strategy for pCMV 3E10-GS3-GAA

Group	Cells	Transfected plasmid
1	COS-7	pCMV
2	COS-7	pCMV-GAA (76 kDa or 70 kDa)
3	COS-7	pCMV Fv3E10-GS3-GAA (76 kDa or 70 kDa)
4	HL-1	pCMV
5	HL-1	pCMV-GAA (76 kDa or 70 kDa)
6	HL-1	pCMV Fv3E10-GS3-GAA (76 kDa or 70 kDa)
7	Myofibers from GAA KO mice $6^{neo}/6^{neo}$	pCMV
8	Myofibers from GAA KO mice $6^{neo}/6^{neo}$	pCMV-GAA (76 kDa or 70 kDa)
9	Myofibers from GAA KO mice $6^{neo}/6^{neo}$	pCMV Fv3E10-GS3-GAA (76 kDa or 70 kDa)
10	Myofibers from WT mice	pCMV (mock)
11	Myofibers from WT mice	pCMV-GAA (76 kDa or 70 kDa)
12	Myofibers from WT mice	pCMV Fv3E10-GS3-GAA (76 kDa or 70 kDa)

ii) Transfection of cells

The strategy to test the expression and glycogen hydrolysis of the 3E10-GS3-mature GAA genetic fusion is described above. The transfection procedure will be the same as described above for transfection of the human GAA cDNA. Transfected cells will be assayed for expression of hGAA and hydrolysis of glycogen.

Production of recombinant 3E10 genetically conjugated to mature GAA

i) Construction of protein expression vectors for pichia. Plasmid construction, transfection, colony selection and culture of Pichia will use kits and manuals per the manufacturer's instructions (Invitrogen). The cDNAs for genetically conjugated 3E10-GS3-mature GAA created and validated as described above will be cloned into two alternative plasmids; PICZ for intracellular expression and PICZalpha for secreted expression. Protein expression from each plasmid is driven by the AOX1 promoter. Transfected pichia will be selected with Zeocin and colonies will be tested for expression of recombinant 3E10-GS3-mature GAA. High expressers will be selected and scaled for purification.

ii) Purification of recombinant 3E10-GS3-mature GAA

cDNA fusions with mAb 3E10 Fv are ligated into the yeast expression vector pPICZA which is subsequently electroporated into the Pichia pastoris X-33 strain. Colonies are selected with Zeocin (Invitrogen, Carlsbad, CA) and identified with anti-his6 antibodies (Qiagen Inc, Valencia, CA X-33 cells are grown in baffled shaker flasks with buffered glycerol/methanol medium, and protein synthesis is induced with 0.5% methanol according to the manufacturer's protocol (EasySelect Pichia Expression Kit, Invitrogen, Carlsbad, CA). The cells are lysed by two passages through a French Cell Press at 20,000 lbs/in2, and recombinant protein is purified from cell pellets solubilized in 9M guanidine HCl and 2% NP40 by immobilized metal ion affinity chromatography (IMAC) on Ni-NTAAgarose (Qiagen, Valencia, CA). Bound protein is eluted in 50 mM NaH2PO4 containing 300 mM NaCl, 500 mM imidazole, and 25% glycerol. Samples of eluted fractions are electrophoresed in 4-20% gradient SDSPAGE (NuSep Ltd, Frenchs Forest, Australia), and recombinant proteins is identified by Western blotting to nitrocellulose membranes developed with cargo-specific mouse antibodies followed by alkalinephosphatase-conjugated goat antibodies to mouse IgG. Alkaline phosphatase activity is measured by the chromogenic substrate, nitroblue tetrazolium chloride/5-bromo-

4-chloro-3-indolylphosphate p-toluidine salt. Proteins are identified in SDS-PAGE gels with GelCode Blue Stain Reagent (Pierce Chemical Co., Rockford, IL). Eluted protein is concentrated, reconstituted with fetal calf serum to 5%, and exchange dialyzed 100-fold in 30,000 MWCO spin filters (Millipore Corp., Billerica, MA) against McCoy's medium (Mediatech, Inc., Herndon, VA) containing 5% glycerol. Although in this example a Pichia expression system is illustrated, protein may alsos be produced in other expression systems, including mammalian expressions systems such as CHO cells. Vectors and methodologies, including contract manufacturing services, for expressing proteins in CHO cells are available, for example, from Lonza.

iii) Quality assessment and formulation

Immunoblot against 3E10 and mature GAA is used to verify the size and identity of recombinant proteins, followed by silver staining to identify the relative purity among preparations of 3E10, GAA and 3E10-GS3-mature GAA. Recombinant material is formulated in a buffer and concentration (~0.5 mg/ml).

iv) In vitro assessment of recombinant material

The activity of 3E10-GS3-mature GAA protein is evaluated using any one or more of the assays detailed in Example 1. Cell penetration and/or enzymatic activity is compared to suitable controls. Moreover, the amount of 3E10-GS3-mature GAA protein needed to alleviate the GAA deficiency is determined using the methods described above. The amounts of GAA activity in mammalian cell-derived and pichia-derived recombinant 3E10-GS3-mature GAA can be tested, for example, on (1) fibroblasts from Pompe disease patients and control patients and (2) myofibers isolated from wildtype and GAA KO mice.

Example 3 In vivo assessment of muscle targeted GAA in GAA KO mice

GAA mouse models for evaluation

Several GAA KO mice have been characterized previously: a GAA KO line (Bijvoet et al., Generalized glycogen storage and cardiomegaly in a knockout mouse model of Pompe disease, Human Molecular Genetics, 7(1): 53-62, 1998); a line in which exon 6 has been replaced with loxP-flanked neo gene ($6^{neo}/6^{neo}$) or deleted ($\Delta6/\Delta6$) (Raben et al., Targeted Disruption of the Acid α-Glucosidase Gene in Mice Causes an Illness with Critical Features of Both Infantile and Adult Human Glycogen Storage Disease Type II, J. Biological Chemistry, 272(30): 19086-19092, 1998); a mouse line in which exon 14 has been deleted ($\Delta14^{neo}/\Delta14^{neo}$) (Raben et al., Modulation of disease severity in mice with

targeted disruption of the acid alpha-glucosiase gene, Neuromuscl. Disord. 10: 283-291, 2000); a GAA KO crossed with an inducible hGAA that restores GAA specifically to the liver or muscles (Raben et al., Conditional tissue-specific expression of the acid alpha-glucosidase (GAA) gene in the GAA knockout mouse: implications for therapy, Hum. Molec. Genet. 10: 2039-2047, 2001); a GAA KO crossed with an inducible hGAA mouse line in which expression of GAA in the liver induces immunological tolerance to GAA; (Raben et al., Induction of tolerance to a recombinant human enzyme, acid alpha-glucosidase, in enzyme deficient knockout mice, Transgenic Research, 12:171-178, 2003); a GAA KO/SCID mouse developed to avoid an immune response to GAA in GAA KO mice (Xu et al., Improved efficacy of gene therapy approaches for Pompe disease using a new, immune-deficient GSD-II mouse model, Gene Therapy, 11:15890-1598, 2004); and a double KO mouse of GAA and glycogen synthase 1, in which the effects of decreased glycogen production are studied (Xu et al., Impaired organization and function of myofilaments in single muscle fibers from a mouse model of Pompe disease, *J Appl Physiol* 108: 1383-1388, 2010).

Of these mice, the $6^{neo}/6^{neo}$ and $\Delta14^{neo}/\Delta14^{neo}$ mice show features of the infantile and adult phenotypes of Pompe disease, while $\Delta6/\Delta6$ shows muscle weakness at a later age. All mice show abnormal glycogen storage in heart and skeletal muscle. Accordingly, all of these models serve as appropriate animal models to test the efficacy of 3E10-mature GAA therapy. If the mice develop an immunological response to high doses of 3E10-mature GAA, then one of the immune tolerant mouse models is used.

Selection of dose of mature GAA

To determine a dosage that treats glycogen accumulation in skeletal muscle without inducing a harmful immune response, weekly doses of 1 mg/kg, 20 mg/kg, or 100 mg/kg of fv3E10*mature GAA or fv3E10-GS3-mature GAA are injected intravenously to GAA KO mice, followed by assessment of changes in glycogen accumulation. The development of anti-3E10-GAA antibodies are also monitored. Assessment of changes in muscle morphology, muscle fiber strength, and decrease in autophagic vacuoles are also evaluated. The below table illustrates exemplary chimeric polypeptides that can be evaluated. As noted above, any of the chimeric polypeptides of the disclosure are similarly made and tested.

Table 4. In vivo dosing plan for chemically and genetically conjugated Fv3E10-GAA

Group	Mouse Genotype	*Age (months)	# of mice	Treatment	Dose (mg/kg)	*Months of treatment
1	GAA KO (or immune tolerant GAA KO line)	1.5	5	Fv3E10*mature GAA (76 kDa or 70 kDa) Chemically conjugated	1, 20, or 100	3
2	GAA KO (or immune tolerant GAA KO line)	1.5	5	Fv3E10 & mature GAA (76 kDa or 70 kDa) Mixed unconjugated	1, 20, or 100	3
3	GAA KO (or immune tolerant GAA KO line)	1.5	5	Fv3E10-GS3-mature GAA (76 kDa or 70 kDa) Genetic conjugate	1, 20, or 100	3
4	GAA KO (or immune tolerant GAA KO line)	1.5	5	Vehicle	N/A	3
5	WT	1.5	5	Fv3E10*mature GAA (76 kDa or 70 kDa) Chemically conjugated	1, 20, or 100	3
6	WT	1.5	5	Fv3E10 & mature GAA (76 kDa or 70 kDa) Mixed unconjugated	1, 20, or 100	3
7	WT	1.5	5	Fv3E10-GS3-mature GAA (76 kDa or 70 kDa) Genetic conjugate	1, 20, or 100	3
8	WT	1.5	5	Vehicle	N/A	3
* Animals at 3.5 months or 6 months will also be tested. The months of treatment for these two groups will be 5 months and 1-2 months, respectively. Note that chemical or genetic conjugates with 3E10 or Fv3E10 are tested.						

