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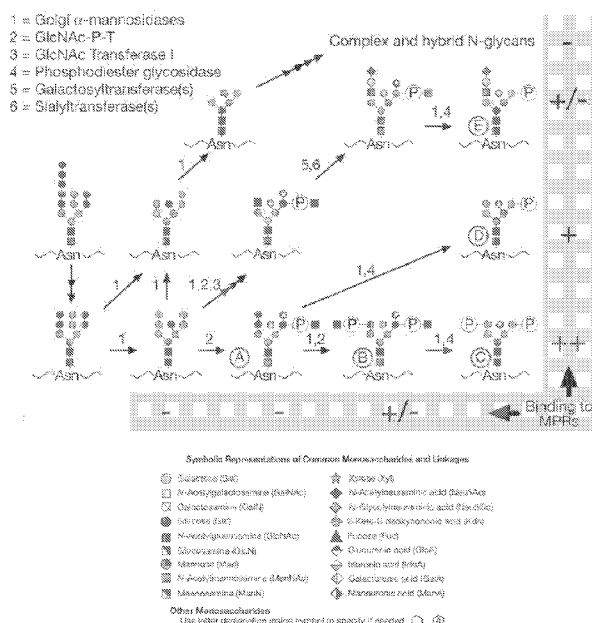
(54) Title: COMPOSITIONS AND METHODS FOR MODIFYING THE GLYCOSYLATION OF LYSOSOMAL STORAGE DIS-  
ORDER THERAPEUTICS

Figure 1

(57) Abstract: The invention generally relates to com-  
positions and methods for producing lysosomal pro-  
teins that have altered glycan structure, such that the  
protein can be delivered efficiently into the lysosomes  
of target cells (e.g., via mannose-6-phosphate mediated  
endocytosis). The lysosomal proteins are produced by  
modifying the glycosylation pathways and/or lysosomal  
targeting in a host cell using an RNA effector mole-  
cule, such as an siRNA. Glycan-modified lysosomal  
proteins produced using the methods described herein  
have improved properties.



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## COMPOSITIONS AND METHODS FOR MODIFYING THE GLYCOSYLATION OF LYSOSOMAL STORAGE DISORDER THERAPEUTICS

### CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/510,437, filed July 21, 2011, U.S. Provisional Application No. 61/532,999, filed September 9, 2011, and U.S. Provisional Application No. 61/617,322, filed March 29, 2012, each of the foregoing applications is incorporated by reference in its entirety.

### BACKGROUND OF THE INVENTION

[0002] The lysosomal storage diseases (LSDs) are a group of inherited metabolic disorders that result from a lysosomal enzyme defect that causes accumulation of a metabolic substrate of the enzyme.

[0003] Gaucher's disease is the most prevalent lysosomal storage disorder. It is caused by a recessive genetic disorder (chromosome 1 q21-q31) resulting in deficiency of glucocerebrosidase, also known as glucosylceramidase, which is a membrane-bound lysosomal enzyme that catalyzes the hydrolysis of the glycosphingolipid glucocerebroside (glucosylceramide, GlcCer) to glucose and ceramide. Gaucher's disease is caused by point mutations in the hGCD (human glucocerebrosidase) gene (GBA), which result in accumulation of GlcCer in the lysosomes of macrophages. The characteristic storage cells, called Gaucher cells, are found in liver, spleen and bone marrow. The associated clinical symptoms include severe hepatosplenomegaly, anemia, thrombocytopenia and skeletal deterioration.

[0004] Another well characterized lysosomal storage disorder is Fabry disease. Fabry disease is an X-linked lysosomal storage disease that is caused by deficient activity of lysosomal enzyme  $\alpha$ -glucosidase A (GAA). Patients with classic Fabry disease typically have GAA activity of less than 1% and often demonstrate the full spectrum of symptoms, including severe pain in the extremities (acroparesthesias), hypohidrosis, corneal and lenticular changes, skin lesions (angiokeratoma), renal failure, cardiovascular disease, pulmonary failure, neurological symptoms and stroke. In atypical Fabry disease, individuals with residual enzyme activity demonstrate symptoms later in life, and the symptoms are usually limited to one or a

few organs. Clinical manifestations in female carriers vary greatly because of random X-chromosome inactivation. Although carriers commonly remain asymptomatic throughout life, many demonstrate clinical symptoms as variable and severe as those of affected males.

[0005] Niemann-Pick types A and B result from a deficiency of acid sphingomyelinase (ASM), leading to the accumulation of lipid substances such as sphingomyelin in the cells of the spleen, liver, lungs, and, in some cases, the bone marrow and lymph nodes. Such accumulation causes these cells to malfunction.

[0006] Enzyme replacement therapy (ERT) has been used successfully to manage symptoms of Gaucher's disease and other lysosomal storage diseases, such as Pompe disease and Fabry disease. Although enzyme replacement therapy is not a cure, such treatments can effectively manage the disorder when administered on a regular basis. In the case of Gaucher's disease, intravenous recombinant glucocerebrosidase administered to patients can decrease liver and spleen size, reduces skeletal abnormalities, and reverses other manifestations.

[0007] Individuals with type I Gaucher disease can be treated with  $\beta$ -glucocerebrosidase prepared from placenta (Ceredase®) or recombinantly (Cerezyme®).

[0008] Pompe disease can be treated by ERT with Myozyme® (Genzyme Corporation, Cambridge, MA), a recombinant human  $\alpha$ -galactosidase A (AGAL).

[0009] Recombinant human acid sphingomyelinase (rhASM) produced in Chinese hamster ovary (CHO) cells is under development as a therapeutic for Niemann-Pick disease. Niemann-Pick disease is an autosomal recessive LSD having four subtypes, A, B, C, and D.

[0010] Lysosomal glycoproteins that comprise a mannose-6-phosphate (M6P) recognition marker are taken up by target cells through cell surface associated M6P Receptor (MPR)-mediated endocytosis. The therapeutic efficacy of an enzyme administered for ERT is often linked in part to the level of M6Ps (one exception is ERT for Gaucher disease, in which  $\beta$ -glucocerebrosidase uptake by macrophages can occur through a mannose receptor). In many instances, however, recombinantly produced enzymes are not expressed with high levels of M6P. For example, rhGAA typically contains approximately one mole of M6P per mole of protein, resulting in a relatively low uptake of the protein mediated by the M6P receptor (and are

therefore not efficiently internalized by cells and directed to the lysosome where they function). Similarly, phosphorylated  $\alpha$ -glucosidase isolated from bovine testis is taken up 200-fold more efficiently by cultured human fibroblasts as compared to poorly phosphorylated  $\alpha$ -glucosidase isolated from human placenta. *See*, Reuser et al., *Exp. Cell Res.* 155:178-189 (1984).

[0011] Several approaches to increase the M6P level of  $\alpha$ -glucosidase and other lysosomal glycoproteins have been reported. U.S. Patent Nos. 6,534,300, 6,670,165, and 6,861,242 disclose the use of recombinant GlcNAc-phosphotransferase and phosphodiester  $\alpha$ -GlcNAcase to create terminal mannose-6-phosphate enzymatically. The method comprises obtaining lysosomal hydrolases that have N-linked high-mannose glycans, and treating the high-mannose hydrolases *in vitro* with GlcNAc-phosphotransferase. Typically, the high-mannose glycans consist of from six to nine molecules of mannose and two molecules of N-acetylglucosamine (GlcNAc).

[0012] U.S. Patent No. 6,800,472 discloses expressing lysosomal hydrolases in cells that express pro-N-acetylglucosamine-1-phosphodiester  $\alpha$ -N-acetyl glucosaminidase. Lysosomal hydrolases expressed in these cells are modified with an N-acetylglucosamine-1-phosphate moiety and is not removed, or removed at a low efficiency. After expression and recovery of the lysosomal hydrolase from these cells, the lysosomal hydrolase can be treated *in vitro* with phosphodiester  $\alpha$ -GlcNAcase, thereby removing the N-acetylglucosamine moiety to yield a highly phosphorylated lysosomal enzyme.

[0013] Enzymatically modifying the glycan structure of a therapeutic protein can be costly. Accordingly, there is a need for improved methods for making therapeutic lysosomal glycoproteins that can be delivered to lysosomes efficiently, in particular by increasing the mannose-6-phosphate levels of the lysosomal proteins.

## SUMMARY OF THE INVENTION

[0014] The invention generally relates to compositions and methods for producing lysosomal proteins that have altered glycan structure, such that the protein can be delivered efficiently into the lysosomes of target cells (*e.g.*, *via* mannose-6-phosphate receptor mediated endocytosis). The lysosomal proteins are produced by modifying the glycosylation and/or

lysosomal targeting pathways in a host cell using an RNA effector molecule, such as an siRNA. Glycan-modified lysosomal proteins produced using the methods described herein have improved properties, including *e.g.*, increased uptake by target cells and increased yield.

## BRIEF DESCRIPTION OF THE FIGURES

[0015] Figure 1 shows the pathways for biosynthesis of N-glycans bearing the mannose-6-phosphate (M6P) recognition marker. Following early N-glycan processing, a single GlcNAc phosphodiester is added to the N-glycans of lysosomal enzymes on one of three mannose (Man) residues on the arm with the  $\alpha$ 1-6 Man linked to the core mannose (structure A). A second phosphodiester can then be added to the other side of the N-glycan (structure B) or onto other mannose residues (alternate sites for phosphorylation are denoted by asterisks). Removal of the outer N-acetylglucosamine residues and further processing of mannose residues give structures C and D. Further mannose removal is restricted by the phosphomonoesters. Thus, C and D represent only two of several possible structures bearing one or two phosphomonoesters. Glycans that are not phosphorylated become typical complex or hybrid N-glycans. Some hybrid N-glycans with M6P are also found (structure E). Binding studies of these N-glycans with purified mannose-6-phosphate receptors (MPRs) have shown the relative affinities indicated in the figure ([++] strong; [+] moderate; [+/-] weak; [-] no binding).

## DETAILED DESCRIPTION OF THE INVENTION

### 1. OVERVIEW

[0016] As described herein, lysosomal glycoproteins that have altered glycan structures can be produced on a commercial scale by transiently reducing the expression of target genes that encode a protein that are involved in glycosylation and/or lysosomal targeting pathways. Transient reduction of target genes in commercial scale bioreactors can be accomplished using RNA effector molecules, such as an siRNA. Lysosomal glycoproteins produced in this way have improved properties, including *e.g.*, increased uptake by target cells and increased yield.

[0017] For example, lysosomal proteins recombinantly produced by mammalian host cells (such as CHO cells) typically do not have high levels of mannose-6-phosphate. siRNAs

are used to transiently reduce the expression of enzymes that are involved in the dephosphorylation of mannose residues in CHO cells. In particular, acid phosphatase 5 (ACP5), an enzyme that is responsible for removing the M6P recognition marker by dephosphorylating M6P, is targeted. Increased amount of lysosomal proteins that comprise the M6P recognition marker are produced upon addition of RNA effectors (e.g., siRNAs) to the cell culture. The glycan-modified lysosomal proteins show increased M6P Receptor-mediated uptake by target cells, as compared to unmodified lysosomal proteins.

**[0018]** Lysosomal proteins are synthesized in the endoplasmic reticulum and are co-translationally glycosylated. As the proteins move through the secretory pathway, the lysosomal proteins are selectively recognized by a phosphotransferase that initiates a two-step reaction that results in the generation of the Man-6-P modification on specific N-linked oligosaccharides (Figure 1). The modified proteins are then recognized by two Man-6-P receptors (MPRs), the cation-dependent MPR and the cation-independent (CI-) MPR. These receptors bind the phosphorylated lysosomal proteins and travel to a pre-lysosomal compartment in which the low pH promotes dissociation of the receptors and ligands. Man-6 phosphorylation is a transient modification of lysosomal proteins. The M6P recognition marker that is removed with a half-life of 1.4 h in mouse lymphoma cells, and also rapidly removed in CHO cells. *See*, Sun et al., PNAS, 2008, 105, 16590-16595. Therefore, inhibiting the activity of ACP5 can enhance the production of lysosomal proteins that bear the M6P recognition marker.

**[0019]** However, the endogenous M6P Receptors of the host cells (e.g., CHO cells), located in the trans-Golgi network and late endosomes, also bind to proteins that have the M6P recognition marker. Binding of M6P to the endogenous M6P Receptors of the host cells can lead to internal trafficking of the protein to host cell lysosomes, and subsequent degradation of the protein. Thus, the endogenous M6P receptors of host cells can also be targeted using an RNA effector (e.g., siRNA). By reducing the expression of host M6P Receptors, greater proportions of synthesized lysosomal glycoproteins can be secreted, thereby improving the yield.

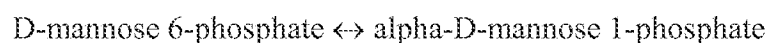
**[0020]** There are two different MPRs, one of about 300kDa and a smaller, dimeric receptor of about 46kDa. The larger receptor is known as the cation-independent mannose 6-phosphate receptor (CI-MPR), while the smaller receptor (CD-MPR) requires divalent cations to

efficiently recognize lysosomal hydrolases. While divalent cations are not essential for ligand binding by the human CD-MPR, the nomenclature has been retained.

[0021] CI-MPR binds to glycans carrying two M6P residues with highest affinity, with a 1:1 stoichiometry (Figure 1, structure C), and poorly to molecules bearing GlcNAc-P-Man phosphodiester (Figure 1, structures A and B). Binding to molecules carrying one M6P (Figure 1, structure D) is intermediate in affinity. The smaller, cation-dependent MPR (CD-MPR) has only one binding site for a single M6P.

[0022] In many cell types, a minority of newly synthesized lysosomal proteins escape sorting (to host lysosomes) and are secreted into the medium even though they carry M6P residues. Such secreted molecules may be recaptured by the same cell or by adjacent cells expressing cell-surface MPRs ("secretion-recapture" pathway). As such, reducing the expression of host cell MPRs not only promotes the secretion of M6P-bearing lysosomal proteins (by reducing the amount of lysosomal proteins that are sorted internally to host lysosomes), but also prevent or reduce the recapture of secreted lysosomal proteins, thereby increasing the yield of production.

[0023] Another protein that can be targeted is phosphomannomutase (PMM). Phosphomannomutase (EC 5.4.2.8) is an enzyme that catalyzes the interconversion between mannose-6-phosphate and mannose-1-phosphate:



[0024] Accordingly, in one aspect, the invention provides a method for producing a glycan-modified lysosomal protein by treating a large scale host cell culture with RNA effector molecules that target a protein that is involved in the removal or recognition of the M6P recognition marker. In particular, acid phosphatase 5 (ACP5), phosphomannomutase (PMM), and the mannose-6-phosphate (M6P) Receptors are targeted, alone or in combination, to increase the production and/or secretion of lysosomal proteins that have the M6P recognition marker. Additional target may include, e.g., inhibitors that inhibit the synthesis of the M6P recognition marker.

[0025] A single species of RNA effector molecule can be used to reduce the expression of a single gene that encodes a protein involved in the removal or recognition of the M6P recognition marker, or a protein that inhibits the synthesis of the M6P recognition marker. Alternatively, two or more different species of RNA effector molecules may be used, to reduce expression of one, two or more genes that encode proteins involved in the removal or recognition of the M6P recognition marker, or a protein that inhibits the synthesis of the M6P recognition marker. For example, ACP5, PMM, and the M6P Receptors may be targeted individually, or simultaneously. Additional gene(s) may also be targeted.

[0026] In another aspect, the invention provides a glycan-modified lysosomal protein that comprises at least one mannose-6-phosphate.