Materials and Methods

i) Injection of chemically and genetically conjugated 3E10-mature GAA

Fv3E10*mature GAA or Fv3E10-GS3-mature GAA is formulated and diluted in a buffer that is consistent with intravenous injection (e.g. sterile saline solution or a buffered solution of 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl). The amount of 3E10*mature GAA or 3E10-GS3-mature GAA given to each mouse is calculated as follows: dose (mg/kg) x mouse weight (kg) x stock concentration (mg/ml) = volume (ml) of stock per mouse, q.s. to

100 µl with vehicle. Exemplary Fv3E10-GS3-mature GAA chimeric polypeptides for use in these experiments are set forth in SEQ ID NO: 11 or 12.

ii) Measurement of anti-GAA antibodies

The immune response to 3E10*mature GAA is determined as described in Raben et al., (Enzyme replacement therapy in the mouse model of Pompe disease, Molecular Genetics and Metabolism, 80: 159-166, 2003). Briefly, blood is drawn from the tail vein at various time points to test the sera for the presence of antibodies to the enzyme by ELISA using 96-well plates. Plates are coated overnight at 4 °C with 5µg/ml of rhGAA in PBS, incubated with blocking solution (0.1% BSA in PBS) for 2 h at 37 °C, and washed with TPBS (0.1% Tween-20 in PBS). Next, the plates are incubated with serial dilutions of test sera (1:100–1:12 800) in triplicates for 1 h at 37 °C, washed extensively, incubated for 1 h at 37 °C with 1:12000 goat-anti-mouse IgG-horseradish peroxidase (Southern Biotechnology, Birmingham, Al), washed again, and developed with trimethylbenzidine (TMB) substrate for 15 min at room temperature (KPL, Gaithersburg, MD). The reaction will be stopped by adding 1N HCL, and the plates will be read in a microplate reader at 450/650 nm.

iii) Tissue collection and preparation

Sampled tissues are obtained from the liver, heart, diaphragm, and lower leg skeletal muscles of 3E10*mature GAA, 3E10-GS3-mature GAA, or control untreated mice at 48-72 hour after the final dose of mature enzyme. Tissues are homogenized for measuring GAA activity, extracts are prepared for glycogen measurements and/or tissues are fixed, embedded, and sectioned into 1 µm sections for staining. For electron microscopy of muscle tissues, clamped muscle specimens are fixed in 2.5% buffered glutaraldehyde in 0.1 M sodium cacodylate buffer for 2-4 h at 4°C, stored in 0.1M cacodylate buffer (pH 7.4) and further processed.

iv) Histological evaluation

For periodic acid Schiff analysis, tissues are fixed in 10% formalin, embedded in paraffin, sectioned, and stained with periodic acid-Schiff (PAS) by standard methods. Additionally, PAS-staining and histochemistry are performed on snap-frozen muscle biopsies. Serial transverse sections (7µm) are stained with alkaline ATPase (pH 10.4) or NADH-TR by standard methods.

v) Immunohistochemistry

To assess proliferation of lysosomes, as described by Raben et al., (Enzyme replacement therapy in the mouse model of Pompe disease, Molecular Genetics and Metabolism, 80: 159-166, 2003), antibodies to the lysosomal membrane protein LAMP are used. In addition, MPR antibodies are used to determine which histochemical muscle fiber types (as assessed by staining for alkaline ATPase or NADH) are able to clear glycogen most effectively. The following primary antibodies may be used: (FITC)-conjugated rat anti-mouse CD107b (LAMP-2; 1:20) (BD Biosciences, San Diego, CA); (FITC)-conjugated rat antimouse CD107a (LAMP-1; 1:20); and rabbit anti-bovine CI-MPR (1:500). Seven micrometer sections of muscle biopsies, snap-frozen in liquid nitrogen cooled isopentane, are collected on cover slips coated with poly-L-lysine and fixed in cold acetone for 10 min followed by re-hydration by immersing in Tris-buffered saline (TBS) for 10 min. Non-specific binding sites are blocked with 10% horse serum in phosphate-buffered saline (PBS) for 1 h in a humidified chamber at room temperature followed by incubation with primary antibodies for 24 h at 4 °C in a humid chamber. After several washes in PBS, the sections are either mounted with antifade mounting reagent mixture (ProLong Antifade kit, Molecular Probes, Inc., Eugene, OR) or developed with phycoerythrin (PE) conjugated goat F (ab')2 anti-rabbit IgG (H+L) (1:200) secondary antibody (Caltag laboratories, Burlingame, CA) (for CI-MPR) prior to mounting.

vi) Tests of muscle function

Assessment of motor ability: locomotor activity in an open field is measured in a Digiscan apparatus (Omnitech Electronics), as described in Raben et al. (Enzyme replacement therapy in the mouse model of Pompe disease, Molecular Genetics and Metabolism, 80: 159-166, 2003). Total distance, horizontal activity, and vertical activity is measured by the total number of photocell beam breaks in 10-min intervals during 1-h sessions. Three independent sessions are conducted for each animal over a period of 1 week. Animals are starved overnight before sacrifice. The student's t test will be used for comparisons between the groups. Differences are considered significant at $p < 0:05$.

In addition, all mice are subjected to a battery of motor tests to determine muscle function, as described in Sidman et al., Temporal Neuropathological and Behavioral Phenotype of $6^{neo}/6^{neo}$ Pompe Disease Mice, J Neuropathol Exp Neurol. 67(8): 803-818, 2008). Briefly, all mice are evaluated once a week from 3 months of age. Motor coordination and balance are measured with a rotating rod apparatus (SmartRod, Accuscan

Instruments, Columbus, OH). For the rocking rotorod test, the rod is programmed to rock backwards and forwards for up to 2.5 sec duration with the overall acceleration in either direction increasing to 25 rpm. Cutoff times are 60 seconds for the accelerating test and 54 seconds for the rocking test. Animals are tested three times on each version of the test with a rest period of at least 5 minutes between measurements. Average fall latency from the rod (or cutoff time) is recorded for each animal and used for statistical analysis. Analyses of locomotor function is performed with the Student *t* test (Prism GraphPad, San Diego, CA). Data are mean ± SEM. $p < 0.05$ is considered as a statistically significant difference. For the Foot Fault Test, each animal is placed on a wire rack with square holes for 60 seconds and the number of times the paws slipped into the holes is recorded. Each animal is tested twice. Mean values are used for statistical analysis. Strength is measured with a Wire Hang Test. The ability to hang upside down from a wire screen placed 60 cm above a large housing cage is measured as a latency to fall into the cage. A score of zero is assigned to animals that fall immediately and a score of 60 seconds is assigned to animals that did not fall. Cut-off time is 60 seconds. Each animal is tested twice and means are used for statistical analyses. Data will be expressed as mean ± SEM. For two groups the Student *t* test is used; for more than two group comparisons, one-way ANOVA is used followed by the post hoc Bonferroni multiple comparison test.

vii) Assessing serum enzyme levels

Blood is collected from tail veins or from the venous sinus from each mouse every three to four days for the duration of the study. Samples are tested for levels of alanine transaminase, aspartate transaminase, alkaline phosphatase, and/or creatine phosphokinase. Decrease in the elevated levels of one or more of these enzymes is indicative of reduction of some of the pathological effects of cytoplasmic glycogen accumulation.

viii) Survival Assessment

Those treated and untreated diseased and control mice that are not sacrificed in the experiments described above will be monitored in a survival study. Specifically, the disease state, treatment conditions and date of death of the animals are recorded. A survival curve will be prepared based on the results of this study.

Example 4: Trials of 3E10*mature GAA and 3E10-GS3-mature GAA in human Pompe disease patients

Following the examples of clinical trials in infantile-onset Pompe disease patients and late-onset Pompe patients (summarized by Schoser et al., Therapeutic approaches in Glycogen Storage Disease type II (GSDII)/Pompe disease, Neurotherapeutics, 5(4): 569-578, 2008), 3E10*mature GAA and/or 3E10-GS3-mature GAA is administered intravenously to patients in, for example, dosages of up to 40 mg/kg weekly (such as chimeric polypeptides comprising a GAA portion comprising mature GAA and an internalizing moiety portion). Patients may receive 10 mg/kg weekly for 52 weeks, or may receive 5-20 mg/kg weekly or biweekly for 153 weeks. Patients are monitored for tolerance of the therapeutic and for improvements in glycogen clearance, tissue morphology, motor function, and/or cardiac function. Muscle biopsies are taken, and analyzed by high-resolution light microscopy, digital histomorphometry, electron microscopy, capillary density, fiber type analysis, and/or confocal microscopy. The left ventricular mass index (LVMI) of infants is monitored. Motor milestones, for example walking or sitting upright, of infants and toddlers undergoing treatment is compared with age-matched subjects not suffering from Pompe disease. Dependence on ventilator support for breathing is also be monitored.

The foregoing experimental schemes will similarly be used to evaluate other chimeric polypeptides. By way of non-limiting example, this scheme will be used to evaluate chemical conjugates and fusion proteins having a GAA portion (or a fragment thereof) and an internalizing moiety portion. By way of further example, the foregoing methods may also be used to evaluate use of compositions comprising a mixture of two or more conjugates, such as a mixture of 3E10*mature GAA (70kDa) and 3E10*mature GAA (76 kDa).

The particular chimeric polypeptides described above for evaluation in examples 1-4 are exemplary of the chimeric polypeptides of the disclosure – any of which can be made and evaluated using, for example, the methods described in examples 1-4. By way of example, chemical and genetic conjugates in the presence or absence of a linker are made and tested. Conjugates in which the mature GAA moiety is located N-terminal to the internalizing moiety, as well as conjugates in which the mature GAA moiety is located C-terminal to the internalizing moiety are made and test. Any of a range of internalizing moieties and linker moieties are used.

By way of non-limiting example the following chimeric polypeptides are made and tested: (a) mature GAA-GS3-3E10, (b) 3E10-GS3-mature GAA, (c) mature GAA-GS3-

Fv3E10, (d) mature GAA-3E10, (e) 3E10-mature GAA, (f) mature GAA-Fv3E10, (g) 3E10*mature GAA, (h) mature GAA*3E10, (i) internalizing moiety*mature GAA, (j) mature GAA*internalizing moiety, (k) internalizing moiety-GS3-mature GAA, (l) mature GAA-GS3-internalizing moiety. Note that throughout the examples, the abbreviation Fv is used to refer to a single chain Fv of 3E10. Similarly, mAb 3E10 and 3E10 are used interchangeably. Similarly, mature GAA refers to a mature GAA protein having a molecular weight of from about 70-76 kDa, such as a mature GAA protein having a molecular weight of about 76 kDa or about 70 kDa. These and other chimeric polypeptides can be tested using, for example, the assays detailed herein.