[0027] The glycan-modified lysosomal proteins described herein can be formulated into a pharmaceutical formulation that is suitable for *in vivo* administration. The invention also relates to the use of the lysosomal proteins described herein, or pharmaceutical compositions comprising the lysosomal proteins, in therapy, and to the use of the lysosomal proteins, or pharmaceutical compositions comprising the lysosomal proteins, for the manufacture of a medicament for use in therapy.

## 2. DEFINITIONS

[0028] The term “about”, as used here, refers to +/- 10% of a value.

[0029] The terms “complementary,” “fully complementary” and “substantially complementary” are used herein to describe the base matching between the sense strand and the antisense strand of a double-stranded RNA (dsRNA), or between the antisense strand of a RNA effector molecule and a target sequence. A nucleotide sequence is “fully complementary” to another nucleotide sequence when there are no mismatched base pairs across the length of the shorter sequence. A nucleotide sequence is “substantially complementary” to another nucleotide sequence when there are no more than 20% of the mismatched base pairs across the length of the shorter sequence (e.g., no more than 5, 4, 3, 2, or 1 mismatched base pair(s) upon hybridization for a duplex up to 30 base pairs). Where two oligonucleotides are designed to form, upon hybridization, one or more single-stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA

comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, can yet be referred to as “fully complementary.”

**[0030]** The term an “exogenous” protein refers to a protein that is not a protein expressed by the host cell’s own genomic sequence. Exogenous proteins include a protein that is expressed by exogenously introduced nucleic acid construct, even though a gene that encodes the protein may also be present in the host cell’s own genomic sequence. If the host cell is engineered such that the gene encoding a protein is activated by an exogenous promoter sequence (therefore, a host cell that does not normally express the lysosomal protein, or expresses the lysosomal protein at low level, is modified to promote the expression of the protein), the protein is also considered as an “exogenous” protein because of the use of exogenous promoter sequence. The term “exogenous” nucleic acid refers to a nucleic acid that is not naturally found in or produced by a host cell; and an “endogenous” nucleic acid refers to a nucleic acid that is naturally found in or produced by a host cell.

**[0031]** The term “glycoform” of a protein refers to a protein comprising a particular glycan structure or structures. It is recognized that a glycoprotein having more than one glycosylation site can have the same glycan species attached to each glycosylation site, or can have different glycan species attached to different glycosylation sites. In this manner, different patterns of glycan attachment yield different glycoforms of a glycoprotein.

**[0032]** The term “isolating” a protein refers to the separation of a protein molecule from its original environment found in nature (e.g., from host cells) with respect to its association with other molecules. Typically, an “isolated” protein is at least about 50% pure, at least about 55% pure, at least about 60% pure, at least about 65% pure, at least about 70% pure, at least about 75% pure, at least about 80% pure, at least about 85% pure, at least about 90% pure, at least about 95% pure, or at least about 99% pure.

**[0033]** A “large scale culture” refers to a culture that is at least about a 10 liter in size, (e.g., a volume of at least about 10L, least about 20L, least about 30L, least about 40L, at least about 50L, least about 60L, least about 70L, least about 80L, least about 90L, at least about

100L, least about 150L, least about 200L, at least about 250L, least about 300L, least about 400L, at least about 500L, least about 600L, least about 700L, least about 800L, least about 900L, at least about 1000 L, at least about 2000 L, at least about 3000 L, at least about 4000 L, at least about 5000 L, at least about 6000 L, at least about 10,000 L, at least about 15,000 L, at least about 20,000 L, at least about 25,000 L, at least about 30,000 L, at least about 35,000 L, at least about 40,000 L, at least about 45,000 L, at least about 50,000 L, at least about 55,000 L, at least about 60,000 L, at least about 65,000 L, at least about 70,000 L, at least about 75,000 L, at least about 80,000 L, at least about 85,000 L, at least about 90,000 L, at least about 95,000 L, at least about 100,000 L, etc.).

**[0034]** The term that an effective amount of an RNA effector “reduces” the expression of a target gene means that an effective amount of an RNA effector is added to a cell culture, such that the expression level of the target gene is reduced by at least about 5%, preferably at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%.

**[0035]** The expression of the target gene in a host cell is “transiently” reduced by an RNA effector molecule when the RNA effector molecule reduces the expression level of the target gene for a defined period of time (e.g., at least about 24 hours, at least about 48 hours, at least about 72 hours, at least about 96 hours, etc), but the reduction in the expression level is not permanent. In other words, the RNA effector, or a nucleic acid construct encoding the RNA effector, does not integrate into the genome of the host cell.

### **3. GLYCAN-MODIFIED LYSOSOMAL PROTEINS**

**[0036]** In one aspect, the invention provides glycan-modified lysosomal proteins that comprise at least one mannose-6-phosphate. “Glycan-modified” or “glycan modification” refer to a change in the glycan structure of a glycoprotein produced by a host cell in the presence of an RNA effector molecule that transiently reduces the expression of a target gene that encodes a protein that is involved in a glycosylation pathway, as compared the glycan structure of the

glycoprotein produced by the host cell under substantially the same conditions but in the absence of the RNA effector.

**[0037]** In one aspect, the invention provides a composition comprising a lysosomal protein, wherein the composition is characterized by: (a) at least about 60% of the lysosomal protein molecules are glycosylated; and (b) at least about 50% of the glycosylated lysosomal protein molecules comprise a glycan that comprises at least one mannose-6-phosphate. For example, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the lysosomal protein molecules are glycosylated; and at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, about 96%, at least about 97%, at least about 98%, or at least about 99% of the glycosylated lysosomal protein molecules comprise at least one mannose-6-phosphate.

**[0038]** In one aspect, the invention provides a composition comprising a lysosomal protein, wherein the composition is characterized by: (a) at least about 60% of the lysosomal protein molecules are glycosylated; and (b) the ratio of mannose-6-phosphate to glycosylated lysosomal protein is at least at about 1:1 (mole:mole). For example, the ratio of mannose-6-phosphate to glycosylated lysosomal protein is at least about 1:1 (mole:mole), at least about 1.25:1 (mole:mole), at least about 1.5:1 (mole:mole), at least about 1.75:1 (mole:mole), at least about 2:1 (mole:mole), at least about 2.25:1 (mole:mole), at least about 2.5:1 (mole:mole), at least about 2.75:1 (mole:mole), at least about 3:1 (mole:mole), at least about 3.25:1 (mole:mole), at least about 3.5:1 (mole:mole), at least about 3.75:1 (mole:mole), at least about 4:1 (mole:mole), at least about 4.25:1 (mole:mole), at least about 4.5:1 (mole:mole), at least about 4.75:1 (mole:mole), at least about 5:1 (mole:mole), at least about 5.25:1 (mole:mole), at least about 5.5:1 (mole:mole), at least about 5.75:1 (mole:mole), at least about 6:1 (mole:mole), at least about 6.25:1 (mole:mole), at least about 6.5:1 (mole:mole), at least about 6.75:1 (mole:mole), at least about 7:1 (mole:mole), at least about 7.25:1 (mole:mole), at least about 7.5:1 (mole:mole), at least about 7.75:1 (mole:mole), at least about 8:1 (mole:mole), at least

about 8.25:1 (mole:mole), at least about 8.5:1 (mole:mole), at least about 8.75:1 (mole:mole), or at least about 9:1 (mole:mole).

**[0039]** In certain embodiments, the lysosomal protein comprises at least one terminal mannose-6-phosphate. A “terminal” mannose-6-phosphate refers to a mannose-6-phosphate at the terminus of a branch of a glycan. A glycan can comprise a chain of residues having several different branch points (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, etc.), resulting in a plurality of “branches.” The terminus of each branch comprises a terminal group (e.g., mannose, galactose, N-acetylglucosamine, sialic acid, *etc.*). Thus, a terminal mannose-6-phosphate refers to a mannose-6-phosphate at the terminus of a single branch. A single glycan can comprise a plurality of terminal mannose-6-phosphates at the termini of a plurality of branches.

**[0040]** In certain embodiments, at least about 5% of the glycosylated lysosomal protein molecules comprise a glycan that comprises at least two mannose-6-phosphate groups (*i.e.*, a bis-phosphorylated glycan). A bis-phosphorylated glycan bind to the M6P Receptor with an affinity that is about 3500-fold higher than a mono-phosphorylated glycan. *See*, Tong, P. Y., et al., (1989), Journal of Biological Chemistry 264: 7962-7969). Therefore, bis-phosphorylated glycans may be more desirable for recombinantly produced lysosomal proteins. For example, , at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%, of the glycosylated lysosomal protein molecules may comprise a glycan that comprises at least two mannose-6-phosphate groups (*i.e.*, a bis-phosphorylated glycan).

**[0041]** Preferably, the glycan-modified lysosomal protein is internalized more efficiently by a target cell than that the corresponding unmodified lysosomal protein. For example, the glycan-modified lysosomal protein may be internalized more efficiently than the corresponding unmodified lysosomal protein by, *e.g.*, at least about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 50%, about 60%, about 70%, about 80%, or about 90% in a given time period. Alternatively, at least about 2, about 3, about 4,

about 5, about 6, about 7, about 8, about 9, or about 10 fold as much of the glycan-modified lysosomal protein may be internalized, relative to the unmodified lysosomal protein, in a given time period. A given time period may be, for example, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 10 hours, 12 hours, 24 hours, 48 hours, 72 hours etc.

**[0042]** In certain embodiments, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the lysosomal protein molecules are N-glycosylated.

**[0043]** In certain embodiments, at least about 50% the glycosylated lysosomal protein molecules comprise a glycan that comprises no more than 5 mannose residues total. It has been reported that in case of glucocerebrosidase, larger oligomannose structures (*e.g.*, Man<sub>9</sub>GlcNAc<sub>2</sub>) increased the binding of glucocerebrosidase to serum mannose-binding lectin (MBL). *See*, Van Patten et al., *Glycobiology* vol. 17, 467-478, (2007). MBL is a collectin involved in the innate immune response and is present in serum at concentrations of 1-5 mg/mL. MBL eliminates intruding microorganisms by binding to carbohydrate structures on their surface, leading to complement system activation, opsonization, and phagocytosis. Therefore, an increase in MBL binding is undesirable and could affect the pharmacokinetic behavior of a lysosomal protein. It has also been reported that MBL could block the uptake of mannosylated liposomes into macrophages. *See*, Van Patten et al. (*supra*).

**[0044]** In certain embodiments, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% of the glycosylated lysosomal protein molecules comprise a glycan that is: Man<sub>2</sub>GlcNAc<sub>2</sub>, Man<sub>2</sub>GlcNAc<sub>2</sub>Fuc, Man<sub>3</sub>GlcNAc<sub>2</sub>, Man<sub>3</sub>GlcNAc<sub>2</sub>Fuc, Man<sub>4</sub>GlcNAc<sub>2</sub>, Man<sub>4</sub>GlcNAc<sub>2</sub>Fuc, Man<sub>5</sub>GlcNAc<sub>2</sub>, or Man<sub>5</sub>GlcNAc<sub>2</sub>Fuc, each may be independently phosphorylated at one or more mannose residues.

**[0045]** In certain embodiments, the lysosomal protein is selected from the group consisting of glucocerebrosidase, glucosidase, galactocerebrosidase, galactosidase, iduronidase,

hexosaminidase, mannosidase, fucosidase, arylsulfatase, V-acetylgalactosamine-6-sulfate sulfatase, acteylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase,  $\alpha$ -glucosaminide-N-acetyltransferase, acetyl-CoA: $\alpha$ -glucosaminide N-acetyltransferase,  $\beta$ -D-glucuronidase, hyaluronidase, mannosidase, neurominidase, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, sialidase and lipoprotein lipase.

[0046] In certain embodiments, the lysosomal protein is selected from the group consisting of: glucocerebrosidase, idursulfase, alglucosidase alfa, galsulfase, agalsidase  $\beta$ , laronidase, acid  $\alpha$ -glucosidase, and a protease inhibitor.

[0047] In certain embodiments, the lysosomal protein is selected from the group consisting of glucocerebrosidase, idursulfase, alglucosidase alfa, galsulfase, agalsidase  $\beta$ , and laronidase.

[0048] In certain embodiment, the lysosomal protein is a glucocerebrosidase.

[0049] In other embodiments, the lysosomal protein is not a glucocerebrosidase.

[0050] Exemplary lysosomal proteins that may be used for treating LSDs are listed in Tables 1-4.

**Table 1: Examples of LSDs in glycoprotein degradation**

| <b>Lysosomal Storage Disorder</b>       | <b>Defective Lysosomal Protein</b>   |
|-----------------------------------------|--------------------------------------|
| $\alpha$ -Mannosidosis (types I and II) | $\alpha$ -mannosidase                |
| $\beta$ -Mannosidosis                   | $\beta$ -mannosidase                 |
| Aspartylglucosaminuria                  | aspartyl-glucosaminidase             |
| Sialidosis (mucopolipidosis I)          | sialidase                            |
| Schindler (types I and II)              | $\alpha$ -N-acetyl-galactosaminidase |
| Galactosialidosis                       | protective protein/cathepsin A       |
| Fucosidosis                             | $\alpha$ -fucosidase                 |
| GM1 gangliosidosis                      | $\beta$ -galactosidase               |
| GM2 gangliosidosis                      | $\beta$ -hexosaminidase              |

**Table 2: Examples of LSDs in glycosaminoglycan degradation - the mucopolysaccharidoses (MPS)**

| Number    | Common Name                   | Defective Lysosomal Protein                                               |
|-----------|-------------------------------|---------------------------------------------------------------------------|
| MPS I H   | Hurler, Hurler/Scheie, Scheie | $\alpha$ -L-iduronidase                                                   |
| MPS II    | Hunter                        | iduronate-2-sulfatase                                                     |
| MPS III A | Sanfilippo A                  | heparan N-sulfatase                                                       |
| MPS III B | Sanfilippo B                  | $\alpha$ -N-acetylglucosaminidase                                         |
| MPS III C | Sanfilippo C                  | acetyl CoA: $\alpha$ -glucosaminide acetyltransferase                     |
| MPS III D | Sanfilippo D                  | N-acetylglucosamine 6-sulfatase                                           |
| MPS IV A  | Morquio A                     | galactose-6-sulfatase                                                     |
| MPS IV B  | Morquio B                     | $\beta$ -galactosidase                                                    |
| MPS VI    | Maroteaux-Lamy                | arylsulfatase B                                                           |
| MPS VII   | Sly                           | $\beta$ -glucuronidase                                                    |
|           | multiple sulfatase deficiency | sulfatase modifying factor converts cysteine $\rightarrow$ formyl glycine |

Table 3: Examples of LSDs in glycolipid degradation

| Lysosomal Storage Disorder    | Defective Lysosomal Protein             |
|-------------------------------|-----------------------------------------|
| Tay-Sachs                     | $\beta$ -hexosaminidase A               |
| Sandhoff                      | $\beta$ -hexosaminidase A and B         |
| GM1 gangliosidosis            | $\beta$ -galactosidase                  |
| Sialidosis                    | sialidase                               |
| Farber                        | acid ceramidase                         |
| Fabry                         | $\alpha$ -galactosidase                 |
| Gaucher's (types 1, 2, and 3) | $\beta$ -glucoceramidase                |
| Krabbe                        | $\beta$ -galactoceramidase              |
| Metachromatic leukodystrophy  | arylsulfatase A (cerebroside sulfatase) |
| Saposin deficiency            | saposin precursor                       |

Table 4: Examples of other types LSDs

| Lysosomal Storage Disorder          | Defective Lysosomal Protein |
|-------------------------------------|-----------------------------|
| Pompe                               | Acid $\alpha$ -glucosidase  |
| Niemann-Pick disease, types A and B | acid sphingomyelinase       |
| Niemann-Pick disease, type C1       | NPC1                        |
| Niemann-Pick disease, type C2       | NPC2                        |
| Salla disease                       | Sialin                      |

[0051] The lysosomal protein may have 1, 2, 3, 4, 5 or more consensus sites for N-linked or O-linked glycosylation, each of which may or may not be glycosylated. For example, human glucocerebrosidase has five N-glycosylation amino acid consensus sequences (Asn-X-Ser/Thr), four of which are normally glycosylated (Asn19, Asn59, Asn146 and Asn270).