By way of further example, the foregoing methods may also be used to evaluate use of compositions comprising a mixture of two or more conjugates, such as a mixture of 3E10*mature GAA (70kDa) and 3E10*mature GAA (76 kDa).

Example 5: Generation and Characterization of 3E10 mAb-GAA and 3E10 Fab-GAA Fusion Constructs

We expressed representative chimeric polypeptides according to the protocol described in Hacker et al., 2013, Protein Expr Purif. 92: 67. Specifically, chimeric polypeptides comprising a GAA polypeptide portion and an internalizing moiety portion were made recombinantly. In this experiment, a GAA polypeptide comprising mature GAA (the GAA polypeptide portion of the chimeric polypeptide) was fused to either a full-length murine monoclonal 3E10 antibody comprising the light chain variable domain set forth in SEQ ID NO: 10 and the heavy chain variable domain set forth in SEQ ID NO: 9 (the internalizing moiety portion), or to a Fab of this 3E10 antibody (see Figure 1). Specifically, in this example, the N-terminus of a GAA polypeptide comprising a mature GAA and having the amino acid sequence of SEQ ID NO: 22 was fused to the C-terminus of either the heavy chain constant region of a murine 3E10 Fab fragment or to the C-terminus of the heavy chain constant region of a full-length murine 3E10 monoclonal antibody (mAb). In this example, the heavy chain of the internalizing moiety comprises murine 3E10 antibody comprises the foregoing VH and a murine heavy chain constant domain comprising CH1, hinge, CH2, and CH3 regions, such as constant domain regions from an IgG1, IgG2a, IgG2b, or IgG4 antibody. In either case, a nucleotide sequence expressing the recombinant heavy chain and 3E10 light chain comprising the foregoing 3E10 VL were inserted into separate vectors and transiently transfected into CHO-DG44

cells in order to produce to produce the recombinant, chimeric protein. Similarly, the nucleotide sequence encoding the heavy and light chains could be expressed from a single vector. The chimeric constructs are shown schematically in Figure 1.

In this example, a linker sequence was used to fuse the GAA polypeptide to the Fab or mAb heavy chains, and that linker had the amino acid sequence of SEQ ID NO: 30.

Exemplary Sequences

SEQ ID NO: 1 = full-length, immature GAA amino acid sequence (952 amino acids; signal sequence indicated in bold/underline)

MGVRHPPCSHRLLAVCALVSLATAALLGHILLHDFLLVPRELSGSSPVLEETHP AHQQGASRPGPRDAQAHPGRPRAVPTQCDVPPNSRFDCAPDKAITQEQCEARGCC YIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTATLTRTTPTFFPKDILT LRLDVMMETENRLHFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSEEPFGVIVR RQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWN RDLAPTPGANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGG ILDVYIFLGPEPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVEN MTRAHFPLDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVD PAISSSGPAGSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWE DMVAEFHDQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAA TICASSHQFLSTHYNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGH WTGDVWSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAF YPFMRNHNSLLSLPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGET VARPLFLEFPKDSSTWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQT VPVEALGSLPPPPAAPREPAIHSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTE SRQQPMALAVALTKGGEARGELFWDDGESLEVLERGAYTQVIFLARNNTIVNELV RVTSEGAGLQLQKVTVLGVATAPQQVLSNGVPVSNFTYSPDTKVLDICVSLLMGE QFLVSWC

SEQ ID NO: 2 = full-length, immature GAA amino acid sequence (957 amino acids; signal sequence indicated in bold/underline)

(GenBank Accession No. EAW89583.1)

MGVRHPPCSHRLLAVCALVSLATAALLGHILLHDFLLVPRELSGSSPVLEETHP
AHQQGASRPGPRDAQAHPGRPRAVPTQCDVPPNSRFDCAPDKAITQEQCEARGCC
YIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTATLTRTTPTFFPKDILT
LRLDVMMETENRLHFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSEEPFGVIVR
RQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWN
RDLAPTPGANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGG
ILDVYIFLGPEPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVEN
MTRAHFPLDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVD
PAISSSGPAGSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWE
DMVAEFHDQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAA
TICASSHQFLSTHYNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGH
WTGDVWSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAF
YPFMRNHNSLLSLPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGET
VARPLFLEFPKDSSTWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQT
VPIEALGSLPPPPAAPREPAIHSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTES
RQQPMALAVALTKGGEARGELFWDDGESLEVLERGAYTQVIFLARNNTIVNELVR
VTSEGAGLQLQKVTVLGVATAPQQVLSNGVPVSNFTYSPDTKARGPRVLDICVSLL
MGEQFLVSWC

SEQ ID NO: 3 = exemplary mature GAA amino acid sequence (corresponding to residues 123-782 of SEQ ID NO: 1; one embodiment of a mature GAA polypeptide)
GQPWCFFPPSYPSYKLENLSSSEMGYTATLTRTTPTFFPKDILTLRLDVMMETENRL
HFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSEEPFGVIVRRQLDGRVLLNTTV
APLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPTPGANLYG
SHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPKS
VVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQW
NDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRP
YDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFHDQVPF
DGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQFLSTH
YNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQ
LASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLS
LPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDS
STWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEA

SEQ ID NO: 4 = exemplary mature GAA amino acid sequence (corresponding to residues 288-782 of SEQ ID NO: 1; one embodiment of a mature GAA polypeptide)
GANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFL
GPEPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPL
DVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPA
GSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFH
DQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQ
FLSTHYNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVW
SSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNH
NSLLSLPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLE
FPKDSSTWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEA

SEQ ID NO: 5 = GS3 linker
GGGGSGGGGSGGGGS

SEQ ID NO: 6 = Linker
GSTSGSGKSSEGKG

SEQ ID NO: 7 = His tag
HHHHHH

SEQ ID NO: 8 = c-Myc tag
EQKLISEEDL

SEQ ID NO: 9 = exemplary 3E10 Variable Heavy Chain (V_H having D31N substitution; see examples)
EVQLVESGGGLVKPGGSRKLSCAASGFTFSNYGMHWVRQAPEKGLEWVAYISSGS
STIYYADTVKGRFTISRDNAKNTLFLQMTSLRSEDTAMYYCARRGLLLDYWGQGT
TLTVSS

SEQ ID NO: 10 = 3E10 Variable Light Chain (V_L)

DIVLTQSPASLAVSLGQRATISCRASKSVSTSSYSYMHWYQQKPGQPPKLLIKYASY
LESGVPARFSGSGSGTDFHLNIHPVEEEDAATYYCQHSREFPWTFGGGTKLELK

SEQ ID NO: 11 = Exemplary chimeric polypeptide, Fv3E10-GAA (123-782)

DIVLTQSPASLAVSLGQRATISCRASKSVSTSSYSYMHWYQQKPGQPPKLLIKYASY
LESGVPARFSGSGSGTDFHLNIHPVEEEDAATYYCQHSREFPWTFGGGTKLELKGG
GGSGGGGSGGGGSEVQLVESGGGLVKPGGSRKLSCAASGFTFSNYGMHWVRQAP
EKGLEWVAYISSGSSTIYYADTVKGRFTISRDNAKNTLFLQMTSLRSEDTAMYYCA
RRGLLLDYWGQGTTLTVSSEQKLSEEDLGSTSGSGKSSEGKGGQPWCFFPPSYPSY
KLENLSSSEMGYTATLTRTTPTFFPKDILTLRLDVMMETENRLHFTIKDPANRRYEV
PLETPRVHSRAPSPLYSVEFSEEPFGVIVHRQLDGRVLLNTTVAPLFFADQFLQLSTS
LPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPTPGANLYGSHPFYLALEDGGSA
HGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPKSVVQQYLDVVGYPF
MPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQWNDLDYMDSRRDFTF
NKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRLYDEGLRRGVFITNET
GQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFHDQVPFDGMWIDMNEPSNFIR
GSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQFLSTHYNLHNLYGLTEAIAS
HRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQLASSVPEILQFNLLG
VPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLSLPQEPYSFSEPAQQA
MRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDSSTWTVDHQLLWGEA
LLITPVLQAGKAEVTGYFPLGTWYDLQTVPIEAHHHHHH

SEQ ID NO:12 = Exemplary chimeric polypeptide, Fv3E10-GAA (288-782)

DIVLTQSPASLAVSLGQRATISCRASKSVSTSSYSYMHWYQQKPGQPPKLLIKYASY
LESGVPARFSGSGSGTDFHLNIHPVEEEDAATYYCQHSREFPWTFGGGTKLELKGG
GGSGGGGSGGGGSEVQLVESGGGLVKPGGSRKLSCAASGFTFSNYGMHWVRQAP
EKGLEWVAYISSGSSTIYYADTVKGRFTISRDNAKNTLFLQMTSLRSEDTAMYYCA
RRGLLLDYWGQGTTLTVSSEQKLSEEDLGSTSGSGKSSEGKGGANLYGSHPFYLAL
EDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPKSVVQQYLDV
VGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQWNDLDYMDS
RRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRLYDEGLRRG
VFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFHDQVPFDGMWIDM
NEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQFLSTHYNLHNLYG

LTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQLASSVPEIL
QFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLSLPQEPYSFS
EPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDSSTWTVDHQ
LLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPIEAHHHHHH

SEQ ID NO: 13 –heavy chain variable domain CDR1 of 3E10 VH (as that VH is defined with reference to SEQ ID NO: 9), in accordance with Kabat system
NYGMH

SEQ ID NO: 14 –heavy chain variable domain CDR2 of 3E10 VH (as that VH is defined with reference to SEQ ID NO: 9), in accordance with Kabat system
YISSGSSTIYYADTVKG

SEQ ID NO: 15 –heavy chain variable domain CDR3 of 3E10 VH (as that VH is defined with reference to SEQ ID NO: 9), in accordance with Kabat system
RGLLLDY

SEQ ID NO: 16 – light chain variable domain CDR1 of 3E10 VL (as that VL is defined with reference to SEQ ID NO: 10), in accordance with Kabat system
RASKSVSTSSYSYMH

SEQ ID NO: 17 – light chain variable domain CDR2 of 3E10 VL (as that VL is defined with reference to SEQ ID NO: 10), in accordance with Kabat system
YASYLES

SEQ ID NO: 18 – light chain variable domain CDR3 of 3E10 VL (as that VL is defined with reference to SEQ ID NO: 10), in accordance with Kabat system
QHSREFPWT

SEQ ID NO: 19
AGIH

SEQ ID NO: 20

SAGIH

SEQ ID NO: 21- Exemplary GAA polypeptide comprising mature GAA (residues 61-952; one embodiment of a GAA polypeptide)