[0052] The lysosomal protein may be an enzyme or a transporter protein that has optimal activity, as measured by an activity assay, at a pH ranging from 1-7, such as, a pH ranging from 1-3, 2-5, 3-6, 4-5, 5-6, or 4-6. Preferably, the lysosomal protein has optimal activity at a pH ranging between 3 to 5.

(1) *Acid  $\alpha$ -glucosidase*

[0053] In certain embodiments, the lysosomal protein is an acid  $\alpha$ -glucosidase (E.C. 3.2.1.20). In certain embodiments, the acid  $\alpha$ -glucosidase is a human acid  $\alpha$ -glucosidase.

[0054] Acid  $\alpha$ -glucosidase is a lysosomal enzyme essential for the degradation of glycogen to glucose in lysosomes, and catalyzes the hydrolysis of  $\alpha$ -1,4- and  $\alpha$ -1,6- glycosidic linkages of glycogen. Human acid  $\alpha$ -glucosidase is encoded by the GAA gene.

[0055] In certain embodiments, the lysosomal protein comprises an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to Alglucosidase alfa (Lumizyme® or Myozyme®; SEQ ID NO: 1). Commercially available Alglucosidase alfa is produced by recombinant DNA technology in a Chinese hamster ovary cell line.

[0056] The glycan-modified acid  $\alpha$ -glucosidase can be used to treat Pompe disease.

(2)  *$\alpha$ -galactosidase A*

[0057] In certain embodiments, the lysosomal protein is an  $\alpha$ -galactosidase A (E.C. 3.2.1.22). In certain embodiments, the  $\alpha$ -galactosidase A is a human  $\alpha$ -galactosidase A.

[0058]  $\alpha$ -galactosidase A ( $\alpha$ -D-galactoside galactohydrolase, E.C. 3.2.1.22) is a lysosomal glycoprotein of about 101 kDa and has a homodimeric structure. It contains a 5-15% N-linked complex and high-mannose oligosaccharide chains. It hydrolyses the terminal  $\alpha$ -

galactosyl moieties from glycolipids and glycoproteins. It predominantly hydrolyzes ceramide trihexoside, and it can catalyze the hydrolysis of melibiose into galactose and glucose.  $\alpha$ -galactosidase A is encoded by the GLA gene.

[0059] In certain embodiments, the lysosomal protein comprises an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to Agalsidase (Replagal® or Fabrazyme®; SEQ ID NO:408).

[0060] Replagal® (Agalsidase alpha) is produced by human fibroblasts that have been modified by gene activation; Fabrazyme® (Agalsidase beta) is produced by CHO cells that are transduced with human  $\alpha$ -galactosidase A cDNA. Agalsidase alpha and beta both have the same amino acid sequence as the native enzyme.

The glycan-modified  $\alpha$ -galactosidase A can be used to treat Fabry's disease.

### (3) *Protease Inhibitors*

[0061] In certain embodiments, the lysosomal protein is protease inhibitor. In certain embodiments, the protease inhibitor is a human protease inhibitor.

[0062] In certain embodiments, the lysosomal protein is a trypsin inhibitor, such as  $\alpha_1$ -antitrypsin (also known as the serum trypsin inhibitor), ovomucoid, basic pancreatic trypsin inhibitor (*e.g.*, aprotinin), a soybean trypsin inhibitor (SBTI, such as a Kunitz trypsin inhibitor, or a Bowman-Birk trypsin and chymotrypsin inhibitor).

[0063] Alpha 1-Antitrypsin (A1AT, or  $\alpha_1$ -antitrypsin) is a protease inhibitor belonging to the serpin superfamily. It protects tissues from enzymes of inflammatory cells, especially neutrophil elastase. In its absence, neutrophil elastase is free to break down elastin, which contributes to the elasticity of the lungs, resulting in respiratory complications such as emphysema, or COPD (chronic obstructive pulmonary disease) in adults and cirrhosis in adults or children. Mature  $\alpha_1$ -antitrypsin is a single-chain glycoprotein of 394 amino acids, and has a number of glycoforms. The three N-linked glycosylations sites are mainly biantennary (complex) N-glycans. However, one particular site, Asparagine 107 (ExPASy amino acid

nomenclature), shows a considerable heterogeneity, since tri- and even tetra-antennary N-glycans can be attached to Asn107. These glycans also carry different amounts of sialic acids. In addition,  $\alpha$ 1,3-linked fucosylated triantennary N-glycans, which forms part of the “Sialyl Lewis x epitope,” also exist.

**[0064]** Three  $\alpha$ 1-antitrypsin products derived from human plasma have been approved by the FDA, Prolastin® (Talecris Biotherapeutics), Zemaira® (Aventis Behring LLC), and Aralast® (Baxter International). All three products showed minor differences compared to the normal human plasma A1AT, and are introduced during the specific purifications procedures. *See*, Kolarich D. et al., *Transfusion* 46 (11): 1959–77 (2006). Recombinant alpha 1-antitrypsin is not yet commercially available.

**[0065]** In certain embodiments, the lysosomal protein comprises an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to  $\alpha$ 1-antitrypsin (SEQ ID NO: 4).

**[0066]** The glycan-modified alpha 1-antitrypsin can be used to treat alpha 1-antitrypsin deficiency.

**[0067]** In certain embodiments, the lysosomal protein is an esterase inhibitor, such as a C1 Esterase Inhibitor, or a Ubiquitin C-Terminal Hydrolase/Esterase (UCH) inhibitor (e.g., UCH-L1 Inhibitor, UCH-L2 Inhibitor, or UCH-L3 Inhibitor).

**[0068]** C1-inhibitor (also called C1 esterase inhibitor) is a protease inhibitor belonging to the serpin superfamily. Its main function is to inhibit the complement system to prevent spontaneous activation. C1-inhibitor has a 2-domain structure. The C-terminal serpin domain is similar to other serpins, and is responsible for its inhibitory activity. The N-terminal domain is not essential for its inhibitory activity. C1-inhibitor is highly glycosylated, bearing both N- and O-glycans. The N-terminal domain is especially heavily glycosylated. Human C1-inhibitor is encoded by the SERPING1 gene.

**[0069]** Deficiency of C1-inhibitor is associated with hereditary angioedema (hereditary angioneurotic edema, or HAE), or swelling due to leakage of fluid from blood vessels into connective tissue.

[0070] In certain embodiments, the lysosomal protein comprises an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to human C1-inhibitor (Cinryze®, SEQ ID NO: 5). Cinryze® is a pharmaceutical-grade C1-inhibitor approved for the use of HAE.

[0071] The glycan-modified C1-inhibitor can be used for treating C1-inhibitor deficiency (Types I, II, III), as well as sepsis, vascular leak syndrome, acute myocardial infarction, pancreatitis, thermal injury, and for the management of xenotransplantation. *See*, Caliezi C, et al., *Pharmacol. Rev.* 52 (1): 91-112 (2008).

(4) *glucocerebrosidase*

[0072] In certain embodiments, the lysosomal protein is glucocerebrosidase. In certain embodiments, the glucocerebrosidase is a human glucocerebrosidase.

[0073] Glucocerebrosidase (also called acid  $\beta$ -glucosidase, D-glucosyl-N-acylsphingosine glucosylhydrolase, or GCase) is an enzyme with glucosylceramidase activity (EC 3.2.1.45) that is needed to cleave the  $\beta$ -glucosidic linkage of the chemical glucocerebroside. Mature human glucocerebrosidase is a 497 amino acid membrane-associated monomeric glycoprotein of about 67 kDa and has a pH optimum for enzymatic activity of about 5.5. Glucocerebrosidase has five N-glycosylation amino acid consensus sequences (Asn-X-Ser/Thr), four of which are normally glycosylated (Asn19, Asn59, Asn146 and Asn270). Recombinant-produced glucocerebrosidase (Cerezyme®; SEQ ID NO:2) differs from placenta glucocerebrosidase (Ceredase®; SEQ ID NO:3) in position 495, in which an arginine is substituted with a histidine.

[0074] In certain embodiments, the lysosomal protein comprises an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to Imiglucerase (Cerezyme®; SEQ ID NO: 2). In certain embodiments, the lysosomal protein comprises an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to Alglucerase (Ceredase®; SEQ ID NO: 3).

[0075] The glycan-modified glucocerebrosidase can be used to treat Gaucher's (types 1, 2, and 3).

#### 4. METHODS FOR MODIFYING PROTEIN GLYCOSYLATION

[0076] The invention also provides methods for producing glycan-modified lysosomal proteins, in particular on a large or commercial scale. The method comprises culturing a host cell in a large scale cell culture in the presence of an RNA effector that targets a gene that encodes a protein that is involved in the removal or recognition of the M6P recognition marker, or a protein that inhibits the synthesis of the M6P recognition marker. The RNA effector transiently reduces the expression level of the target gene, thereby improving the yield of M6P recognition marker on the lysosomal protein.

[0077] In one aspect, the invention provides a method for producing a composition comprising a lysosomal protein, the method comprises: culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule; (a) wherein the host cell (i) expresses the lysosomal protein, and (ii) comprises a target gene that encodes acid phosphatase 5 (ACP5); (b) wherein the RNA effector is substantially complementary to the target gene that encodes ACP5, and reduces or prevents the expression of the target gene; and (c) wherein the host cell is cultured for a period of time sufficient for the production of the lysosomal protein.

[0078] In certain embodiments, the lysosomal protein is not iduronate 2-sulfatase (IDS) or arylsulfatase A.

[0079] ACP5 (also known as Tartrate-resistant acid phosphatase, or TRAP) is an enzyme that is responsible for removing the M6P recognition marker by dephosphorylating M6P. The sequence of hamster gene that encodes ACP5 is provided in Appendix II.

[0080] If desired, two or more genes may be targeted. For example, the host cell may further comprises a second target gene that encodes a Mannose-6-Phosphate receptor (MPR); and the method may further comprise: culturing the large scale host cell culture in a medium that comprises an effective amount of a second RNA effector molecule, wherein the second RNA effector is substantially complementary to the second target gene that encodes

MPR, and wherein the RNA effector reduces or prevents the expression of the second target gene that encodes MPR.

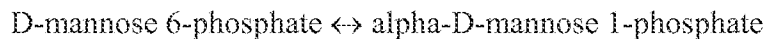
[0081] In certain embodiments, only one of the MPRs is targeted. In other embodiments, both MPRs are targeted. Under normal conditions, only the CI-MPR is responsible for lysosomal enzyme endocytosis from the cell surface (the “secretion-recapture” pathway). However, when the CD-MPR is strongly overexpressed, it is capable of mediating uptake from the plasma membrane. Therefore, in some cases, targeting CI-MPR alone may be sufficient to improve yield; in other cases, it may be desirable to target both types of MPRs.

[0082] In one aspect, the invention provides a method for producing a composition comprising a lysosomal protein, the method comprises: culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule; (a) wherein the host cell (i) expresses the lysosomal protein, and (ii) comprises a target gene that encodes a Mannose-6-Phosphate receptor (MPR); (b) wherein the RNA effector is substantially complementary to the target gene that encodes MPR, and reduces or prevents the expression of the target gene; and (c) wherein the host cell is cultured for a period of time sufficient for the production of the lysosomal protein.

[0083] The sequences of hamster gene that encodes CD-MPR and CI-MPR are provided in Appendix II. In certain embodiments, CI-MPR is targeted. In certain embodiments, CD-MPR is targeted. In certain embodiments, both CI-MPR and CD-MPR are targeted.

[0084] In another aspect, the invention provides a method for producing a composition comprising a lysosomal protein, the method comprises: culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule; (a) wherein the host cell (i) expresses the lysosomal protein, and (ii) comprises a target gene that encodes Phosphomannomutase (PMM); (b) wherein the RNA effector is substantially complementary to the target gene that encodes PMM, and reduces or prevents the expression of the target gene; and (c) wherein the host cell is cultured for a period of time sufficient for the production of the lysosomal protein.

[0085] Phosphomannomutase (EC 5.4.2.8) is an enzyme that catalyzes the interconversion between mannose-6-phosphate and mannose-1-phosphate:



PMM belongs to the family of isomerases, specifically the phosphotransferases (phosphomutases), which transfer phosphate groups within a molecule. PMM is also referred to as:  $\alpha$ -D-mannose 1,6-phosphomutase, mannose phosphomutase, phosphomannose mutase, and D-mannose 1,6-phosphomutase.

**[0086]** In certain embodiment, the lysosomal protein is a human lysosomal protein. When the lysosomal protein is a human lysosomal protein, and the host cell is not a human host cell, it may be desirable to also reduce or prevent the expression of the corresponding host lysosomal protein. For example, if CHO cells (or CHO-derived cells) are used to express human acid  $\alpha$ -glucosidase, it may be desirable to also reduce or prevent the expression of the hamster acid  $\alpha$ -glucosidase. Otherwise, the endogenous hamster acid  $\alpha$ -glucosidase expressed by CHO cells could co-purify with exogenous human acid  $\alpha$ -glucosidase.

**[0087]** Therefore, in one aspect, the invention provides a method for producing a composition comprising an exogenous lysosomal protein, the method comprises: culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule; (a) wherein the host cell comprises (i) an exogenous nucleic acid that expresses the exogenous lysosomal protein, and (ii) an endogenous target gene that encodes an ortholog of the lysosomal protein; (b) wherein the RNA effector is substantially complementary to the endogenous target gene that encodes the ortholog, and reduces or prevents the expression of the endogenous target gene; and (c) wherein the host cell is cultured for a period of time sufficient for the production of the lysosomal protein. In certain embodiments, the host cell is a CHO cell, or CHO-derived cell. In certain embodiments, the exogenous lysosomal protein is a human lysosomal protein, and the endogenous gene encodes the hamster ortholog of the human lysosomal protein. In certain embodiments, the invention provides a method for producing a composition comprising an human lysosomal protein, the method comprises: culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule; (a) wherein the host cell is a CHO cell or CHO-derived cell, and wherein the host cell comprises (i) an exogenous nucleic acid that expresses the human lysosomal protein, and (ii) an endogenous target gene that encodes the hamster ortholog of the human lysosomal protein; (b) wherein the RNA effector is substantially complementary to the endogenous target gene that

encodes the ortholog, and reduces or prevents the expression of the endogenous target gene; and (c) wherein the host cell is cultured for a period of time sufficient for the production of the human lysosomal protein.

[0088] In certain embodiments, the exogenous lysosomal protein is human acid  $\alpha$ -glucosidase, and the endogenous target gene encodes hamster acid  $\alpha$ -glucosidase. In certain embodiments, the exogenous lysosomal protein is human  $\alpha$ -galactosidase A, and the endogenous target gene encodes hamster  $\alpha$ -galactosidase A. In certain embodiments, the exogenous lysosomal protein is a human trypsin inhibitor, and the endogenous target gene encodes the corresponding hamster trypsin inhibitor. In certain embodiments, the exogenous lysosomal protein is a human esterase inhibitor, and the endogenous target gene encodes the corresponding hamster esterase inhibitor. In certain embodiments, the exogenous lysosomal protein is human glucocerebrosidase, and the endogenous target gene encodes hamster glucocerebrosidase.