SRPGPRDAQAHPGRPRAVPTQCDVPPNSRFDCAPDKAITQEQCEARGCCYIPAKQG
LQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTATLTRTTPTFFPKDILTLRLDVM
METENRLHFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSEEPFGVIVRRQLDGR
VLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPT
PGANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIF
LGPEPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFP
LDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGP
AGSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEF
HDQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSH
QFLSTHYNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDV
WSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMR
NHNSLLSLPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLF
LEFPKDSSTWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEAL
GSLPPPPAAPREPAIHSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTESRQQPM
ALAVALTKGGEARGELFWDDGESLEVLERGAYTQVIFLARNNTIVNELVRVTSEG
AGLQLQKVTVLGVATAPQQVLSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSW
C

SEQ ID NO: 22- Exemplary GAA polypeptide comprising mature GAA (residues 67-952; one embodiment of a GAA polypeptide)

DAQAHPGRPRAVPTQCDVPPNSRFDCAPDKAITQEQCEARGCCYIPAKQGLQGAQ
MGQPWCFFPPSYPSYKLENLSSSEMGYTATLTRTTPTFFPKDILTLRLDVMMETEN
RLHFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSEEPFGVIVRRQLDGRVLLNTT
VAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPTPGANLY
GSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPK
SVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQW
NDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRP
YDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFHDQVPF
DGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQFLSTH

YNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQ
LASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLS
LPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDS
STWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEALGSLPPPP
AAPREPAIHSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTESRQQPMALAVAL
TKGGEARGELFWDDGESLEVLERGAYTQVIFLARNNTIVNELVRVTSEGAGLQLQ
KVTVLGVATAPQQVLSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSWC

SEQ ID NO: 23- Exemplary GAA polypeptide comprising mature GAA (residues 70-952; one embodiment of a GAA polypeptide)

AHPGRPRAVPTQCDVPPNSRFDCAPDKAITQEQCEARGCCYIPAKQGLQGAQMGQ
PWCFFPPSYPSYKLENLSSSEMGYTATLTRTTPTFFPKDILTLRLDVMMETENRLHF
TIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSEEPFGVIVRRQLDGRVLLNTTVAP
LFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPTPGANLYGSH
PFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPKSVV
QQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQWNDL
DYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRPYDE
GLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFHDQVPFDGM
WIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQFLSTHYNL
HNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQLAS
SVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLSLPQ
EPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDSSTW
TVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEALGSLPPPPAAPR
EPAIHSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTESRQQPMALAVALTKGG
EARGELFWDDGESLEVLERGAYTQVIFLARNNTIVNELVRVTSEGAGLQLQKVTVL
GVATAPQQVLSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSWC

SEQ ID NO: 24 – heavy chain variable (VH) domain CDR1 of exemplary 3E10 V_H (as that VH is defined with reference to SEQ ID NO: 9), in accordance with the IMGT system

GFTFSNYG

SEQ ID NO: 25 – heavy chain variable (VH) domain CDR2 of exemplary 3E10 V_H (as that VH is defined with reference to SEQ ID NO: 9), in accordance with the IMGT system

ISSGSSTI

SEQ ID NO: 26 – heavy chain variable (VH) domain CDR3 of exemplary 3E10 V_H (as that VH is defined with reference to SEQ ID NO: 9), in accordance with the IMGT system
ARRGLLLDY

SEQ ID NO: 27 – light chain variable (VL) domain CDR1 of exemplary 3E10 V_L (as that VL is defined with reference to SEQ ID NO: 10), in accordance with the IMGT system
KSVSTSSYSY

SEQ ID NO: 28 – light chain variable (VL) domain CDR2 of exemplary 3E10 V_L (as that VL is defined with reference to SEQ ID NO: 10), in accordance with the IMGT system
YAS

SEQ ID NO: 29 – light chain variable (VL) domain CDR3 of exemplary 3E10 V_L (as that VL is defined with reference to SEQ ID NO: 10), in accordance with the IMGT system
QHSREFPWT

SEQ ID NO: 30- linker sequence
GGSGGGSGGGSGG

SEQ ID NO: 31- full linker region (residues 57-78 of GAA)
HILLHDFLLVPRELSGSSPVLEETHPAH

SEQ ID NO: 32- bovine GAA precursor protein (GenBank Accession No. NP_776338.1)

MMRWPPCSRPLLGVCTLLSLALLGHILLHDLEVVPRELRGFSQDEIHQACQPGASSP
ECRGSPRAAPTQCDLPPNSRFDCAPDKGITPQQCEARGCCYMPAEWPPDAQMGQP
WCFFPPSYPSYRLENLTTTETGYTATLTRAVPTFFPKDIMTLRLDMLMETESRLHFT
IKDPANRRYEVPLETPRVYSQAPFTLYSVEFSEEPFGVVVRRKLDGRVLLNTTVAPL
FFADQFLQLSTSLPSQHITGLAEHLGSLMLSTNWTKITLWNRDIAPEPNVNLYGSHP
FYLVLEDGGLAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPKSVVQ
QYLDVVGYPFMPPYWGLGFHLCRWGYSTSAITRQVVENMTRAYFPLDVQWNDLD

YMDARRDFTFNKDHFGDFPAMVQELHQGGRRYIMIVDPAISSSGPAGTYRPYDEG
LRRGVFITNETGQPLIGQVWPGLTAFPDFTNPETLDWWQDMVTEFHAQVPFDGM
WIDMNEPSNFVRGSVDGCPDNSLENPPYLPGVVGGTLRAATICASSHQFLSTHYDL
HNLYGLTEALASHRALVKARGMRPFVISRSTFAGHGRYSGHWTGDVWSNWEQLS
YSVPEILLFNLLGVPLVGADICGFLGNTSEELCVRWTQLGAFYPFMRNHNALNSQP
QEPYRFSETAQQAMRKAFTLRYVLLPYLYTLFHRAHVRGETVARPLFLEFPEDPST
WTVDRQLLWGEALLITPVLEAEKVEVTGYFPQGTWYDLQTVPMEAFGSLPPPAPL
TSVIHSKGQWVTLSAPLDTINVHLRAGHIIPMQGPALTTTESRKQHMALAVALTAS
GEAQGELFWDDGESLGVLDGGDYTQLIFLAKNNTFVNKLVHVSSEGASLQLRNVT
VLGVATAPQQVLCNSVPVSNFTFSPDTETLAIPVSLTMGEQFVISWS

SEQ ID NO: 33- KFERQ

KFERQ

SEQ ID NO: 34- $(G_4S)_n$, wherein n is an integer from 1-10

$(GGGGS)_n$

SEQ ID NO: 35- ASSLNIA homing peptide

ASSLNIA

SEQ ID NO: 36- Arg7 Peptide

RRRRRRR

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject disclosure have been discussed, the above specification is illustrative and not restrictive. Many variations of the disclosure will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the disclosure should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

WE CLAIM:

1. A chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety;
wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

2. A chimeric polypeptide comprising: (i) a GAA polypeptide comprising a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety that binds DNA with a K_D of less than 100 nM and/or promotes transit across cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter;
wherein the chimeric polypeptide does not comprise the full length, GAA precursor polypeptide set forth in SEQ ID NO: 1.

3. The chimeric polypeptide of claim 1 or 2, wherein the chimeric polypeptide does not comprise the portion of GAA polypeptide set forth in residues 1-56 of SEQ ID NO: 1 or 2.

4. The chimeric polypeptide of any of claims 1-3, wherein the chimeric polypeptide does not comprise the portion of GAA polypeptide set forth in residues 1-57 of SEQ ID NO: 1 or 2.

5. The chimeric polypeptide of any of claims 1-4, wherein the chimeric polypeptide has acid alpha-glucosidase activity.

6. The chimeric polypeptide of any of claims 1-5, wherein the chimeric polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2.

7. The chimeric polypeptide of any of claims 1-6, wherein neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-60 of SEQ ID NO: 1 or 2.

8. The chimeric polypeptide of claim 7, wherein the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 21.

9. The chimeric polypeptide of any of claims 1-7, wherein neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-66 of SEQ ID NO: 1 or 2.

10. The chimeric polypeptide of claim 9, wherein the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22.

11. The chimeric polypeptide of any of claims 1-7, wherein neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-69 of SEQ ID NO: 1 or 2.

12. The chimeric polypeptide of claim 11, wherein the chimeric polypeptide or GAA polypeptide comprises the sequence of SEQ ID NO: 23.

13. The chimeric polypeptide of any of claims 1-12, wherein the mature GAA polypeptide has a molecular weight of approximately 70-76 kilodaltons.

14. The chimeric polypeptide of any of claims 1-13, wherein the mature GAA polypeptide has a molecular weight of approximately 70 kilodaltons.

15. The chimeric polypeptide of any of claims 1-13, wherein the mature GAA polypeptide has a molecular weight of approximately 76 kilodaltons.

16. The chimeric polypeptide of any of claims 1-5 or 13-15, wherein the mature GAA polypeptide consists of an amino acid sequence selected from residues 122-782 of SEQ ID NO: 1 or residues 204-782 of SEQ ID NO: 1.

17. The chimeric polypeptide of any of claims 1-16, wherein the mature GAA polypeptide is glycosylated.

18. The chimeric polypeptide of any of claims 1-16, wherein the mature GAA polypeptide is not glycosylated.

19. The chimeric polypeptide of any of claims 1-16, wherein the mature GAA polypeptide has a glycosylation pattern that differs from that of naturally occurring human GAA.

20. The chimeric polypeptide of any of claims 1-19, wherein the internalizing moiety promotes delivery of the chimeric polypeptide into cytoplasm of cells.

21. The chimeric polypeptide of any of claims 1-20, wherein the chimeric polypeptide is capable of being taken up by an autophagic vacuole.

22. The chimeric polypeptide of any of claims 1-21, wherein the internalizing moiety promotes transport of said chimeric polypeptide into muscle cells.

23. The chimeric polypeptide of any of claims 1-22, wherein the internalizing moiety promotes transport of said chimeric polypeptide into hepatocytes.

24. The chimeric polypeptide of any of claims 1-22, wherein the internalizing moiety promotes transport of said chimeric polypeptide into neurons.

25. The chimeric polypeptide of any of claims 1-24, wherein the chimeric polypeptide comprises *N*-linked oligosaccharide chains modified with M6P residues.

26. The chimeric polypeptide of any of claims 1-25, wherein the chimeric polypeptide further comprises one or more polypeptide portions that enhance one or more of in vivo stability, in vivo half life, uptake/administration, production, or purification.

27. The chimeric polypeptide of any of claims 1-26, wherein the internalizing moiety comprises an antibody or antigen binding fragment that can transit a cellular membrane via an equilibrative nucleoside transporter 2 (ENT2) transporter and/or binds DNA with a K_D of less than 100 nM.

28. The chimeric polypeptide of claim 27, wherein said antibody is a monoclonal antibody or fragment thereof.

29. The chimeric polypeptide of claim 27 or 28, wherein said antibody or antigen binding fragment is a monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing.

30. The chimeric polypeptide of any of claims 27-29, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains the cell penetrating activity of 3E10, or an antigen binding fragment of 3E10 or said 3E10 variant.

31. The chimeric polypeptide of any of claims 27-30, wherein the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment.

32. The chimeric polypeptide of any of claims 27-31, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof.

33. The chimeric polypeptide of any of claims 27-32, wherein the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof.

34. The chimeric polypeptide of any of claims 27-33, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9, or a humanized variant thereof, and a light chain

variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof.

35. The chimeric polypeptide of any of claims 27-34, wherein the antibody or antigen binding fragment comprises
a VH CDR1 having the amino acid sequence of SEQ ID NO 13;
a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;
a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;
a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;
a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and
a VL CDR3 having the amino acid sequence of SEQ ID NO: 18;
which CDRs are according to Kabat.

36. The chimeric polypeptide of any of claims 27-34, wherein the antibody or antigen binding fragment comprises
a VH CDR1 having the amino acid sequence of SEQ ID NO 24;
a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;
a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;
a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;
a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and
a VL CDR3 having the amino acid sequence of SEQ ID NO: 29;
which CDRs are according to the IMGT system.

37. The chimeric polypeptide of any of claims 27-36, wherein the antibody or antigen binding fragment is an scFv.

38. The chimeric polypeptide of any of claims 27-36, wherein the antibody or antigen binding fragment is an Fab.

39. The chimeric polypeptide of any of claims 27-36, wherein the antibody or antigen binding fragment is an antibody.

40. The chimeric polypeptide of claim 38, wherein the N-terminus of the GAA polypeptide is fused to the C-terminus of the heavy chain of the Fab.

41. The chimeric polypeptide of claim 39, wherein the N-terminus of the GAA polypeptide is fused to the C-terminus of the heavy chain of the antibody.

42. The chimeric polypeptide of claim 40, wherein the N-terminus of the GAA polypeptide is fused to the C-terminus of the heavy chain of the Fab by means of a linker.

43. The chimeric polypeptide of claim 41, wherein the N-terminus of the GAA polypeptide is fused to the C-terminus of the heavy chain of the antibody by means of a linker.

44. The chimeric polypeptide of claim 42 or 43, wherein the linker comprises the amino acid sequence of SEQ ID NO: 30.

45. The chimeric polypeptide of any of claims 1-44, wherein the internalizing moiety is capable of binding DNA with a K_D of less than 1 μM.

46. The chimeric polypeptide of any of claims 1-45, wherein the internalizing moiety binds DNA with a K_D of less than 50 nM.

47. The chimeric polypeptide of any of claims 1-27, wherein the internalizing moiety comprises a homing peptide.

48. The chimeric polypeptide of any of claims 1-47, wherein the chimeric polypeptide is a chemical conjugate of GAA polypeptide to the internalizing moiety.

49. The chimeric polypeptide of any of claims 1-47, wherein the chimeric polypeptide is a recombinant, co-translational fusion protein comprising the GAA polypeptide and the internalizing moiety.

50. The chimeric polypeptide of any of claims 1-49, wherein the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 1 (ENT1), ENT2, ENT3, or ENT4 transporter.

51. The chimeric polypeptide of claim 50, wherein the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter.

52. The chimeric polypeptide of any of claims 1-51, wherein the chimeric polypeptide is a fusion protein.

53. The chimeric polypeptide of claim 52, wherein the chimeric polypeptide is produced in a prokaryotic or eukaryotic cell.

54. The chimeric polypeptide of claim 53, wherein the eukaryotic cell is selected from a yeast cell, an avian cell, an insect cell, or a mammalian cell.

55. The chimeric polypeptide of any of claims 1-54, wherein the chimeric polypeptide comprises a linker that conjugates or joins, directly or indirectly, the GAA polypeptide to the internalizing moiety.

56. The chimeric polypeptide of any of claims 1-54, wherein the chimeric polypeptide does not include a linker interconnecting the GAA polypeptide to the internalizing moiety.

57. The chimeric polypeptide of claim 55, wherein the linker is a cleavable linker.

58. The chimeric polypeptide of any of claims 55-57, wherein the internalizing moiety is N-terminal to the GAA polypeptide.

59. The chimeric polypeptide of any of claims 55-57, wherein the internalizing moiety is conjugated or joined to an internal amino acid of the GAA polypeptide.

60. A nucleic acid construct, comprising a nucleotide sequence that encodes the chimeric polypeptide of any of claims 1-59.

61. A nucleic acid construct, comprising a nucleotide sequence that encodes a mature GAA polypeptide, operably linked to a nucleotide sequence that encodes a internalizing moiety, wherein the nucleic acid construct encodes a chimeric polypeptide having the internalizing activity of the internalizing moiety, and wherein the nucleic acid construct does not encode a chimeric polypeptide comprising a GAA precursor polypeptide of approximately 110 kilodaltons.

62. A nucleic acid construct, comprising a nucleotide sequence that encodes a GAA polypeptide, operably linked to a nucleotide sequence that encodes a internalizing moiety, wherein the nucleic acid construct encodes a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety that promotes transit across cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter and/or that binds DNA with a K_D of less than 100 nM;
wherein the chimeric polypeptide does not comprise the full length, GAA polypeptide set forth in SEQ ID NO: 1.

63. The nucleic acid construct of claim 61 or 62, wherein the chimeric polypeptide has acid alpha-glucosidase activity.

64. The nucleic acid construct of claim 61 or 62, wherein the chimeric polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2.

65. The nucleic acid construct comprising a nucleotide sequence encoding the chimeric polypeptide of any of claims 6-12.

66. The nucleic acid construct of any of claims 61-65, wherein the internalizing moiety promotes delivery into muscle cells.

67. The nucleic acid construct of any of claims 61-66, wherein the internalizing moiety promotes delivery into hepatocytes.

68. The nucleic acid construct of any of claims 61-67, wherein the internalizing moiety promotes delivery into neurons.

69. The nucleic acid construct of any of claims 60-68, wherein the internalizing moiety transits cellular membranes via an ENT1, ENT2, ENT3 or ENT4 transporter.

70. The nucleic acid construct of claim 69, wherein the internalizing moiety transits cellular membranes via an ENT2 transporter.

71. The nucleic acid construct of any of claims 61-70, wherein the nucleotide sequence that encodes the GAA polypeptide encodes a mature GAA polypeptide having a molecular weight of approximately 70-76 kilodaltons.

72. The nucleic acid construct of any of claims 61-71, wherein the nucleotide sequence that encodes the GAA polypeptide encodes a mature GAA polypeptide having a molecular weight of approximately 70 kilodaltons.

73. The nucleic acid construct of any claims 61-70, wherein the nucleotide sequence that encodes the GAA polypeptide encodes a mature GAA polypeptide having a molecular weight of approximately 76 kilodaltons.

74. The nucleic acid construct of any of claims 61-73, wherein the nucleotide sequence that encodes the GAA polypeptide encodes a mature GAA polypeptide consisting of an amino acid sequence selected from residues 122-782 of SEQ ID NO: 1 or residues 204-782 of SEQ ID NO: 1.

75. The nucleic acid construct of any of claims 61-74, further comprising a nucleotide sequence that encodes a linker.

76. The nucleic acid construct of any of claims 61-75, wherein the internalizing moiety is an antibody or an antigen binding fragment that can transit a cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter and/or can bind DNA with a K_D of less than 100 nM.

77. The nucleic acid construct of claim 76, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing.

78. The nucleic acid construct of claim 76 or 77, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains the cell penetrating activity of 3E10, or an antigen binding fragment of 3E10 or said 3E10 variant.

79. The nucleic acid construct of any of claims 76-78, wherein the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment.

80. The nucleic acid construct of any of claims 76-79, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof.

81. The nucleic acid construct of any of claims 76-80, wherein the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof.

82. The nucleic acid construct of any of claims 76-81, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9, or a humanized variant thereof, and a light chain

variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof.

83. The nucleic acid construct of any of claims 76-82, wherein the antibody or antigen binding fragment comprises
a VH CDR1 having the amino acid sequence of SEQ ID NO 13;
a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;
a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;
a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;
a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and
a VL CDR3 having the amino acid sequence of SEQ ID NO: 18;
which CDRs are according to Kabat.

84. The nucleic acid construct of any of claims 76-82, wherein the antibody or antigen binding fragment comprises
a VH CDR1 having the amino acid sequence of SEQ ID NO 24;
a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;
a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;
a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;
a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and
a VL CDR3 having the amino acid sequence of SEQ ID NO: 29;
which CDRs are according to the IMGT system.

85. The nucleic acid construct of any of claims 76-84, wherein the antibody or antigen binding fragment is a Fab.

86. The nucleic acid construct of any of claims 76-84, wherein the antibody or antigen binding fragment is an antibody.

87. The nucleic acid construct of any of claims 61-86, wherein the internalizing moiety is a homing peptide.

88. A vector comprising the nucleic acid construct of any of claims 60-87.

89. A host cell comprising the vector of claim 88.

90. A host cell comprising and capable of expressing the vector of claim 89.

91. A method of producing a chimeric polypeptide comprising culturing the host cell of claim 89 or 90 under appropriate conditions to allow expression of the polypeptide to occur.

92. A composition comprising the chimeric polypeptide of any of claims 1-59, and a pharmaceutically acceptable carrier.

93. The composition of claim 92, wherein said composition is substantially pyrogen-free.

94. A method of treating Pompe disease in a subject in need thereof, comprising administering to the subject an effective amount of the chimeric polypeptide of any of claims 1-59.

95. A method of treating Pompe disease in a subject in need thereof, comprising administering to the subject an effective amount of the composition of claim 92 or 93.

96. The method of claim 94 or 95, wherein said subject in need thereof is a subject whose disease has been refractory to one or more previous enzyme replacement therapies.