[0089] The RNA effector can be an siRNA, shRNA, or antisense RNA.

[0090] Additional genes that encode a protein that is involved in lysosome targeting may also be targeted, such as, *e.g.*, a protein that is involved in the synthesis of terminal mannose. For example, it has been reported that removal of “blocking” GlcNAc residues from molecules carrying two M6P-GlcNAc residues improves binding to both M6P receptors. Therefore, enzymes or transporter proteins involved in addition of GlcNAc residues to glycans may be targeted to further improve MPR targeting.

[0091] In certain embodiments, the RNA effector transiently reduces the expression of its target gene.

[0092] In certain embodiments, the method further comprises harvesting said glycoprotein from said large scale culture.

## **B. RNA effector molecules**

[0093] RNA effector molecules that are suitable for modifying glycosylation process of a host cell has been disclosed in detail in WO 2011/005786, and is described brief below.

[0094] RNA effector molecules are ribonucleotide agents that are capable of reducing or preventing the expression of a target gene within a host cell, or ribonucleotide agents capable of forming a molecule that can reduce the expression level of a target gene within a host cell. A portion of a RNA effector molecule, wherein the portion is at least 10, at least 12, at least 15, at least 17, at least 18, at least 19, or at least 20 nucleotide long, is substantially complementary to the target gene. The complementary region may be the coding region, the promoter region, the 3' untranslated region (3'-UTR), and/or the 5'-UTR of the target gene. Preferably, at least 16 contiguous nucleotides of the RNA effector molecule are complementary to the target sequence (e.g., at least 17, at least 18, at least 19, or more contiguous nucleotides of the RNA effector molecule are complementary to the target sequence). The RNA effector molecules interact with RNA transcripts of target genes and mediate their selective degradation or otherwise prevent their translation.

[0095] RNA effector molecules can comprise a single RNA strand or more than one RNA strand. Examples of RNA effector molecules include, e.g., double stranded RNA (dsRNA), microRNA (miRNA), antisense RNA, promoter-directed RNA (pdRNA), Piwi-interacting RNA (piRNA), expressed interfering RNA (eiRNA), short hairpin RNA (shRNA), antagomirs, decoy RNA, DNA, plasmids and aptamers. The RNA effector molecule can be single-stranded or double-stranded. A single-stranded RNA effector molecule can have double-stranded regions and a double-stranded RNA effector can have single-stranded regions. Preferably, the RNA effector molecules are double-stranded RNA, wherein the antisense strand comprises a sequence that is substantially complementary to the target gene.

[0096] Complementary sequences within a RNA effector molecule, e.g., within a dsRNA (a double-stranded ribonucleic acid) may be fully complementary or substantially complementary. Generally, for a duplex up to 30 base pairs, the dsRNA comprises no more than 5, 4, 3 or 2 mismatched base pairs upon hybridization, while retaining the ability to regulate the expression of its target gene.

[0097] In some embodiments, the RNA effector molecule comprises a single-stranded oligonucleotide that interacts with and directs the cleavage of RNA transcripts of a target gene. For example, single stranded RNA effector molecules comprise a 5' modification including one or more phosphate groups or analogs thereof to protect the effector molecule from

nuclease degradation. The RNA effector molecule can be a single-stranded antisense nucleic acid having a nucleotide sequence that is complementary to a “sense” nucleic acid of a target gene, e.g., the coding strand of a double-stranded cDNA molecule or a RNA sequence, e.g., a pre-mRNA, mRNA, miRNA, or pre-miRNA. Accordingly, an antisense nucleic acid can form hydrogen bonds with a sense nucleic acid target.

[0098] Given a coding strand sequence (e.g., the sequence of a sense strand of a cDNA molecule), antisense nucleic acids can be designed according to the rules of Watson-Crick base pairing. The antisense nucleic acid can be complementary to the coding or noncoding region of a RNA, e.g., the region surrounding the translation start site of a pre-mRNA or mRNA, e.g., the 5' UTR. An antisense oligonucleotide can be, for example, about 10 to 25 nucleotides in length (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length). In some embodiments, the antisense oligonucleotide comprises one or more modified nucleotides, e.g., phosphorothioate derivatives and/or acridine substituted nucleotides, designed to increase its biological stability of the molecule and/or the physical stability of the duplexes formed between the antisense and target nucleic acids. Antisense oligonucleotides can comprise ribonucleotides only, deoxyribonucleotides only (e.g., oligodeoxynucleotides), or both deoxyribonucleotides and ribonucleotides. For example, an antisense agent consisting only of ribonucleotides can hybridize to a complementary RNA and prevent access of the translation machinery to the target RNA transcript, thereby preventing protein synthesis. An antisense molecule including only deoxyribonucleotides, or deoxyribonucleotides and ribonucleotides, can hybridize to a complementary RNA and the RNA target can be subsequently cleaved by an enzyme, e.g., RNase H, to prevent translation. The flanking RNA sequences can include 2'-O-methylated nucleotides, and phosphorothioate linkages, and the internal DNA sequence can include phosphorothioate internucleotide linkages. The internal DNA sequence is preferably at least five nucleotides in length when targeting by RNaseH activity is desired.

[0099] In certain embodiments, the RNA effector comprises a double-stranded ribonucleic acid (dsRNA), wherein said dsRNA (a) comprises a sense strand and an antisense strand that are substantially complementary to each other; and (b) wherein said antisense strand comprises a region of complementarity that is substantially complementary to one of the target genes, and wherein said region of complementarity is from 10 to 30 nucleotides in length.

**[00100]** In some embodiments, RNA effector molecule is a double-stranded oligonucleotide. Typically, the duplex region formed by the two strands is small, about 30 nucleotides or less in length. Such dsRNA is also referred to as siRNA. For example, the siRNA may be from 15 to 30 nucleotides in length, from 10 to 26 nucleotides in length, from 17 to 28 nucleotides in length, from 18 to 25 nucleotides in length, or from 19 to 24 nucleotides in length, etc.

**[00101]** The duplex region can be of any length that permits specific degradation of a desired target RNA through a RISC pathway, but will typically range from 9 to 36 base pairs in length, e.g., 15 to 30 base pairs in length. For example, the duplex region may be 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, or 36 base pairs in length, or any sub-range there between, including, e.g., 15 to 30 base pairs, 15 to 26 base pairs, 15 to 23 base pairs, 15 to 22 base pairs, 15 to 21 base pairs, 15 to 20 base pairs, 15 to 19 base pairs, 15 to 18 base pairs, 15 to 17 base pairs, 18 to 30 base pairs, 18 to 26 base pairs, 18 to 23 base pairs, 18 to 22 base pairs, 18 to 21 base pairs, 18 to 20 base pairs, 19 to 30 base pairs, 19 to 26 base pairs, 19 to 23 base pairs, 19 to 22 base pairs, 19 to 21 base pairs, 19 to 20 base pairs, 20 to 30 base pairs, 20 to 26 base pairs, 20 to 25 base pairs, 20 to 24 base pairs, 20 to 23 base pairs, 20 to 22 base pairs, 20 to 21 base pairs, 21 to 30 base pairs, 21 to 26 base pairs, 21 to 25 base pairs, 21 to 24 base pairs, 21 to 23 base pairs, or 21 to 22 base pairs.

**[00102]** The two strands forming the duplex structure of a dsRNA can be from a single RNA molecule having at least one self-complementary region, or can be formed from two or more separate RNA molecules. Where the duplex region is formed from two strands of a single molecule, the molecule can have a duplex region separated by a single stranded chain of nucleotides (a “hairpin loop”) between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure. The hairpin loop can comprise at least one unpaired nucleotide; in some embodiments the hairpin loop can comprise at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 23 or more unpaired nucleotides. Where the two substantially complementary strands of a dsRNA are formed by separate RNA strands, the two strands can be optionally covalently linked. Where the two strands are connected covalently by means other than a hairpin loop, the connecting structure is referred to as a “linker.”

[00103] It is known that dsRNAs having a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference. Elbashir et al., 20 EMBO 6877-88 (2001).

[00104] A double-stranded oligonucleotide can include one or more single-stranded nucleotide overhangs, which are one or more unpaired nucleotide that protrudes from the terminus of a duplex structure of a double-stranded oligonucleotide, e.g., a dsRNA. A double-stranded oligonucleotide can comprise an overhang of at least one nucleotide; alternatively the overhang can comprise at least two nucleotides, at least three nucleotides, at least four nucleotides, at least five nucleotides or more. The overhang(s) can be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5' end, 3' end, or both ends of either an antisense or sense strand of a dsRNA.

[00105] In one embodiment, at least one end of a dsRNA has a single-stranded nucleotide overhang of 1 to 4, generally 1 or 2 nucleotides.

[00106] The overhang can comprise a deoxyribonucleoside or a nucleoside analog. Further, one or more of the internucleoside linkages in the overhang can be replaced with a phosphorothioate. In some embodiments, the overhang comprises one or more deoxyribonucleoside or the overhang comprises one or more dT, e.g., the sequence 5'-dTdT-3' or 5'-dTdTdT-3'. In some embodiments, overhang comprises the sequence 5'-dT\*dT-3, wherein \* is a phosphorothioate internucleoside linkage.

[00107] An RNA effector molecule as described herein can contain one or more mismatches to the target sequence. Preferably, a RNA effector molecule as described herein contains no more than three mismatches. If the antisense strand of the RNA effector molecule contains one or more mismatches to a target sequence, it is preferable that the mismatch(s) is (are) not located in the center of the region of complementarity, but are restricted to be within the last 5 nucleotides from either the 5' or 3' end of the region of complementarity. For example, for a 23-nucleotide RNA effector molecule agent RNA, the antisense strand generally does not contain any mismatch within the central 13 nucleotides.

[00108] The dsRNA can be synthesized by standard methods known in the art as further discussed below, e.g., by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch Technologies (Novato, CA).

[00109] In some embodiments, the RNA effector molecule is a promoter-directed RNA (pdRNA) which is substantially complementary to a noncoding region of an mRNA transcript of a target gene. In one embodiment, the pdRNA is substantially complementary to the promoter region of a target gene mRNA at a site located upstream from the transcription start site, e.g., more than 100, more than 200, or more than 1,000 bases upstream from the transcription start site. In another embodiment, the pdRNA is substantially complementary to the 3'-UTR of a target gene mRNA transcript. In one embodiment, the pdRNA comprises dsRNA of 18-28 bases optionally having 3' di- or tri-nucleotide overhangs on each strand. In another embodiment, the pdRNA comprises a gapmer consisting of a single stranded polynucleotide comprising a DNA sequence which is substantially complementary to the promoter or the 3'-UTR of a target gene mRNA transcript, and flanking the polynucleotide sequences (e.g., comprising the 5 terminal bases at each of the 5' and 3' ends of the gapmer) comprises one or more modified nucleotides, such as 2' MOE, 2'OMe, or Locked Nucleic Acid bases (LNA), which protect the gapmer from cellular nucleases.

[00110] pdRNA can be used to selectively increase, decrease, or otherwise modulate expression of a target gene. Without being limited to theory, it is believed that pdRNAs modulate expression of target genes by binding to endogenous antisense RNA transcripts which overlap with noncoding regions of a target gene mRNA transcript, and recruiting Argonaute proteins (in the case of dsRNA) or host cell nucleases (e.g., RNase H) (in the case of gapmers) to selectively degrade the endogenous antisense RNAs. In some embodiments, the endogenous antisense RNA negatively regulates expression of the target gene and the pdRNA effector molecule activates expression of the target gene. Thus, in some embodiments, pdRNAs can be used to selectively activate the expression of a target gene by inhibiting the negative regulation of target gene expression by endogenous antisense RNA. Methods for identifying antisense transcripts encoded by promoter sequences of target genes and for making and using promoter-directed RNAs are known, *see, e.g.*, WO 2009/046397.

[00111] In some embodiments, the RNA effector molecule comprises an aptamer which binds to a non-nucleic acid ligand, such as a small organic molecule or protein, e.g., a transcription or translation factor, and subsequently modifies (e.g., inhibits) activity. An aptamer can fold into a specific structure that directs the recognition of a targeted binding site on the non-nucleic acid ligand. Aptamers can contain any of the modifications described herein.

[00112] In some embodiments, the RNA effector molecule comprises an antagomir. Antagomirs are single stranded, double stranded, partially double stranded or hairpin structures that target a microRNA. An antagomir consists essentially of or comprises at least 10 or more contiguous nucleotides substantially complementary to an endogenous miRNA and more particularly a target sequence of an miRNA or pre-miRNA nucleotide sequence. Antagomirs preferably have a nucleotide sequence sufficiently complementary to a miRNA target sequence of about 12 to 25 nucleotides, such as about 15 to 23 nucleotides, to allow the antagomir to hybridize to the target sequence. More preferably, the target sequence differs by no more than 1, 2, or 3 nucleotides from the sequence of the antagomir. In some embodiments, the antagomir includes a non-nucleotide moiety, e.g., a cholesterol moiety, which can be attached, e.g., to the 3' or 5' end of the oligonucleotide agent.

[00113] In some embodiments, antagomirs are stabilized against nucleolytic degradation by the incorporation of a modification, e.g., a nucleotide modification. For example, in some embodiments, antagomirs contain a phosphorothioate comprising at least the first, second, and/or third internucleotide linkages at the 5' or 3' end of the nucleotide sequence. In further embodiments, antagomirs include a 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA). In some embodiments, antagomirs include at least one 2'-O-methyl-modified nucleotide.

[00114] In some embodiments, the RNA effector molecule is a promoter-directed RNA (pdRNA) which is substantially complementary to a noncoding region of an mRNA transcript of a target gene. The pdRNA can be substantially complementary to the promoter region of a target gene mRNA at a site located upstream from the transcription start site, e.g., more than 100, more than 200, or more than 1,000 bases upstream from the transcription start

site. Also, the pdRNA can be substantially complementary to the 3'-UTR of a target gene mRNA transcript. For example, the pdRNA comprises dsRNA of 18 to 28 bases optionally having 3' di- or tri-nucleotide overhangs on each strand. The dsRNA is substantially complementary to the promoter region or the 3'-UTR region of a target gene mRNA transcript. In another embodiment, the pdRNA comprises a gapmer consisting of a single stranded polynucleotide comprising a DNA sequence which is substantially complementary to the promoter or the 3'-UTR of a target gene mRNA transcript, and flanking the polynucleotide sequences (e.g., comprising the five terminal bases at each of the 5' and 3' ends of the gapmer) comprising one or more modified nucleotides, such as 2'MOE, 2'OMe, or Locked Nucleic Acid bases (LNA), which protect the gapmer from cellular nucleases.

[00115] Expressed interfering RNA (eiRNA) can be used to selectively increase, decrease, or otherwise modulate expression of a target gene. Typically, for eiRNA, the dsRNA is expressed in the first transfected cell from an expression vector. In such a vector, the sense strand and the antisense strand of the dsRNA can be transcribed from the same nucleic acid sequence using e.g., two convergent promoters at either end of the nucleic acid sequence or separate promoters transcribing either a sense or antisense sequence. Alternatively, two plasmids can be cotransfected, with one of the plasmids designed to transcribe one strand of the dsRNA while the other is designed to transcribe the other strand. Methods for making and using eiRNA effector molecules are known in the art. *See, e.g.*, WO 2006/033756; U.S. Patent Pubs. No. 2005/0239728 and No. 2006/0035344.

[00116] In some embodiments, the RNA effector molecule comprises a small single-stranded Piwi-interacting RNA (piRNA effector molecule) which is substantially complementary to a target gene, and which selectively binds to proteins of the Piwi or Aubergine subclasses of Argonaute proteins. A piRNA effector molecule can be about 10 to 50 nucleotides in length, about 25 to 39 nucleotides in length, or about 26 to 31 nucleotides in length. *See, e.g.*, U.S. Patent Application Pub. No. 2009/0062228.

[00117] MicroRNAs are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Pre-microRNAs are processed into miRNAs. Processed microRNAs are single stranded ~17 to 25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing

complex (RISC) and have been identified as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. MicroRNAs cause post-transcriptional silencing of specific target genes, e.g., by inhibiting translation or initiating degradation of the targeted mRNA. In some embodiments, the miRNA is completely complementary with the target nucleic acid. In other embodiments, the miRNA has a region of noncomplementarity with the target nucleic acid, resulting in a "bulge" at the region of non-complementarity. In some embodiments, the region of noncomplementarity (the bulge) is flanked by regions of sufficient complementarity, e.g., complete complementarity, to allow duplex formation. For example, the regions of complementarity are at least 8 to 10 nucleotides long (e.g., 8, 9, or 10 nucleotides long).

[00118] miRNA can inhibit gene expression by, e.g., repressing translation, such as when the miRNA is not completely complementary to the target nucleic acid, or by causing target RNA degradation, when the miRNA binds its target with perfect or a high degree of complementarity. In further embodiments, the RNA effector molecule can include an oligonucleotide agent which targets an endogenous miRNA or pre-miRNA. For example, the RNA effector can target an endogenous miRNA which negatively regulates expression of a target gene, such that the RNA effector alleviates miRNA-based inhibition of the target gene.

[00119] The miRNA can comprise naturally occurring nucleobases, sugars, and covalent internucleotide (backbone) linkages, or comprise one or more non-naturally-occurring features that confer desirable properties, such as enhanced cellular uptake, enhanced affinity for the endogenous miRNA target, and/or increased stability in the presence of nucleases. In some embodiments, an miRNA designed to bind to a specific endogenous miRNA has substantial complementarity, e.g., at least 70%, 80%, 90%, or 100% complementary, with at least 10, 20, or 25 or more bases of the target miRNA. Exemplary oligonucleotide agents that target miRNAs and pre-miRNAs are described, for example, in U.S. Patent Pubs. No. 20090317907, No. 20090298174, No. 20090291907, No. 20090291906, No. 20090286969, No. 20090236225, No. 20090221685, No. 20090203893, No. 20070049547, No. 20050261218, No. 20090275729, No. 20090043082, No. 20070287179, No. 20060212950, No. 20060166910, No. 20050227934,

No. 20050222067, No. 20050221490, No. 20050221293, No. 20050182005, and No. 20050059005.

[00120] A miRNA or pre-miRNA can be 10 to 200 nucleotides in length, for example from 16 to 80 nucleotides in length. Mature miRNAs can have a length of 16 to 30 nucleotides, such as 21 to 25 nucleotides, particularly 21, 22, 23, 24, or 25 nucleotides in length. miRNA precursors can have a length of 70 to 100 nucleotides and can have a hairpin conformation. In some embodiments, miRNAs are generated *in vivo* from pre-miRNAs by the enzymes cDicer and Drosha. miRNAs or pre-miRNAs can be synthesized *in vivo* by a cell-based system or can be chemically synthesized. miRNAs can comprise modifications which impart one or more desired properties, such as superior stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell-type, and/or cell permeability, e.g., by an endocytosis-dependent or -independent mechanism. Modifications can also increase sequence specificity, and consequently decrease off-site targeting.

[00121] Upon contact with a cell expressing the target gene, the RNA effector molecule inhibits the expression of the target gene by at least 10%, as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by western blot. Expression of a target gene in cell culture can be assayed by measuring target gene mRNA levels, e.g., by bDNA or TAQMAN® assay, or by measuring protein levels, e.g., by immunofluorescence analysis or quantitative immunoblot.

#### ***RNA Effector Modification***

[00122] Optionally, an RNA effector may be chemically modified to enhance stability or other beneficial characteristics.

[00123] Oligonucleotides can be modified to prevent rapid degradation of the oligonucleotides by endo- and exo-nucleases and avoid undesirable off-target effects. The nucleic acids featured in the invention can be synthesized and/or modified by methods well established in the art, such as those described in CURRENT PROTOCOLS IN NUCLEIC ACID CHEMISTRY (Beaucage et al., eds., John Wiley & Sons, Inc., NY). Modifications include, for example, (a) end modifications, e.g., 5' end modifications (phosphorylation, conjugation, inverted linkages, etc.), or 3' end modifications (conjugation, DNA nucleotides, inverted

linkages, etc.); (b) base modifications, e.g., replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases; (c) sugar modifications (e.g., at the 2' position or 4' position) or replacement of the sugar; as well as (d) internucleoside linkage modifications, including modification or replacement of the phosphodiester linkages. Specific examples of oligonucleotide compounds useful in this invention include, but are not limited to RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. Specific examples of oligonucleotide compounds useful in this invention include, but are not limited to oligonucleotides containing modified or non-natural internucleoside linkages. Oligonucleotides having modified internucleoside linkages include, among others, those that do not have a phosphorus atom in the internucleoside linkage.

**[00124]** Modified internucleoside linkages include (e.g., RNA backbones) include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

**[00125]** Representative patents that teach the preparation of the above phosphorus-containing linkages include, e.g., U.S. Patents No. 3,687,808; No. 4,469,863; No. 4,476,301; No. 5,023,243; No. 5,177,195; No. 5,188,897; No. 5,264,423; No. 5,276,019; No. 5,278,302; No. 5,286,717; No. 5,321,131; No. 5,399,676; No. 5,405,939; No. 5,453,496; No. 5,455,233; No. 5,466,677; No. 5,476,925; No. 5,519,126; No. 5,536,821; No. 5,541,316; No. 5,550,111; No. 5,563,253; No. 5,571,799; No. 5,587,361; No. 5,625,050; No. 6,028,188; No. 6,124,445; No. 6,160,109; No. 6,169,170; No. 6,172,209; No. 6,239,265; No. 6,277,603; No. 6,326,199; No. 6,346,614; No. 6,444,423; No. 6,531,590; No. 6,534,639; No. 6,608,035; No. 6,683,167;

No. 6,858,715; No. 6,867,294; No. 6,878,805; No. 7,015,315; No. 7,041,816; No. 7,273,933; No. 7,321,029; and No. RE39464.

**[00126]** Additionally, both the sugar and the internucleoside linkage may be modified, i.e., the backbone, of the nucleotide units are replaced with novel groups. One such oligomeric compound, a RNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). See, e.g., U.S. Patents No. 5,539,082; No. 5,714,331; and No. 5,719,262. Further teaching of PNA compounds can be found, for example, in Nielsen et al., 254 Science 1497-1500 (1991).

**[00127]** Modified oligonucleotides can also contain one or more substituted sugar moieties. The RNA effector molecules, e.g., dsRNAs, can include one of the following at the 2' position: H (deoxyribose); OH (ribose); F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Exemplary suitable modifications include O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m are from 1 to 10, inclusive. In some embodiments, oligonucleotides include one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, a RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide (e.g., a RNA effector molecule), or a group for improving the pharmacodynamic properties of an oligonucleotide (e.g., a RNA effector molecule), and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., 78 Helv. Chim. Acta 486-504 (1995)), i.e., an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminoethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>.

**[00128]** Other modifications include 2'-methoxy (2'-OCH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and 2'-fluoro (2'-F). Similar modifications can also be made at other

positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patents No. 4,981,957; No. 5,118,800; No. 5,319,080; No. 5,359,044; No. 5,393,878; No. 5,446,137; No. 5,466,786; No. 5,514,785; No. 5,519,134; No. 5,567,811; No. 5,576,427; No. 5,591,722; No. 5,597,909; No. 5,610,300; No. 5,627,053; No. 5,639,873; No. 5,646,265; No. 5,658,873; No. 5,670,633; and No. 5,700,920, certain of which are commonly owned with the instant application.

**[00129]** An oligonucleotide (e.g., a RNA effector molecule) can also include nucleobase (often referred to in the art simply as “base”) modifications or substitutions. As used herein, “unmodified” or “natural” nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 2-(halo)adenine, 2-(alkyl)adenine, 2-(propyl)adenine, 2 (amino)adenine, 2-(aminoalkyl)adenine, 2 (aminopropyl)adenine, 2 (methylthio) N6 (isopentenyl)adenine, 6 (alkyl)adenine, 6 (methyl)adenine, 7 (deaza)adenine, 8 (alkenyl)adenine, 8-(alkyl)adenine, 8 (alkynyl)adenine, 8 (amino)adenine, 8-(halo)adenine, 8-(hydroxyl)adenine, 8 (thioalkyl)adenine, 8-(thiol)adenine, N6-(isopentyl)adenine, N6 (methyl)adenine, N6, N6 (dimethyl)adenine, 2-(alkyl)guanine, 2 (propyl)guanine, 6-(alkyl)guanine, 6 (methyl)guanine, 7 (alkyl)guanine, 7 (methyl)guanine, 7 (deaza)guanine, 8 (alkyl)guanine, 8-(alkenyl)guanine, 8 (alkynyl)guanine, 8-(amino)guanine, 8 (halo)guanine, 8-(hydroxyl)guanine, 8 (thioalkyl)guanine, 8-(thiol)guanine, N (methyl)guanine, 2-(thio)cytosine, 3 (deaza) 5 (aza)cytosine, 3-(alkyl)cytosine, 3 (methyl)cytosine, 5-(alkyl)cytosine, 5-(alkynyl)cytosine, 5 (halo)cytosine, 5 (methyl)cytosine, 5 (propynyl)cytosine, 5 (propynyl)cytosine, 5 (trifluoromethyl)cytosine, 6-(azo)cytosine, N4 (acetyl)cytosine, 3 (3 amino-3 carboxypropyl)uracil, 2-(thio)uracil, 5 (methyl) 2 (thio)uracil, 5 (methylaminomethyl)-2 (thio)uracil, 4-(thio)uracil, 5 (methyl) 4 (thio)uracil, 5 (methylaminomethyl)-4 (thio)uracil, 5 (methyl) 2,4 (dithio)uracil, 5 (methylaminomethyl)-2,4 (dithio)uracil, 5 (2-aminopropyl)uracil, 5-(alkyl)uracil, 5-(alkynyl)uracil, 5-(allylamino)uracil, 5 (aminoallyl)uracil, 5 (aminoalkyl)uracil, 5 (guanidiniumalkyl)uracil, 5 (1,3-diazole-1-alkyl)uracil,

5-(cyanoalkyl)uracil, 5-(dialkylaminoalkyl)uracil, 5 (dimethylaminoalkyl)uracil, 5-(halo)uracil, 5-(methoxy)uracil, uracil-5 oxyacetic acid, 5 (methoxycarbonylmethyl)-2-(thio)uracil, 5 (methoxycarbonyl-methyl)uracil, 5 (propynyl)uracil, 5 (propynyl)uracil, 5 (trifluoromethyl)uracil, 6 (azo)uracil, dihydrouracil, N3 (methyl)uracil, 5-uracil (i.e., pseudouracil), 2 (thio)pseudouracil, 4 (thio)pseudouracil, 2,4-(dithio)pseudouracil, 5-(alkyl)pseudouracil, 5-(methyl)pseudouracil, 5-(alkyl)-2-(thio)pseudouracil, 5-(methyl)-2-(thio)pseudouracil, 5-(alkyl)-4 (thio)pseudouracil, 5-(methyl)-4 (thio)pseudouracil, 5-(alkyl)-2,4 (dithio)pseudouracil, 5-(methyl)-2,4 (dithio)pseudouracil, 1 substituted pseudouracil, 1 substituted 2(thio)-pseudouracil, 1 substituted 4 (thio)pseudouracil, 1 substituted 2,4-(dithio)pseudouracil, 1 (aminocarbonylethylenyl)-pseudouracil, 1 (aminocarbonylethylenyl)-2(thio)-pseudouracil, 1 (aminocarbonylethylenyl)-4 (thio)pseudouracil, 1 (aminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-pseudouracil, 1 (aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-substituted 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-substituted 1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 1,3,5-(triaz)-2,6-(diox)-naphthalene, inosine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, inosinyl, 2-aza-inosinyl, 7-deaza-inosinyl, nitroimidazolyl, nitropyrazolyl, nitrobenzimidazolyl, nitroindazolyl, aminoindolyl, pyrrolopyrimidinyl, 3-(methyl)isocarbostyrilyl, 5-(methyl)isocarbostyrilyl, 3-(methyl)-7-(propynyl)isocarbostyrilyl, 7-(aza)indolyl, 6-(methyl)-7-(aza)indolyl, imidizopyridinyl, 9-(methyl)-imidizopyridinyl, pyrrolopyrizinyl, isocarbostyrilyl, 7-(propynyl)isocarbostyrilyl, propynyl-7-(aza)indolyl, 2,4,5-(trimethyl)phenyl,

4-(methyl)indolyl, 4,6-(dimethyl)indolyl, phenyl, naphthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenyl, tetracenyl, pentacenyl, difluorotolyl, 4-(fluoro)-6-(methyl)benzimidazole, 4-(methyl)benzimidazole, 6-(azo)thymine, 2-pyridinone, 5 nitroindole, 3 nitropyrrole, 6-(aza)pyrimidine, 2 (amino)purine, 2,6-(diamino)purine, 5 substituted pyrimidines, N2-substituted purines, N6-substituted purines, O6-substituted purines, substituted 1,2,4-triazoles, pyrrolo-pyrimidin-2-on-3-yl, 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, ortho-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-ortho-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl, 2-oxo-pyridopyrimidine-3-yl, or any O-alkylated or N-alkylated derivatives thereof. Modified nucleobases also include natural bases that comprise conjugated moieties, e.g., a ligand.

**[00130]** The oligonucleotides can also be modified to include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively “locks” the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to oligonucleotide molecules has been shown to increase oligonucleotide molecule stability in serum, and to reduce off-target effects. Elmen et al., 33 Nucl. Acids Res. 439-47 (2005); Mook et al., 6 Mol. Cancer Ther. 833-43 (2007); Grunweller et al., 31 Nucl. Acids Res. 3185-93 (2003); U.S. Patents No. 6,268,490; No. 6,670,461; No. 6,794,499; No. 6,998,484; No. 7,053,207; No. 7,084,125; and No. 7,399,845.

**[00131]** In certain instances, the oligonucleotides of a RNA effector molecule can be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to oligonucleotides in order to enhance the activity, cellular distribution or cellular uptake of the oligonucleotides, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Kubo et al., 365 Biochem. Biophys. Res. Comm. 54-61 (2007)); Letsinger et al., 86 PNAS 6553 (1989)); cholic acid (Manoharan et al., 1994); a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., 1992; Manoharan et al., 1993); a thiocholesterol (Oberhauser et al., 1992); an aliphatic chain,

e.g., dodecandiol or undecyl residues (Saison-Belmoaras et al., 1991; Kabanov et al., 259 FEBS Lett. 327 (1990); Svinarchuk et al., 75 Biochimie 75 (1993)); a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., 1995); Shea et al., 18 Nucl. Acids Res. 3777 (1990)); a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995); or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995); a palmityl moiety (Mishra et al., 1995); or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., 1996). Representative United States patents that teach the preparation of such RNA conjugates have been listed herein. Typical conjugation protocols involve the synthesis of an oligonucleotide bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction can be performed either with the RNA still bound to the solid support or following cleavage of the RNA, in solution phase. Purification of the RNA conjugate by HPLC typically affords the pure conjugate.