97. The method of any of claims 94-96, wherein said subject in need thereof is a subject having pathologic cytoplasmic glycogen accumulation prior to initiation of treatment with said chimeric polypeptide.

98. The method of any of claims 94-97, wherein said subject in need thereof is a subject diagnosed with Pompe disease greater than six months prior to initiation of treatment with said chimeric polypeptide.

99. The method of claim 98, wherein said subject in need thereof is a subject diagnosed with Pompe disease at least one year prior to initiation of treatment with said chimeric polypeptide.

100. The method of any of claims 94-98, wherein said subject in need thereof is a subject in whom the onset of symptoms of Pompe disease occurred greater than six months prior to initiation of treatment with said chimeric polypeptide.

101. The method of claim 100, wherein said subject in need thereof is a subject in whom the onset of symptoms of Pompe disease occurred at least one year prior to initiation of treatment with said chimeric polypeptide.

102. A method of treating Pompe disease in a subject in need thereof, comprising administering to the subject an effective amount of a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety;
wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

103. A method of increasing acid alpha-glucosidase enzyme activity in a cell, comprising contacting the cell with a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety;
wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

104. A method of increasing acid alpha-glucosidase enzyme activity in a cell, comprising contacting the cell with a chimeric polypeptide comprising: (i) an acid alpha-glucosidase (GAA) polypeptide comprising a mature GAA polypeptide and (ii) an

internalizing moiety that promotes transit across cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter and/or binds to DNA with a K_D of less than 100 nM;
wherein the chimeric polypeptide does not comprise the full length, GAA polypeptide set forth in SEQ ID NO: 1.

105. The method of claim 103 or 104, wherein the chimeric polypeptide has acid alpha-glucosidase activity.

106. The method of any of claims 103-105, wherein the chimeric polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2.

107. A method of increasing acid alpha-glucosidase enzyme activity in a cell, comprising contacting the cell with the chimeric polypeptide of any of claims 1-59.

108. The method of any of claims 102-107, wherein the mature GAA polypeptide has a molecular weight of approximately 70-76 kilodaltons.

109. The method of any of claims 102-108, wherein the mature GAA polypeptide consists of an amino acid sequence selected from residues 122-782 of SEQ ID NO: 1 or residues 204-782 of SEQ ID NO: 1.

110. The method of any of claims 102-109, wherein the internalizing moiety promotes delivery of the chimeric polypeptide into cytoplasm of cells.

111. The method of any of claims 103-110, wherein the cell is a cell in a subject in need thereof.

112. The method of any of claims 102-111, wherein the internalizing moiety promotes transport of said chimeric polypeptide into muscle cells.

113. The method of any of claims 102-112, wherein the internalizing moiety promotes delivery of said chimeric polypeptide into hepatocytes.

114. The method of any of claims 102-113, wherein the internalizing moiety promotes delivery of said chimeric polypeptide into neurons.

115. The method of any of claims 94-114, wherein the chimeric polypeptide is also delivered to lysosomes.

116. The method of any of claims 94-115, wherein said chimeric polypeptide reduces cytoplasmic glycogen accumulation.

117. The method of any of claims 94-116, wherein said chimeric polypeptide reduces lysosomal glycogen accumulation.

118. The method of any of claims 94-117, wherein said chimeric polypeptide reduces glycogen accumulation in autophagic vacuoles.

119. The method of any of claims 94-118, wherein said chimeric polypeptide reduces glycogen accumulation in cytoplasm, lysosomes, and autophagic vacuoles.

120. The method of any of claims 118-119, wherein the GAA polypeptide is glycosylated.

121. The method of any of claims 118-119, wherein the GAA polypeptide is not glycosylated.

122. The method of any of claims 118-119, wherein the GAA polypeptide has a glycosylation pattern that differs from that of naturally occurring human GAA.

123. The method of any of claims 118-122, wherein the chimeric polypeptide comprises *N*-linked oligosaccharide chains modified with M6P residues.

124. The method of any of claims 118-123, wherein the chimeric polypeptide comprises a KFERQ-like motif.

125. The method of any of claims 118-124, wherein said subject in need thereof is a subject whose disease has been refractory to one or more previous enzyme replacement therapies.

126. The method of any of claims 118-127, wherein said subject in need thereof is a subject having pathologic cytoplasmic glycogen accumulation prior to initiation of treatment with said chimeric polypeptide.

127. The method of any of claims 118-126, wherein said subject in need thereof is a subject diagnosed with Pompe disease greater than six months prior to initiation of treatment with said chimeric polypeptide.

128. The method of claim 127, wherein said subject in need thereof is a subject diagnosed with Pompe disease at least one year prior to initiation of treatment with said chimeric polypeptide.

129. The method of any of claims 118-128, wherein said subject in need thereof is a subject in whom the onset of symptoms of Pompe disease occurred greater than six months prior to initiation of treatment with said chimeric polypeptide.

130. The method of claim 129, wherein said subject in need thereof is a subject in whom the onset of symptoms of Pompe disease occurred at least one year prior to initiation of treatment with said chimeric polypeptide.

131. The method of any of claims 118-130, wherein the internalizing moiety comprises an antibody or antigen binding fragment that can transit a cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter and/or bind DNA with a K_D of less than 100 nM.

132. The method of claim 131, wherein said antibody is a monoclonal antibody or fragment thereof.

133. The method of claim 131-132, wherein said antibody or antigen binding fragment is 3E10, or an antigen binding fragment thereof.

134. The method of any of claims 118-133, wherein the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 1 (ENT1), ENT2, ENT3 or ENT4 transporter.

135. The method of claim 134, wherein the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter.

136. The method of any of claims 131-133, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing.

137. The method of claim 136, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains the cell penetrating activity of 3E10, or an antigen binding fragment of 3E10 or said 3E10 variant.

138. The method of any of claims 131-137, wherein the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment.

139. The method of any of claims 131-138, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof.

140. The method of any of claims 131-139, wherein the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof.

141. The method of any of claims 131-140, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9, or a humanized variant thereof, and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof.

142. The method of any of claims 131-141, wherein the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 13;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 18;

which CDRs are according to Kabat.

143. The method of any of claims 131-141, wherein the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 24;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 29;

which CDRs are according to the IMGT system.

144. The method of any of claims 131-143, wherein the antibody or antigen binding fragment is an Fab.

145. The method of any of claims 131-143, wherein the antibody or antigen binding fragment is an antibody.

146. The method of any of claims 131-145, wherein the chimeric polypeptide is the chimeric polypeptide of any of claims 36-46.

147. The method of any of claims 65-146, wherein the chimeric polypeptide comprises a linker that conjugates or joins, directly or indirectly, the GAA polypeptide to the internalizing moiety.

148. The method of any of claims 131-147, wherein the chimeric polypeptide does not include a linker, and the GAA polypeptide is conjugated or joined directly to the internalizing moiety.

149. The method of claim 147, wherein the linker is a cleavable linker.

150. The method of any of claims 131-149, wherein the chimeric polypeptide is formulated with a pharmaceutically acceptable carrier.

151. The method of any of claims 131-150, wherein the chimeric polypeptide is administered systemically.

152. The method of claim 151, wherein the chimeric polypeptide is administered intravenously.

153. A method of treating Pompe disease in a subject in need thereof, comprising administering to the subject an effective amount of a chimeric polypeptide, nucleic acid construct, or composition of any of claims 1-93.

154. Use of the chimeric polypeptide of any of claims 1-59 in the manufacture of a medicament for treating Pompe disease.

155. Use of the chimeric polypeptide of any of claims 1-59 for treating Pompe disease.

156. Use of the nucleic acid construct of any of claims 60-91 in the manufacture of a medicament for treating Pompe disease.

157. Use of the nucleic acid construct of any of claims 60-91 for treating Pompe disease.

158. Use of the composition of claim 92 or 93 for treating Pompe disease.

159. A method of decreasing glycogen accumulation in cytoplasm of muscle cells, comprising:
contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprises (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety;
wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

160. A method of decreasing glycogen accumulation in cytoplasm and lysosomes of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprises (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety;
wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

161. A method of decreasing glycogen accumulation in cytoplasm, lysosomes, and autophagic vacuoles of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprises (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety;
wherein the chimeric polypeptide has acid alpha-glucosidase activity, and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

162. A method of decreasing glycogen accumulation in cytoplasm and lysosomes of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprising: (i) an acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety that promotes transit across cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter;
wherein the chimeric polypeptide does not comprise the full length, GAA precursor polypeptide set forth in SEQ ID NO: 1.

163. A method of decreasing glycogen accumulation in cytoplasm, lysosomes, and autophagic vacuoles of muscle cells, comprising contacting muscle cells with a chimeric polypeptide, which chimeric polypeptide comprising: (i) an acid alpha-glucosidase (GAA) polypeptide and (ii) an internalizing moiety that promotes transit across cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter;
wherein the chimeric polypeptide does not comprise the full length, GAA precursor polypeptide set forth in SEQ ID NO: 1.

164. The method of claim 162 or 163, wherein the chimeric polypeptide has acid alpha-glucosidase activity.

165. The method of claim 162 or 163, wherein the chimeric polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2.

166. A method of decreasing glycogen accumulation in cytoplasm and lysosomes of muscle cells, comprising contacting the cell with the chimeric polypeptide of any of claims 6-12.

167. A method of decreasing glycogen accumulation in cytoplasm, lysosomes, and autophagic vacuoles of muscle cells, comprising contacting the cell with the chimeric polypeptide of any of claims 6-12.

168. The method of any of claims 159-167, wherein the mature GAA polypeptide has a molecular weight of approximately 70-76 kilodaltons.

169. The method of any of claims 159-168, wherein the mature GAA polypeptide has a molecular weight of approximately 70 kilodaltons.

170. The method of any of claims 159-169, wherein the mature GAA polypeptide has a molecular weight of approximately 76 kilodaltons.

171. The method of any of claims 159-170, wherein the mature GAA polypeptide consists of an amino acid sequence selected from: residues 122-782 of SEQ ID NO: 1, residues 123-782 of SEQ ID NO: 1, or residues 204-782 of SEQ ID NO: 1.

172. The method of any of claims 159-171, wherein the GAA polypeptide is glycosylated.

173. The method of any of claims 159-171, wherein the GAA polypeptide is not glycosylated.

174. The method of any of claims 159-171, wherein the GAA polypeptide has a glycosylation pattern that differs from that of naturally occurring human GAA.