#### *Delivery Methods of RNA Effector Molecules*

**[00132]** The delivery of RNA effector molecules to cells can be achieved in a number of different ways. Several suitable delivery methods are well known in the art. For example, the skilled person is directed to WO 2011/005786, which discloses exemplary delivery methods that can be used in this invention at pages 187-219, the teachings of which are incorporated herein by reference. For example, delivery can be performed directly by administering a composition comprising a RNA effector molecule, e.g., an siRNA, into cell culture. Alternatively, delivery can be performed indirectly by administering into the cell one or more vectors that encode and direct the expression of the RNA effector molecule.

**[00133]** A reagent that facilitates RNA effector molecule uptake may be used. For example, an emulsion, a cationic lipid, a non-cationic lipid, a charged lipid, a liposome, an anionic lipid, a penetration enhancer, a transfection reagent or a modification to the RNA effector molecule for attachment, e.g., a ligand, a targeting moiety, a peptide, a lipophilic group, etc.

[00134] For example, RNA effector molecules can be delivered using a drug delivery system such as a nanoparticle, a dendrimer, a polymer, a liposome, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of a RNA effector molecule (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient cellular uptake. Cationic lipids, dendrimers, or polymers can either be bound to RNA effector molecules, or induced to form a vesicle, liposome, or micelle that encases the RNA effector molecule. *See, e.g.,* Kim et al., 129 J. Contr. Release 107-16 (2008). Methods for making and using cationic-RNA effector molecule complexes are well within the abilities of those skilled in the art. *See e.g.,* Sorensen et al 327 J. Mol. Biol. 761-66 (2003); Verma et al., 9 Clin. Cancer Res. 1291-1300 (2003); Arnold et al., 25 J. Hypertens. 197-205 (2007).

[00135] In one embodiment, the reagent that facilitates RNA effector molecule uptake used herein comprises a charged lipid as described in International Application Ser. No. PCT/US10/59206, filed 7 December 2010.

[00136] The RNA effector molecules described herein can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, the RNA effector molecules can be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C1-20 alkyl ester (e.g., isopropylmyristate IPM), monoglyceride, diglyceride, or acceptable salts thereof.

[00137] In one embodiment, the RNA effector molecules are fully encapsulated in the lipid formulation (e.g., to form a SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle). The term "SNALP" refers to a stable nucleic acid-lipid particle: a vesicle of lipids coating a reduced aqueous interior comprising a nucleic acid such as a RNA effector molecule or a plasmid from which a RNA effector molecule is transcribed. SNALPs are described, e.g., in U.S. Patent Pubs. No. 2006/0240093, No. 2007/0135372; No. 2009/0291131; U.S. Patent Applications Ser. No. 12/343,342; No.12/424,367. The term "SPLP" refers to a nucleic acid-lipid particle comprising plasmid DNA encapsulated within a lipid vesicle. SNALPs and SPLPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the

particle (e.g., a PEG-lipid conjugate). SPLPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in WO 00/03683. The particles in this embodiment typically have a mean diameter of about 50 nm to about 150 nm, or about 60 nm to about 130 nm, or about 70 nm to about 110 nm, or typically about 70 nm to about 90 nm, inclusive, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid-lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are reported in, e.g., U.S. Patents No. 5,976,567; No. 5,981,501; No. 6,534,484; No. 6,586,410; No. 6,815,432; and WO 96/40964.

**[00138]** The lipid to RNA ratio (mass/mass ratio) (e.g., lipid to dsRNA ratio) can be in ranges of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1, inclusive.

**[00139]** A cationic lipid of the formulation can comprise at least one protonatable group having a pKa of from 4 to 15. The cationic lipid can be, for example, N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), 1,2-DiLinoleoyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleoxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-Dilinoleoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMA), 1,2-Dilinoleoxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleoxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxyp propane (DLin-EG-DMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane, or a mixture

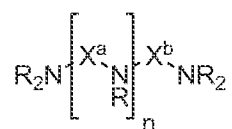
thereof. The cationic lipid can comprise from about 20 mol% to about 70 mol%, inclusive, or about 40 mol% to about 60 mol%, inclusive, of the total lipid present in the particle. In one embodiment, cationic lipid can be further conjugated to a ligand.

**[00140]** A non-cationic lipid can be an anionic lipid or a neutral lipid, such as distearoyl-phosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoyl-phosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoyl-phosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1- carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1 -stearoyl-2-oleoyl- phosphatidylethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid can be from about 5 mol% to about 90 mol%, inclusive, of about 10 mol%, to about 58 mol%, inclusive, if cholesterol is included, of the total lipid present in the particle.

**[00141]** The lipid that inhibits aggregation of particles can be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkylxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA can be, for example, a PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmitoxypropyl (C16), or a PEG- distearyloxypropyl (C18). The lipid that prevents aggregation of particles can be from 0 mol % to about 20 mol % or about 2 mol % of the total lipid present in the particle. In one embodiment, PEG lipid can be further conjugated to a ligand.

**[00142]** In some embodiments, the nucleic acid-lipid particle further includes a steroid such as, cholesterol at, e.g., about 10 mol% to about 60 mol%, inclusive, or about 48 mol% of the total lipid present in the particle.

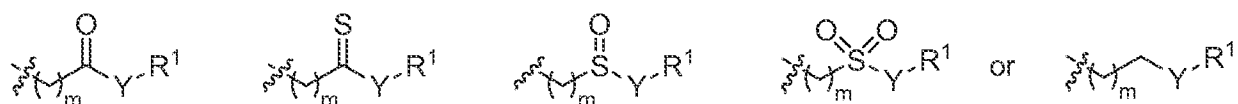
**[00143]** In one embodiment, the lipid particle comprises a steroid, a PEG lipid and a cationic lipid of formula (I):



formula (I)

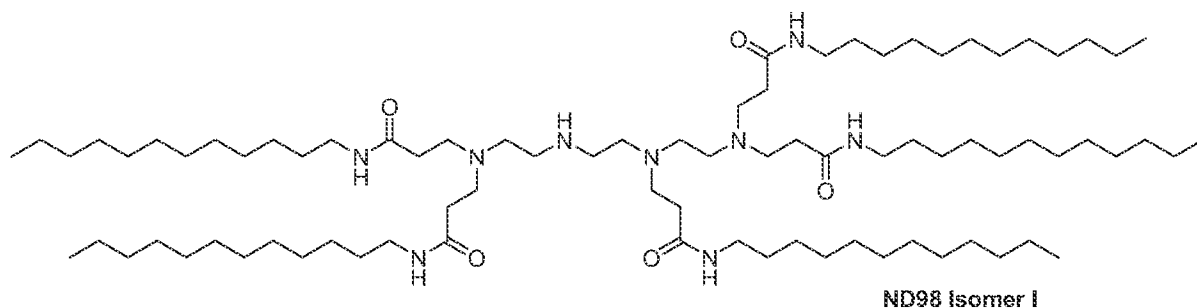
wherein each Xa and Xb, for each occurrence, is independently C1-6 alkylene;

n is 0, 1, 2, 3, 4, or 5; each R is independently H,



m is 0, 1, 2, 3 or 4; Y is absent, O, NR<sup>2</sup>, or S; R<sup>1</sup> is alkyl alkenyl or alkynyl; each of which is optionally substituted with one or more substituents; and R<sup>2</sup> is H, alkyl alkenyl or alkynyl; each of which is optionally substituted each of which is optionally substituted with one or more substituents.

**[00144]** In one example, the lipidoid ND98·4HCl (MW 1487) (Formula 2), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid RNA effector molecule nanoparticles (e.g., LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/mL; Cholesterol, 25 mg/mL, PEG-Ceramide C16, 100 mg/mL. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in, e.g., a 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous RNA effector molecule (e.g., in sodium acetate pH 5) such that the final ethanol concentration is about 35% to 45% and the final sodium acetate concentration is about 100 mM to 300 mM, inclusive. Lipid RNA effector molecule nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (e.g., 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, e.g., about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.



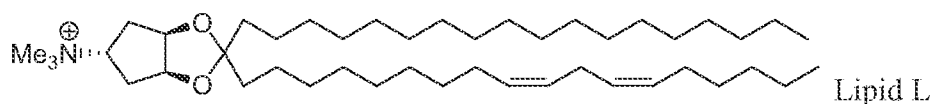
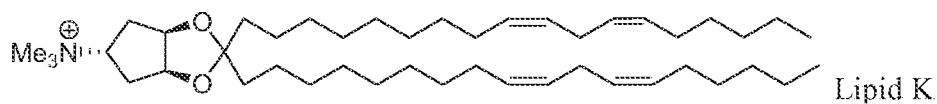
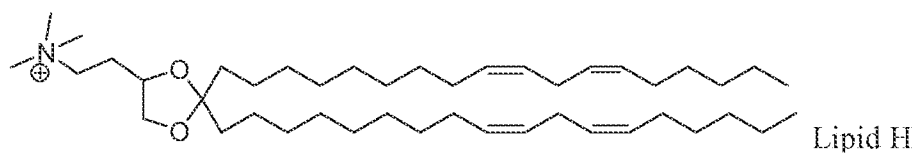
Formula 2

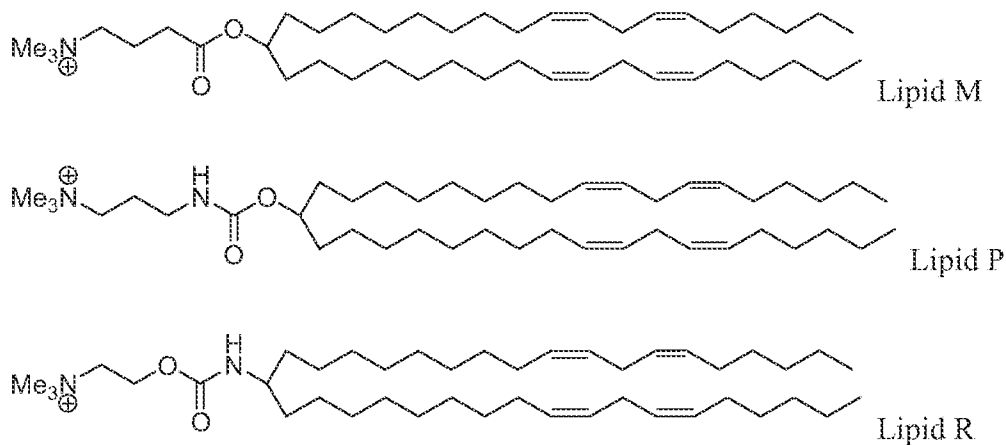
[00145] In some embodiments, the nucleic acid-lipid particle further includes a steroid such as, cholesterol at, e.g., about 10 mol% to about 60 mol%, inclusive, or about 48 mol% of the total lipid present in the particle.

[00146] LNP01 formulations are described elsewhere, e.g., WO 2008/042973.

[00147] In one embodiment, the reagent that facilitates RNA effector molecule uptake used herein comprises a cationic lipid as described in e.g., in International Application Ser. No. PCT/US10/59206, filed 7 December 2010.

[00148] In various embodiments, the RNA effector molecule composition described herein comprises a cationic lipid selected from the group consisting of: "Lipid H", "Lipid K"; "Lipid L", "Lipid M"; "Lipid P"; or "Lipid R", whose formulas are indicated as follows:





**[00149]** Also contemplated herein are various formulations of the lipids described above, such as, e.g., K8, P8 and L8 which refer to formulations comprising Lipid K, P, and L, respectively. Some exemplary lipid formulations for use with the methods and compositions described herein are found in e.g., Table 5:

| Table 5. Example lipid formulations |                       |                      |        |               |
|-------------------------------------|-----------------------|----------------------|--------|---------------|
| Formulation Number                  | Cationic Lipid Number | Cationic Lipid Mol % | DOPE % | Cholesterol % |
| 1                                   | 200 (Lipid H)         | 48.08                | 51.92  | -             |
| 2                                   | 200 (Lipid H)         | 47.94                | 47.06  | 5             |
| 3                                   | 201 (Lipid K)         | 45.56                | 54.44  | -             |
| 4 (K8)                              | 201 (Lipid K)         | 47.94                | 47.06  | 5             |
| 5 (L8)                              | 202 (Lipid L)         | 47.94                | 47.06  | 5             |
| 6                                   | 203 (Lipid M)         | 53.01                | 44.49  | 2.5           |
| 7                                   | 203 (Lipid M)         | 47.94                | 47.06  | 5             |
| 8 (P8)                              | 204 (Lipid P)         | 47.94                | 47.06  | 5             |
| 9                                   | 205 (Lipid R)         | 47.94                | 47.06  | 5             |

**[00150]** In another embodiment, the RNA effector molecule composition described herein further comprises a lipid formulation comprising a lipid selected from the group consisting of Lipid H, Lipid K, Lipid L, Lipid M, Lipid P, and Lipid R, and further comprises a neutral lipid and a sterol. In particular embodiments, the lipid formulation comprises between approximately 25 mol % - 100 mol% of the lipid. In another embodiment, the lipid formulation comprises between 0 mol% - 50 mol% cholesterol. In still another embodiment, the lipid formulation comprises between 30 mol% - 65 mol% of a neutral lipid. In particular

embodiments, the lipid formulation comprises the relative mol% of the components as listed in Table 6 as follows:

| Table 6. Example lipid formulae |              |       |      |
|---------------------------------|--------------|-------|------|
| Series                          | Lipid (Mol%) | DOPE  | Chol |
| 1                               | 45.56        | 54.44 | 0    |
| 2                               | 48.08        | 51.92 | 0    |
| 3                               | 50.60        | 49.40 | 0    |
| 4                               | 53.10        | 46.90 | 0    |
| 5                               | 52.73        | 37.27 | 10   |
| 6                               | 52.92        | 42.08 | 5    |
| 7                               | 53.01        | 44.49 | 2.5  |
| 8                               | 47.94        | 47.06 | 5    |

[00151] Additional exemplary lipid-siRNA formulations are as shown below in Table

7.

TABLE 7: lipid-siRNA formulations

|           | Cationic Lipid                                             | cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate<br>Lipid:siRNA ratio | Process        |
|-----------|------------------------------------------------------------|----------------------------------------------------------------------------------------|----------------|
| SNALP     | 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)      | DLinDMA/DPPC/Cholesterol/PEG-cDMA<br>(57.1/7.1/34.4/1.4)<br>lipid:siRNA ~ 7:1          |                |
| SNALP-XTC | 2,2-Dilinoyleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC) | XTC/DPPC/Cholesterol/PEG-cDMA<br>57.1/7.1/34.4/1.4<br>lipid:siRNA ~ 7:1                |                |
| LNP05     | 2,2-Dilinoyleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC) | XTC/DSPC/Cholesterol/PEG-DMG<br>57.5/7.5/31.5/3.5<br>lipid:siRNA ~ 6:1                 | Extrusion      |
| LNP06     | 2,2-Dilinoyleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC) | XTC/DSPC/Cholesterol/PEG-DMG<br>57.5/7.5/31.5/3.5<br>lipid:siRNA ~ 11:1                | Extrusion      |
| LNP07     | 2,2-Dilinoyleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC) | XTC/DSPC/Cholesterol/PEG-DMG<br>60/7.5/31/1.5,<br>lipid:siRNA ~ 6:1                    | In-line mixing |
| LNP08     | 2,2-Dilinoyleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC) | XTC/DSPC/Cholesterol/PEG-DMG<br>60/7.5/31/1.5,<br>lipid:siRNA ~ 11:1                   | In-line mixing |
| LNP09     | 2,2-Dilinoyleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC) | XTC/DSPC/Cholesterol/PEG-DMG<br>50/10/38.5/1.5<br>Lipid:siRNA 10:1                     | In-line mixing |

|       |                                                                                                                                         |                                                                        |                |
|-------|-----------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|----------------|
| LNP10 | (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100)                 | ALN100/DSPC/Cholesterol/PEG-DMG<br>50/10/38.5/1.5<br>Lipid:siRNA 10:1  | In-line mixing |
| LNP11 | (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3)                                                 | MC-3/DSPC/Cholesterol/PEG-DMG<br>50/10/38.5/1.5<br>Lipid:siRNA 10:1    | In-line mixing |
| LNP12 | 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyldidodecan-2-ol (Tech G1) | Tech G1/DSPC/Cholesterol/PEG-DMG<br>50/10/38.5/1.5<br>Lipid:siRNA 10:1 | In-line mixing |

[00152] LNP09 formulations and XTC comprising formulations are described, *e.g.*, in PCT Publication No. WO 2010/088537, which is hereby incorporated by reference.

[00153] LNP11 formulations and MC3 comprising formulations are described, *e.g.*, in PCT Publication No. WO 2010/144740, which is hereby incorporated by reference.

[00154] LNP12 formulations and TechG1 comprising formulations are described, *e.g.*, in International Application Ser. No. PCT/US10/ 33777, filed May 5, 2010, which is hereby incorporated by reference.

[00155] Formulations prepared by either the standard or extrusion-free method can be characterized in similar manners. For example, formulations are typically characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles can be measured by light scattering using, for example, a Malvern Zetasizer Nano ZS (Malvern, PA). Particles should be about 20-300 nm, such as 40-100 nm in size. The particle size distribution should be unimodal. The total dsRNA effector molecule concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated RNA effector molecule can be incubated with a RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, *e.g.*, 0.5% Triton-X100. The total RNA effector molecule in the formulation can be determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the “free” RNA effector molecule content (as measured by the signal in the absence of surfactant) from the total RNA effector molecule content. Percent entrapped RNA effector molecule is typically >85%. For lipid nanoparticle formulation, the particle size is

at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, or at least 120 nm. The suitable range is typically about at least 50 nm to about at least 110 nm, about at least 60 nm to about at least 100 nm, or about at least 80 nm to about at least 90 nm, inclusive.

[00156] Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*. In order to cross intact cell membranes, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

[00157] Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; and liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation. *See, e.g.,* Wang et al., DRUG DELIV. PRINCIPLES & APPL. (John Wiley & Sons, Hoboken, NJ, 2005); Rosoff, 1988. Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

[00158] Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act. Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

[00159] Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged polynucleotide molecules to form a stable complex. The positively charged polynucleotide/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm. Wang et al., 147 *Biochem. Biophys. Res. Commun.*, 980-85 (1987).

[00160] Liposomes which are pH-sensitive or negatively-charged, entrap polynucleotide rather than complex with it. Because both the polynucleotide and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some polynucleotide is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells. Zhou et al., 19 *J. Controlled Rel.* 269-74 (1992).

[00161] One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

[00162] Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside GM1, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation

half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES). Allen et al., 223 FEBS Lett. 42 (1987); Wu et al., 53 Cancer Res. 3765 (1993).

**[00163]** Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (507 Ann. NY Acad. Sci. 64 (1987)), reported the ability of monosialoganglioside GM1, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (85 PNAS 6949 (1988)). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside GM1 or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

**[00164]** Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (53 Bull. Chem. Soc. Jpn. 2778 (1980)) described liposomes comprising a nonionic detergent, 2C1215G, that contains a PEG moiety. Illum et al. (167 FEBS Lett. 79 (1984)), noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent No. 4,426,330 and No. 4,534,899). In addition, antibodies can be conjugated to a polyalkylene derivatized liposome (see e.g., PCT Application US 2008/0014255). Klibanov et al. (268 FEBS Lett. 235 (1990)), described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (1029 Biochim. Biophys. Acta 1029, (1990)), extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. 0445131 B1 and WO 90/04384 to Fisher.

**[00165]** Liposome compositions containing 1-20 mol% of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patents No. 5,013,556;

No. 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804; European Patent No. 0 496813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 and in WO 94/20073. Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391. U.S. Patents No. 5,540,935 and No. 5,556,948 describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces. Methods and compositions relating to liposomes comprising PEG can be found in, e.g., U.S. Patents No. 6,049,094; No. 6,224,903; No. 6,270,806; No. 6,471,326; No. 6,958,241.

**[00166]** As noted above, liposomes can optionally be prepared to contain surface groups, such as antibodies or antibody fragments, small effector molecules for interacting with cell-surface receptors, antigens, and other like compounds, and these groups can facilitate delivery of liposomes and their contents to specific cell populations. Such ligands can be included in the liposomes by including in the liposomal lipids a lipid derivatized with the targeting molecule, or a lipid having a polar-head chemical group that can be derivatized with the targeting molecule in preformed liposomes. Alternatively, a targeting moiety can be inserted into preformed liposomes by incubating the preformed liposomes with a ligand-polymer-lipid conjugate.

**[00167]** Lipids can be derivatized using a variety of targeting moieties, such as ligands, cell surface receptors, glycoproteins, vitamins (e.g., riboflavin) and monoclonal antibodies by covalently attaching the ligand to the free distal end of a hydrophilic polymer chain, which is attached at its proximal end to a vesicle-forming lipid. There are a wide variety of techniques for attaching a selected hydrophilic polymer to a selected lipid and activating the free, unattached end of the polymer for reaction with a selected ligand, and as noted above, the hydrophilic polymer polyethyleneglycol (PEG) has been studied widely. Allen et al., 1237 Biochem. Biophys. Acta 99-108 (1995); Zalipsky, 4 Bioconj. Chem. 296-99 (1993); Zalipsky et al., 353 FEBS Lett. 1-74 (1994); Zalipsky et al., Bioconj. Chem. 705-08 (1995); Zalipsky, *in* STEALTH LIPOSOMES (Lasic & Martin, eds. CRC Press, Boca Raton, FL, 1995).

**[00168]** A number of liposomes comprising nucleic acids are known in the art, such as methods for encapsulating high molecular weight nucleic acids in liposomes. WO 96/40062. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that

the contents of such liposomes can include a dsRNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes.

WO 97/04787 to Love et al. discloses liposomes comprising dsRNAs targeted to the *raf* gene. In addition, methods for preparing a liposome composition comprising a nucleic acid can be found in, e.g., U.S. Patents No. 6,011,020; No. 6,074,667; No. 6,110,490; No. 6,147,204; No. 6,271,206; No. 6,312,956; No. 6,465,188; No. 6,506,564; No. 6,750,016; No. 7,112,337.

[00169] Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes can be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g., they are self-optimizing, self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition.

[00170] Encapsulated nanoparticles can also be used for delivery of RNA effector molecules. Examples of such encapsulated nanoparticles include those created using yeast cell wall particles (YCWP). For example, glucan-encapsulated siRNA particles (GeRPs) are payload delivery systems made up of a yeast cell wall particle (YCWP) exterior and a multilayered nanoparticle interior, wherein the multilayered nanoparticle interior has a core comprising a payload complexed with a trapping agent. Glucan-encapsulated delivery systems, such as those described in U.S. Patent Applications Ser. No. 12/260,998, filed October 29, 2008, can be used to deliver siRNA duplexes to achieve silencing *in vitro* and *in vivo*.

### C. Cell Cultures

[00171] Methods described herein use host cells to produce glycosylated lysosomal proteins having modified glycans. A host cell can be derived from a yeast, insect, amphibian, fish, reptile, bird, mammal or human, or can be a hybrid cell, such as a hybridoma cell. Host cells can be unmodified cells or cell lines, or cell lines which have been genetically modified (e.g., to facilitate production of a biological product). In some embodiments, the host cell is a cell line that has been modified to allow for growth under desired conditions, such as in serum-free media, in cell suspension culture, or in adherent cell culture.

[00172] A mammalian host cell can be advantageous where the glycoprotein is a mammalian glycoprotein, particularly if the glycoprotein is a biotherapeutic agent or is otherwise intended for administration to or consumption by humans. In some embodiments, the host cell is a CHO cell, which is a cell line used for the expression of many recombinant proteins. Additional mammalian cell lines used commonly for the expression of recombinant proteins include 293HEK cells, HeLa cells, COS cells, NIH/3T3 cells, Jurkat Cells, NSO cells, and HUVEC cells.

[00173] In some embodiments, the host cell is a CHO cell derivative that has been modified genetically to facilitate production of recombinant proteins. For example, various CHO cell strains have been developed which permit stable insertion of recombinant DNA into a specific gene or expression region of the cells, amplification of the inserted DNA, and selection of cells exhibiting high level expression of the recombinant protein. Examples of CHO cell derivatives useful in methods provided herein include, but are not limited to, CHO-K1 cells, CHO-DUKX, CHO-DUKX B1, CHO-DG44 cells, CHO-ICAM-1 cells, and CHO-hlFN $\gamma$  cells. Methods for expressing recombinant proteins in CHO cells are known in the art and are described, e.g., in U.S. Patents No. 4,816,567 and No. 5,981,214.

[00174] Examples of human cell lines useful in methods provided herein include the cell lines 293T (embryonic kidney), 786-0 (renal), A498 (renal), A549 (alveolar basal epithelial), ACHN (renal), BT-549 (breast), BxPC-3 (pancreatic), CAKI-1 (renal), Capan-1 (pancreatic), CCRF-CEM (leukemia), COLO 205 (colon), DLD-1 (colon), DMS 114 (small cell lung), DU145 (prostate), EKVX (non-small cell lung), HCC-2998 (colon), HCT-15 (colon), HCT-116 (colon), HT29 (colon), HT-1080 (fibrosarcoma), HEK 293 (embryonic kidney), HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), HL-60(TB) (leukemia), HOP-62 (non-small cell lung), HOP-92 (non-small cell lung), HS 578T (breast), HT-29 (colon adenocarcinoma), IGR-OV1 (ovarian), IMR32 (neuroblastoma), Jurkat (T lymphocyte), K-562 (leukemia), KM12 (colon), KM20L2 (colon), LAN5 (neuroblastoma), LNCap.FGC (Caucasian prostate adenocarcinoma), LOX IMVI (melanoma), LXFL 529 (non-small cell lung), M14 (melanoma), M19-MEL (melanoma), MALME-3M (melanoma), MCF10A (mammary epithelial), MCF7 (mammary), MDA-MB-453 (mammary epithelial), MDA-MB-468 (breast), MDA-MB-231 (breast), MDA-N (breast), MOLT-4 (leukemia), NCI/ADR-RES (ovarian), NCI-

H226 (non-small cell lung), NCI-H23 (non-small cell lung), NCI-H322M (non-small cell lung), NCI-H460 (non-small cell lung), NCI-H522 (non-small cell lung), OVCAR-3 (ovarian), OVCAR-4 (ovarian), OVCAR-5 (ovarian), OVCAR-8 (ovarian), P388 (leukemia), P388/ADR (leukemia), PC-3 (prostate), PERC6® (E1-transformed embryonal retina), RPMI-7951 (melanoma), RPMI-8226 (leukemia), RXF 393 (renal), RXF-631 (renal), Saos-2 (bone), SF-268 (CNS), SF-295 (CNS), SF-539 (CNS), SHP-77 (small cell lung), SH-SY5Y (neuroblastoma), SK-BR3 (breast), SK-MEL-2 (melanoma), SK-MEL-5 (melanoma), SK-MEL-28 (melanoma), SK-OV-3 (ovarian), SN12K1 (renal), SN12C (renal), SNB-19 (CNS), SNB-75 (CNS), SNB-78 (CNS), SR (leukemia), SW-620 (colon), T-47D (breast), THP-1 (monocyte-derived macrophages), TK-10 (renal), U87 (glioblastoma), U293 (kidney), U251 (CNS), UACC-257 (melanoma), UACC-62 (melanoma), UO-31 (renal), W138 (lung), and XF 498 (CNS).

**[00175]** Examples of non-human primate cell lines useful in methods provided herein include the cell lines monkey kidney (CVI-76), African green monkey kidney (VERO-76), green monkey fibroblast (COS-1), and monkey kidney (CVI) cells transformed by SV40 (COS-7). Additional mammalian cell lines are known to those of ordinary skill in the art and are catalogued at the American Type Culture Collection catalog (Manassas, VA).

**[00176]** Examples of rodent cell lines useful in methods provided herein include the cell lines baby hamster kidney (BHK) (e.g., BHK21, BHK TK), mouse Sertoli (TM4), buffalo rat liver (BRL 3A), mouse mammary tumor (MMT), rat hepatoma (HTC), mouse myeloma (NS0), murine hybridoma (Sp2/0), mouse thymoma (EL4), Chinese Hamster Ovary (CHO) and CHO cell derivatives, murine embryonic (NIH/3T3, 3T3 L1), rat myocardial (H9c2), mouse myoblast (C2C12), and mouse kidney (miMCD-3).

**[00177]** In some embodiments, the host cell is a multipotent stem cell or progenitor cell. Examples of multipotent cells useful in methods provided herein include murine embryonic stem (ES-D3) cells, human umbilical vein endothelial (HuVEC) cells, human umbilical artery smooth muscle (HuASMC) cells, human differentiated stem (HKB-II) cells, human mesenchymal stem (hMSC) cells, and induced pluripotent stem (iPS) cells.

[00178] In some embodiments, the host cell is an insect cell, such as Sf9 cell line (derived from pupal ovarian tissue of *Spodoptera frugiperda*); Hi-5 (derived from *Trichoplusia ni* egg cell homogenates); or S2 cells (from *Drosophila melanogaster*).

[00179] In some embodiments, the host cells are suitable for growth in suspension cultures. Suspension-competent host cells are generally monodisperse or grow in loose aggregates without substantial aggregation. Suspension-competent host cells include cells that are suitable for suspension culture without adaptation or manipulation (e.g., hematopoietic cells, lymphoid cells) and cells that have been made suspension-competent by modification or adaptation of attachment-dependent cells (e.g., epithelial cells, fibroblasts).

[00180] In some embodiments, the host cell is an attachment dependent cell which is grown and maintained in adherent culture. Examples of human adherent cell lines useful in methods provided herein include the cell lines human neuroblastoma (SH-SY5Y, IMR32, and LAN5), human cervical carcinoma (HeLa), human breast epithelial (MCF10A), human embryonic kidney (293T), and human breast carcinoma (SK-BR3).

[00181] In some embodiments, the host cell is a cell line that has been modified to allow for growth under desired conditions, such as in serum-free media, in cell suspension culture, or in adherent cell culture. The host cell can be, for example, a human Namalwa Burkitt lymphoma cell (BLcl-kar-Namalwa), baby hamster kidney fibroblast (BHK), CHO cell, Murine myeloma cell (NS0, SP2/0), hybridoma cell, human embryonic kidney cell (293 HEK), human retina-derived cell (PER.C6® cells, U.S. Patent No. 7,550,284), insect cell line (Sf9, derived from pupal ovarian tissue of *Spodoptera frugiperda*; or Hi-5, derived from *Trichoplusia ni* egg cell homogenates; *see also* U.S. Patent No. 7,041,500), Madin-Darby canine kidney cell (MDCK), primary mouse brain cells or tissue, primary calf lymph cells or tissue, primary monkey kidney cells, embryonated chicken egg, primary chicken embryo fibroblast (CEF), Rhesus fetal lung cell (FRhL-2), Human fetal lung cell (WI-38, MRC-5), African green monkey kidney epithelial cell (Vero, CV-1), Rhesus monkey kidney cell (LLC-MK2), or yeast cell. Additional mammalian cell lines commonly used for the expression of recombinant proteins include, but are not limited to, HeLa cells, COS cells, NIH/3T3 cells, Jurkat Cells, and human umbilical vein endothelial cells (HUVEC) cells.