175. The method of any of claims 159-174, wherein the internalizing moiety promotes transport of the chimeric polypeptide into cytoplasm of cells.

176. The method of any of claims 159-175, wherein the chimeric polypeptide comprises *N*-linked oligosaccharide chains modified with M6P residues.

177. The method of any of claims 159-176, wherein the chimeric polypeptide comprises a KFERQ-like motif.

178. The method of any of claims 159-177, wherein the cell is a cell in a subject in need of treatment for Pompe disease, and contacting said cell comprising administering the chimeric polypeptide to the subject.

179. The method of claim 178, wherein said subject is a subject whose Pompe disease has been refractory to one or more previous enzyme replacement therapies.

180. The method of claim 178 or 179, wherein said subject is a subject having pathologic cytoplasmic glycogen accumulation prior to initiation of treatment with said chimeric polypeptide.

181. The method of any of claims 178-180, wherein said subject is a subject diagnosed with Pompe disease greater than six months prior to initiation of treatment with said chimeric polypeptide.

182. The method of claim 181, wherein said subject is a subject diagnosed with Pompe disease at least one year prior to initiation of treatment with said chimeric polypeptide.

183. The method of any of claims 178-182, wherein said subject is a subject in whom the onset of symptoms of Pompe disease occurred greater than six months prior to initiation of treatment with said chimeric polypeptide.

184. The method of claim 183, wherein said subject is a subject in whom the onset of symptoms of Pompe disease occurred at least one year prior to initiation of treatment with said chimeric polypeptide.

185. The method of any of claims 159-184, wherein the internalizing moiety comprises an antibody or antigen binding fragment.

186. The method of claim 185, wherein said antibody is a monoclonal antibody or fragment thereof.

187. The method of claim 185 or 186, wherein said antibody is monoclonal antibody 3E10, or an antigen binding fragment thereof.

188. The method of any of claims 159-187, wherein the internalizing moiety transits cellular membranes via an equilibrative nucleoside transporter 1 (ENT1), ENT2, ENT3 or ENT4 transporter.

189. The method of claim 188, wherein the internalizing moiety transits cellular membranes via an ENT2 transporter.

190. The method of any of claims 185-188, wherein said antibody or antigen binding fragment is a monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing.

191. The method of claim 190, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains the cell penetrating activity of 3E10, or an antigen binding fragment of 3E10 or said 3E10 variant.

192. The method of any of claims 185-188, wherein the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment.

193. The method of any of claims 185-192, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof.

194. The method of any of claims 185-193, wherein the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof.

195. The method of any of claims 185-194, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9, or a humanized variant thereof, and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof.

196. The method of any of claims 185-195, wherein the antibody or antigen binding fragment comprises:
a VH CDR1 having the amino acid sequence of SEQ ID NO 13;
a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;
a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;
a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;
a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and
a VL CDR3 having the amino acid sequence of SEQ ID NO: 18.

197. The method of any of claims 185-195, wherein the antibody or antigen binding fragment comprises
a VH CDR1 having the amino acid sequence of SEQ ID NO 24;
a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;
a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;
a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;
a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and
a VL CDR3 having the amino acid sequence of SEQ ID NO: 29.

198. The method of any of claims 185-197, wherein the antibody or antigen binding fragment is a Fab.

199. The method of any of claims 185-197, wherein the antibody or antigen binding fragment is an antibody.

200. The method of any of claims 185-197, wherein the chimeric polypeptide is the chimeric polypeptide of any of claims 35-46.

201. The method of any of claims 159-200, wherein the chimeric polypeptide comprises a linker that conjugates or joins, directly or indirectly, the GAA polypeptide to the internalizing moiety.

202. The method of any of claims 159-200, wherein the chimeric polypeptide does not include a linker, and the GAA polypeptide is conjugated or joined directly to the internalizing moiety.

203. The method of claim 201, wherein the linker is a cleavable linker.

204. The method of any of claims 159-203, wherein the chimeric polypeptide is formulated with a pharmaceutically acceptable carrier.

205. Use of a chimeric polypeptide of any of claims 1-59 to decrease cytoplasmic glycogen accumulation in cells.

206. Use of a chimeric polypeptide of any of claims 1-59 in the manufacture of a medicament for decreasing cytoplasmic glycogen accumulation in cells.

207. Use of a chimeric polypeptide of any of claims 1-59 to decrease cytoplasmic and lysosomal glycogen accumulation in cells.

208. Use of a chimeric polypeptide of any of claims 1-59 to decrease autophagic vacuole glycogen accumulation in cells.

209. Use of a chimeric polypeptide of any of claims 1-59 in the manufacture of a medicament for decreasing cytoplasmic and lysosomal glycogen accumulation in cells.

210. Use of a chimeric polypeptide of any of claims 1-59 in the manufacture of a medicament for decreasing autophagic vacuole glycogen accumulation in cells.

211. A chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) an antibody or antigen binding fragment the promotes delivery of the chimeric polypeptide into cells;
and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

212. A chimeric polypeptide comprising: (i) an acid alpha-glucosidase (GAA) polypeptide and (ii) an antibody or antigen binding fragment the promotes delivery of the chimeric polypeptide into cells and can transit a cellular membranes via an equilibrative nucleoside transporter 2 (ENT2) transporter;
wherein the chimeric polypeptide does not comprise the full length, GAA precursor polypeptide set forth in SEQ ID NO: 1.

213. The chimeric polypeptide of claim 211 or 212, wherein the chimeric polypeptide has acid alpha-glucosidase activity.

214. The chimeric polypeptide of claim 211-213, wherein the chimeric polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2.

215. The chimeric polypeptide of claim 211 or 212, wherein neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-60 of SEQ ID NO: 1 or 2.

216. The chimeric polypeptide of claim 215, wherein the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 21.

217. The chimeric polypeptide of claim 211 or 212, wherein neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-66 of SEQ ID NO: 1 or 2.

218. The chimeric polypeptide of claim 217, wherein the chimeric polypeptide or GAA polypeptide comprises the amino acid sequence of SEQ ID NO: 22.

219. The chimeric polypeptide of claim 211 or 212, wherein neither the GAA polypeptide nor the chimeric polypeptide comprise a contiguous amino acid sequence corresponding to the amino acids 1-69 of SEQ ID NO: 1 or 2.

220. The chimeric polypeptide of claim 219, wherein the chimeric polypeptide or GAA polypeptide comprises the sequence of SEQ ID NO: 23.

221. The chimeric polypeptide of any of claims 211-220, wherein the antibody or antigen binding fragment promotes delivery of the chimeric polypeptide into cytoplasm of cells.

222. The chimeric polypeptide of any of claims 211-221, wherein the antibody or antigen binding fragment promotes delivery of said chimeric polypeptide into muscle cells.

223. The chimeric polypeptide of any one of claims 211-222, wherein the antibody or antigen binding fragment promotes delivery of said chimeric polypeptide into hepatocytes.

224. The chimeric polypeptide of any one of claims 211-223, wherein the antibody or antigen binding fragment promotes delivery of said chimeric polypeptide into neurons.

225. The chimeric polypeptide of any of claims 211-224, wherein the GAA polypeptide comprises a mature GAA having a molecular weight of approximately 70-76 kilodaltons.

226. The chimeric polypeptide of any of claims 211-225, wherein the GAA polypeptide comprises a mature GAA having a molecular weight of approximately 70 kilodaltons.

227. The chimeric polypeptide of any of claims 211-225, wherein the GAA polypeptide comprises a mature GAA having a molecular weight of approximately 76 kilodaltons.

228. The chimeric polypeptide of any of claims 225-227, wherein the GAA polypeptide consists of an amino acid sequence selected from: residues 122-782 of SEQ ID NO: 1, residues 123-782 of SEQ ID NO: 1, or residues 204-782 of SEQ ID NO: 1.

229. The chimeric polypeptide of any of claims 211-228, wherein said antibody is monoclonal antibody 3E10, or an antigen binding fragment thereof.

230. The chimeric polypeptide of any of claims 211-229, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains cell penetrating activity, or a variant thereof that binds the same epitope as 3E10, or an antibody that has substantially the same cell penetrating activity as 3E10 and binds the same epitope as 3E10, or an antigen binding fragment of any of the foregoing.

231. The chimeric polypeptide of claim 230, wherein said antibody or antigen binding fragment is monoclonal antibody 3E10, or a variant thereof that retains the cell penetrating activity of 3E10, or an antigen binding fragment of 3E10 or said 3E10 variant.

232. The chimeric polypeptide of any of claims 211-231, wherein the antibody or antigen binding fragment is a chimeric, humanized, or fully human antibody or antigen binding fragment.

233. The chimeric polypeptide of any of claims 211-232, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 9, or a humanized variant thereof.

234. The chimeric polypeptide of any of claims 211-233, wherein the antibody or antigen binding fragment comprises a light chain variable domain comprising an amino acid sequence at least 95% identical to SEQ ID NO: 10, or a humanized variant thereof.

235. The chimeric polypeptide of any of claims 211-234, wherein the antibody or antigen binding fragment comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO: 9, or a humanized variant thereof, and a light chain variable domain comprising the amino acid sequence of SEQ ID NO: 10, or a humanized variant thereof.

236. The chimeric polypeptide of any of claims 211-235, wherein the antibody or antigen binding fragment comprises:

a VH CDR1 having the amino acid sequence of SEQ ID NO 13;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 14;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 15;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 16;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 17; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 18,

which CDRs are according to Kabat.

237. The chimeric polypeptide of any of claims 211-235, wherein the antibody or antigen binding fragment comprises

a VH CDR1 having the amino acid sequence of SEQ ID NO 24;

a VH CDR2 having the amino acid sequence of SEQ ID NO: 25;

a VH CDR3 having the amino acid sequence of SEQ ID NO: 26;

a VL CDR1 having the amino acid sequence of SEQ ID NO: 27;

a VL CDR2 having the amino acid sequence of SEQ ID NO: 28; and

a VL CDR3 having the amino acid sequence of SEQ ID NO: 29,

which CDRs are according to the IMGT system.

238. The chimeric polypeptide of any of claims 211-237, wherein the antibody or antigen binding fragment is a Fab.

239. The chimeric polypeptide of any of claims 211-237, wherein the antibody or antigen binding fragment is an antibody.

240. The chimeric polypeptide of any of claims 211-237, wherein the antigen binding fragment comprises a single chain Fv.

241. A composition comprising
(a) a first chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide having a molecular weight of approximately 76 kDa and (ii) a internalizing moiety that promotes transport into cytoplasm of cells; and
(b) a second chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide having a molecular weight of approximately 70 kDa and (ii) a internalizing moiety that promotes transport into cytoplasm of cells; wherein the first chimeric polypeptide and the second chimeric polypeptide each have acid alpha-glucosidase activity, and wherein neither the first chimeric polypeptide nor the second chimeric polypeptide comprise a GAA precursor polypeptide of approximately 110 kilodaltons.

242. The composition of claim 182, further comprising a polypeptide comprising a precursor GAA polypeptide having a molecular weight of about 110 kDa.

243. A composition comprising
(a) a chimeric polypeptide comprising: (i) a mature acid alpha-glucosidase (GAA) polypeptide and (ii) a internalizing moiety that promotes transport into cytoplasm of cells; and wherein the chimeric polypeptide does not comprise a GAA precursor polypeptide of approximately 110 kilodaltons; and
(b) a polypeptide comprising a precursor GAA polypeptide having a molecular weight of about 110 kDa.

244. A composition comprising a chimeric polypeptide comprising: (i) an acid alpha-glucosidase (GAA) polypeptide and (ii) an antibody or antigen binding fragment that binds to DNA and that promotes delivery of the chimeric polypeptide into cells;

wherein the chimeric polypeptide does not comprise the full length, GAA precursor polypeptide set forth in SEQ ID NO: 1.

245. The composition of claim 243 or 244, wherein the chimeric polypeptide has acid alpha-glucosidase activity.

246. The composition of any of claims 243-245, wherein the chimeric polypeptide lacks at least a portion of the GAA full linker region, wherein the full linker region corresponds to the amino acids 57-78 of SEQ ID NOs: 1 or 2.

247. The composition of any of claims 243-246, wherein the chimeric polypeptide comprises a mature GAA polypeptide having a molecular weight of approximately 70-76 kilodaltons.

248. The composition of any of claims 243-246, wherein the mature GAA polypeptide has a molecular weight of approximately 70 kilodaltons.

249. The composition of claim any of claims 211-246, wherein the mature GAA polypeptide has a molecular weight of approximately 76 kilodaltons.

250. The composition of any of claims 211-249, wherein the mature GAA polypeptide consists of an amino acid sequence selected from: residues 122-782 of SEQ ID NO: 1, residues 123-782 of SEQ ID NO: 1, or residues 204-782 of SEQ ID NO: 1.

251. A chimeric polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 11 or the amino acid sequence set forth in SEQ ID NO: 11, in the absence of one or more epitope tags.

252. A chimeric polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 12 or the amino acid sequence set forth in SEQ ID NO: 12, in the absence of one or more epitope tags.

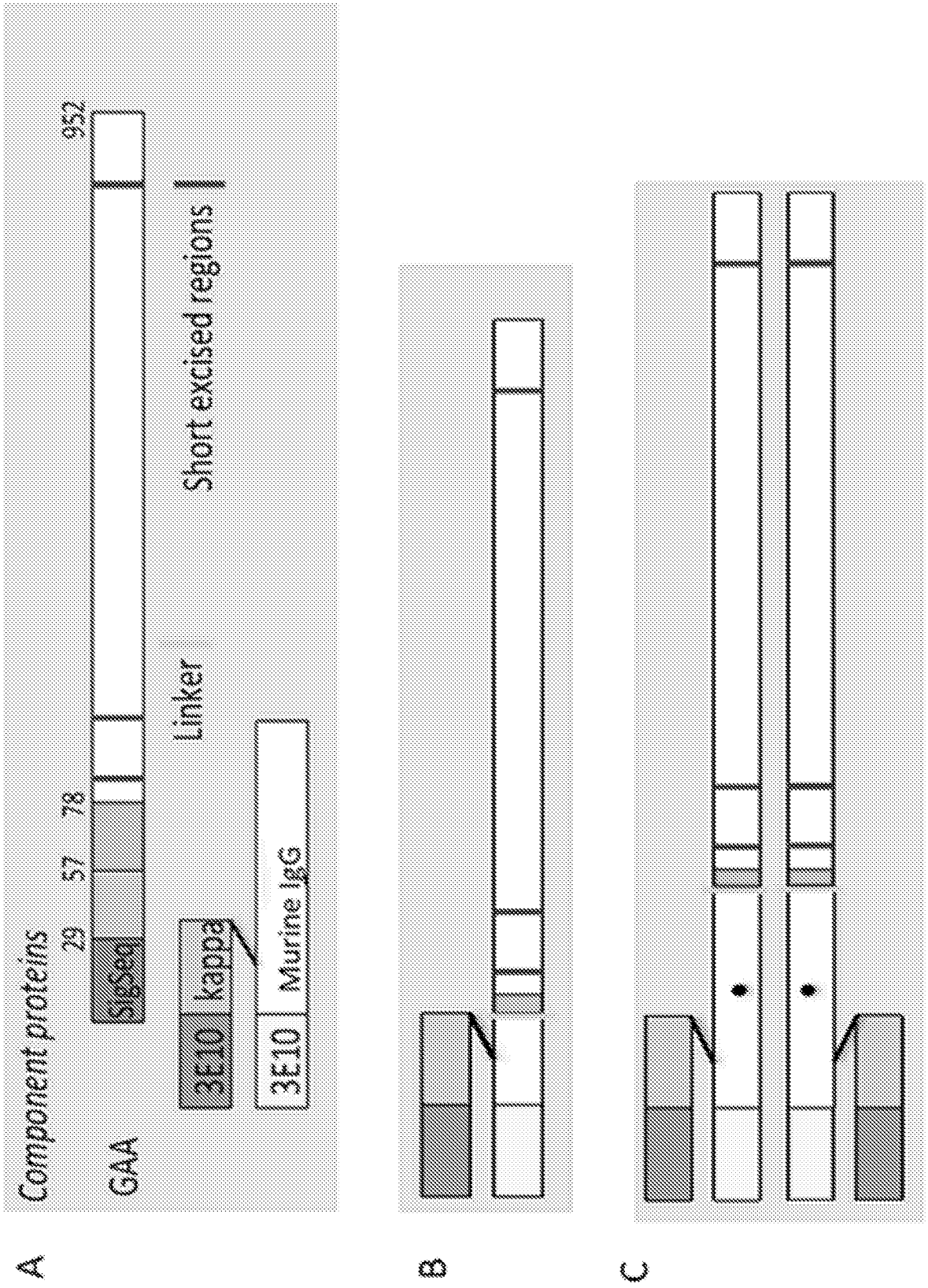
253. The chimeric polypeptide of claim 251 or 252, wherein the chimeric polypeptide further comprises, at the N-terminus, the amino acid sequence set forth in SEQ ID NO: 19 or 20.

254. A method of treating Pompe disease in a subject in need thereof comprising administering to said subject the chimeric polypeptide of any of claims 251-253.

255. The chimeric polypeptide of any of claims 1-15, wherein the chimeric polypeptide comprises residues 122-782 of SEQ ID NO: 1 or residues 204-782 of SEQ ID NO: 1.

256. The chimeric polypeptide of any of claims 55-57, wherein the internalizing moiety is C-terminal to the GAA polypeptide.

Figure 1


A
Component proteins
GAA
29
57
78
952
SigSeq
Linker
Short excised regions
3E10
kappa
3E10
Murine IgG
B
C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/017483

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/47 (2006.01) C07K 19/00 (2006.01) A61K 38/47 (2006.01) A61K 39/395 (2006.01) A61P 43/00 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

WPI, EPODOC, MEDLINE, CAPLUS and BIOSIS databases searched using the following keywords: Pompe disease, glycogen storage disease type 2, acid maltase deficiency, glycogenosis type 2, acid alpha glucosidase, GAA, lysosomal alpha glucosidase, alpha glucosidase, internalizing moiety, cell penetration, cell transport, homing peptide, CPP, transcytosis, 3E10 antibody, equilibrative nucleoside transport and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 June 2014	18 June 2014

Name and mailing address of the ISA/AU	**Authorised officer**
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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2014/017483**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/002515 A2 (BIOMARIN PHARMACEUTICAL INC.) 13 January 2005 see paragraph bridging pages 3 and 4; page 6, lines 20-31; paragraph bridging pages 49-50; Figure 3 and SEQ ID NO: 7; page 101, line 18 - page 102, line 28; see page 5, lines 25-31 and page 45, lines 3-9; page 78, lines 14-20 and paragraph bridging pages 49-50; paragraph bridging pages 6 and 7; page 66, lines 19-30; page 43, lines 21-30; page 44, lines 14-21; paragraph bridging page 98-99; SEQ ID NO: 6; page 26, lines 18-25; page 66, lines 1-12;page 77, lines 22-29; Example 7	1, 3-7, 9, 11-12, 17, 19, 22-24, 26, 48-49, 52-58, 60-61, 63-68, 75, 88-95, 102-103, 105-107, 111-115, 117, 153-158 and 255
X	WO 2008/148063 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA; DEPARTMENT OF VETERANS AFFAIRS) 04 December 2008 see paragraph [0043]; Figures 3 and 4; paragraph [0191]; claim 38; paragraphs [0023]-[0024]; paragraph [0057]; paragraphs [0128]-[0129]; claims 30 and 39; paragraphs [0161] and [0127]	1-256

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2014/017483**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2005/002515 A2	13 January 2005	AU 2004253471 A1	13 Jan 2005
		AU 2004253471 A2	13 Jan 2005
		AU 2004253471 B2	13 May 2010
		CA 2525236 A1	13 Jan 2005
		EP 1638605 A2	29 Mar 2006
		EP 1638605 B1	08 Jan 2014
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		JP 2011207889 A	20 Oct 2011
		US 2006029609 A1	09 Feb 2006
		US 7560431 B2	14 Jul 2009
		US 2010028370 A1	04 Feb 2010
		US 7829537 B2	09 Nov 2010
		US 2005026823 A1	03 Feb 2005
		US 2005042227 A1	24 Feb 2005
		US 7569544 B2	04 Aug 2009
		US 2011142763 A1	16 Jun 2011
		US 8609103 B2	17 Dec 2013
WO 2008/148063 A1	04 December 2008	AU 2008256644 A1	04 Dec 2008
		CA 2688240 A1	04 Dec 2008
		EP 2164875 A1	24 Mar 2010
		JP 2010527618 A	19 Aug 2010
		US 2008292618 A1	27 Nov 2008

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.