[00182] Host cells can be unmodified or genetically modified (e.g., a cell from a transgenic animal). For example, CEFs from transgenic chicken eggs can have one or more genes essential for the IFN pathway, e.g., interferon receptor, STAT1, etc., has been disrupted, i.e., is a “knockout.” *See, e.g.,* Sang, 12 Trends Biotech. 415 (1994); Perry et al., 2 Transgenic Res. 125 (1993); Stern, 212 Curr Top Micro. Immunol. 195-206 (1996); Shuman, 47 Experientia 897 (1991). Also, the cell can be modified to allow for growth under desired conditions, e.g., incubation at 30°C.

[00183] The host cells may express the glycoprotein of interest endogenously, or alternatively, the host cell may be engineered to express an exogenous glycoprotein. For example, a host cell may be transfected with one or more expression vectors that encode the glycoprotein. The nucleic acid molecule encoding the glycoprotein may be transiently introduced into the host cell, or stably integrated into the genome of the host cell. For example, one or more recombinant expression vectors encoding a lysosomal protein may be transfected, such that the lysosomal protein is expressed in the host cell. Alternatively, the host cell can be engineered such that the gene encoding the lysosomal protein is activated by an exogenous promoter. This way, a host cell that does not normally express the lysosomal protein (or expresses the lysosomal protein at low level) can be modified to promote the production the lysosomal protein.

[00184] If desired, the glycoprotein may be secreted into the medium in which the host cell is cultured, from which medium the glycoprotein can be recovered.

[00185] Standard recombinant DNA methodologies may be used to obtain a nucleic acid that encodes a glycoprotein, incorporate the nucleic acid into an expression vector and introduce the vector into a host cell, such as those described in Sambrook, et al. (eds), Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor, (2001); Ausubel, F. M. et al. (eds. ) Current Protocols in Molecular Biology, John Wiley & Sons (1995). A nucleic acid encoding the glycoprotein may be inserted into an expression vector or vectors such that the nucleic acids are operably linked to transcriptional and translational control sequences. The expression vector and expression control sequences are generally chosen to be compatible with the expression host cell used.

[00186] For example, to express a lysosomal protein, nucleic acids encoding the lysosomal protein may be first obtained. These nucleic acids can be obtained by amplification and modification of a gene that encodes the lysosomal protein, using e.g., PCR.

[00187] In addition to the nucleic acid that encodes the glycoprotein, the expression vector may additionally carry regulatory sequences that control the expression of the glycoprotein in a host cell, such as promoters, enhancers or other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the nucleic acid(s). Such regulatory sequences are known in the art (see, e.g., Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press (1990)). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Exemplary regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (e.g., the adenovirus major late promoter (AdMLP)) and polyoma virus.

[00188] In addition to sequences encoding the glycoprotein and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes.

[00189] The expression vector(s) encoding the glycoprotein may be transfected into a host cell by standard techniques, such as electroporation, calcium-phosphate precipitation, or DEAE-dextran transfection. If desired, viral vectors, such as retro-viral vectors, may also be used to generate stable cell lines (as a source of a continuous supply of the glycoprotein).

[00190] The methods described herein can be applied to any size of cell culture flask and/or bioreactor. For example, the methods can be applied in bioreactors or cell cultures of 10 L, 30 L, 50 L, 100 L, 150 L, 200 L, 300 L, 500 L, 1000 L, 2000 L, 3000 L, 4000 L, 5000 L, 10,000 L or larger. In some embodiments, the cell culture size can range from 10 L to 5000 L,

from 10 L to 10,000 L, from 10 L to 20,000 L, from 10 L to 50,000 L, from 40 L to 50,000 L, from 100 L to 50,000 L, from 500 L to 50,000 L, from 1000 L to 50,000 L, from 2000 L to 50,000 L, from 3000 L to 50,000 L, from 4000 L to 50,000 L, from 4500 L to 50,000 L, from 1000 L to 10,000 L, from 1000 L to 20,000 L, from 1000 L to 25,000 L, from 1000 L to 30,000 L, from 15 L to 2000 L, from 40 L to 1000 L, from 100 L to 500 L, from 200 L to 400 L, or any integer there between.

[00191] Media components include, e.g., buffer, amino acid content, vitamin content, salt content, mineral content, serum content, carbon source content, lipid content, nucleic acid content, hormone content, trace element content, ammonia content, co-factor content, indicator content, small molecule content, hydrolysate content and enzyme modulator content. Preferably, the growth medium is a chemically defined media such as Biowhittaker® POWERCHO® (Lonza, Basel, Switzerland), HYCLONE PF CHO™ (Thermo Scientific, Fisher Scientific), GIBCO® CD DG44 (Invitrogen, Carlsbad, CA), Medium M199 (Sigma-Aldrich), OPTIPRO™ SFM (Gibco), etc.).

#### **D. Regulation of Host Cell Gene Expression.**

[00192] One or more RNA effector molecules are added to the cell culture to regulate the expression level(s) of target gene(s). If more than two or more RNA effector molecules are used, they may be provided at the same concentration, or different concentrations. The RNA effectors may be added simultaneously into the cell culture, or added at different times into the cell culture.

[00193] An effective amount of an RNA effector is added to the cell culture to allow sufficient reduction of the expression of a target gene. For example, an effective amount of an RNA effector is added to the cell culture such that the expression level of its target gene is reduced by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%.

[00194] In general, a suitable dose of RNA effector molecule will be in the range of 0.001 to 200.0 milligrams per unit volume per day. For example, the RNA effector molecule may be provided in the range of 0.001 nM to 200 nM per day, generally in the range of 0.1 nM to 500 nM. For example, a dsRNA can be administered at 0.01 nM, 0.05 nM, 0.1 nM, 0.5 nM, 0.75 nM, 1 nM, 1.5 nM, 2 nM, 3 nM, 10 nM, 20 nM, 30 nM, 40 nM, 50 nM, 100 nM, 200 nM, 400 nM, or 500 nM per single dose. In one embodiment, the RNA effector molecule is administered a cell culture at a concentration less than about 50nM.

[00195] The composition can be added to the cell culture once daily, or the RNA effector molecule can be added as two, three, or more sub-doses at appropriate intervals throughout the day or delivery through a controlled release formulation. In that case, the RNA effector molecule contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, e.g., using a conventional sustained release formulation, which provides sustained release of the RNA effector molecule over a several-day-period.

[00196] The effect of a single dose on target gene transcript levels can be long-lasting, such that subsequent doses are administered at not more than 3-, 4-, or 5-day intervals, or at not more than 1-, 2-, 3-, or 4-week intervals.

[00197] The administration of the RNA effector molecule may be ceased at least 6 hr, at least 12 hr, at least 18 hr, at least 36 hr, at least 48 hr, at least 60 hr, at least 72 hr, at least 96 hr, or at least 120 hr, or at least 1 week, before isolation of the biological product. Thus in one embodiment, contacting a host cell (e.g., in a large scale host cell culture) with a RNA effector molecule is complete at least 6 hr, at least 12 hr, at least 18 hr, at least 36 hr, at least 48 hr, at least 60 hr, at least 72 hr, at least 96 hr, or at least 120 hr, or at least 1 week, before isolation of the biological product.

[00198] Sometimes, it may be beneficial to provide a RNA effector molecule to the host cell cultures in a way that a constant number (or at least a minimum number) of RNA effector molecules per each cell is maintained. Maintaining the levels of the RNA effector molecule as such can ensure that modulation of target gene expression is maintained even at high cell densities.

**[00199]** The amount of a RNA effector molecule can also be administered according to the cell density. In such embodiments, the RNA effector molecule(s) is added at a concentration of at least 0.01 fmol/ $10^6$  cells, at least 0.1 fmol/ $10^6$  cells, at least 0.5 fmol/ $10^6$  cells, at least 0.75 fmol/ $10^6$  cells, at least 1 fmol/ $10^6$  cells, at least 2 fmol/ $10^6$  cells, at least 5 fmol/ $10^6$  cells, at least 10 fmol/ $10^6$  cells, at least 20 fmol/ $10^6$  cells, at least 30 fmol/ $10^6$  cells, at least 40 fmol/ $10^6$  cells, at least 50 fmol/ $10^6$  cells, at least 60 fmol/ $10^6$  cells, at least 100 fmol/ $10^6$  cells, at least 200 fmol/ $10^6$  cells, at least 300 fmol/ $10^6$  cells, at least 400 fmol/ $10^6$  cells, at least 500 fmol/ $10^6$  cells, at least 700 fmol/ $10^6$  cells, at least 800 fmol/ $10^6$  cells, at least 900 fmol/ $10^6$  cells, or at least 1 pmol/ $10^6$  cells, or more.

**[00200]** For example, the RNA effector molecule may be administered at a dose of at least 10 molecules per cell, at least 20 molecules per cell (molecules/cell), at least 30 molecules/cell, at least 40 molecules/cell, at least 50 molecules/cell, at least 60 molecules/cell, at least 70 molecules/cell, at least 80 molecules/cell, at least 90 molecules/cell at least 100 molecules/cell, at least 200 molecules/cell, at least 300 molecules/cell, at least 400 molecules/cell, at least 500 molecules/cell, at least 600 molecules/cell, at least 700 molecules/cell, at least 800 molecules/cell, at least 900 molecules/cell, at least 1000 molecules/cell, at least 2000 molecules/cell, at least 5000 molecules/cell or more, inclusive.

**[00201]** In some embodiments, the RNA effector molecule is administered at a dose within the range of 10-100 molecules/cell, 10-90 molecules/cell, 10-80 molecules/cell, 10-70 molecules/cell, 10-60 molecules/cell, 10-50 molecules/cell, 10-40 molecules/cell, 10-30 molecules/cell, 10-20 molecules/cell, 90-100 molecules/cell, 80-100 molecules/cell, 70-100 molecules/cell, 60-100 molecules/cell, 50-100 molecules/cell, 40-100 molecules/cell, 30-100 molecules/cell, 20-100 molecules/cell, 30-60 molecules/cell, 30-50 molecules/cell, 40-50 molecules/cell, 40-60 molecules/cell, or any range there between.

**[00202]** In one embodiment, the RNA effector molecule is administered as a sterile aqueous solution. In one embodiment, the RNA effector molecule is formulated in a non-lipid formulation. In another embodiment, the RNA effector molecule is formulated in a cationic or non-cationic lipid formulation. In still another embodiment, the RNA effector molecule is formulated in a cell medium suitable for culturing a host cell (e.g., a serum-free medium).

## **E. Purification of Lysosomal Glycoproteins**

**[00203]** The lysosomal glycoproteins produced in accordance with the methods described herein can be harvested from host cells, and purified using any suitable methods. For example, methods for purifying polypeptides by immune-affinity chromatography are known in the art. *Ruiz-Arguello et al., J. Gen. Virol., 85:3677-3687 (2004)*. Suitable methods for purifying desired lysosomal glycoprotein including precipitation and various types of chromatography, such as hydrophobic interaction, ion exchange, affinity, chelating and size exclusion are well-known in the art. Suitable purification schemes can be created using two or more of these or other suitable methods. If desired, the glycoprotein can include a “tag” that facilitates purification, such as an epitope tag or a HIS tag. Such tagged polypeptides can conveniently be purified, for example from conditioned media, by chelating chromatography or affinity chromatography. Optionally, the tag sequence may be cleaved post-purification.

**[00204]** For example, normal phase liquid chromatography can be used to separate glycans and/or glycoproteins based on polarity. Reverse-phase chromatography can be used, e.g., with derivatized sugars. Anion-exchange columns can be used to purify sialylated, phosphorylated, and sulfated sugars. Other methods include high pH anion exchange chromatography and size exclusion chromatography can be used and is based on size separation.

**[00205]** Affinity based methods can be selected that preferentially bind certain chemical units and glycan structures. Matrices such as m-aminophenylboronic acid, immobilized lectins and antibodies can bind particular glycan structures. M-aminophenylboronic acid matrices can form a temporary covalent bond with any molecule (such as a carbohydrate) that contains a 1,2-cis-diol group. The covalent bond can be subsequently disrupted to elute the protein of interest. Lectins are a family of carbohydrate-recognizing proteins that exhibit affinities for various monosaccharides. Lectins bind carbohydrates specifically and reversibly. Primary monosaccharides recognized by lectins include mannose/glucose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and sialic acid (QProteome Glycoarray Handbook, Qiagen, September 2005, available at: [http://wolfson.huji.ac.il/urification/PDF/Lectins/QIAGEN\\_GlycoArrayHa-ndbook.pdf](http://wolfson.huji.ac.il/urification/PDF/Lectins/QIAGEN_GlycoArrayHa-ndbook.pdf)) or similar references. Lectin matrices (e.g., columns or arrays) can consist of a number of lectins with varying and/or overlapping specificities to bind glycoproteins with specific glycan

compositions. Some lectins commonly used to purify glycoproteins include concavalin A (often coupled to Sepharose or agarose) and Wheat Germ. Anti-glycan antibodies can also be generated by methods known in the art and used in affinity columns to bind and purify glycoproteins.

**[00206]** The interaction of a lectin or antibody with a ligand, such as a glycoprotein, allows for the formation of cross-linked complexes, which are often insoluble and can be identified as precipitates (Varki et al., ed., "Protein-Glycan Interactions" in *Essentials of Glycobiology* available at world wide web at <http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=glyco.section.269>) or similar references. In this technique, a fixed amount of lectin or antibody (receptor) is titrated with a glycoprotein or a glycan, and at a precise ratio of ligand to receptor, a precipitate is formed (Varki et al.). Such precipitation is highly specific to the affinity constant of the ligand to the receptor (Varki et al.). Another precipitation approach takes advantage of the fact that a complex between a lectin and a glycan can be "salted" out or precipitated by ammonium sulfate (Varki et al.).

## **F. Analysis of Lysosomal glycoproteins**

### **1. Analysis of the structure and composition of glycans**

**[00207]** The glycan structure of the lysosomal glycoproteins described herein can be determined using art-known methods for analyzing glycan structures of glycoproteins, such as chromatography, mass spectrometry (MS), chromatography followed by MS, electrophoresis, electrophoresis followed by MS, nuclear magnetic resonance (NMR), and any combinations thereof. A preferred technique is Liquid chromatography-mass spectrometry (LC-MS, or alternatively HPLC-MS). See, e.g., Mulloy et al., *Structural Analysis of Glycans, ESSENTIALS OF GLYCOBIOLOGY*, 2nd edition (Varki et al., eds.), Chapter 47 (2009); Cold Spring Harbor Laboratory Press

**[00208]** For example, an enzyme, such as an N-glycanase (e.g., N-glycanase F, N-glycanase-A), can be used to cleave the N-glycan moiety from a glycoprotein. Further, exoglycosidases (e.g., sialidase, galactosidase, hexosaminidase, fucosidase, mannosidase etc.) can be used to cleave terminal glycosidic bonds from the non-reducing end of glycans. Alternatively, acid hydrolysis (e.g., trifluoroacetic acid) can be used to release neutral saccharides (e.g., galactose, mannose, fucose) or amino saccharides (e.g., N-acetylglucosamine)

from a glycan. The cleaved or hydrolyzed saccharides can be analyzed using chromatography spectrometry, or electrophoresis methods described above.

**[00209]** For example, glycan structure and composition can be analyzed by chromatography, including, e.g., liquid chromatography (LC), high performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC), thin layer chromatography (TLC), amide column chromatography, or combinations thereof.

**[00210]** Another method to analyze glycan structure and composition is mass spectrometry (MS), including, e.g., tandem MS, LC-MS, LC-MS/MS, matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS), Fourier transform mass spectrometry (FTMS), ion mobility separation with mass spectrometry (IMS-MS), electron transfer dissociation (ETD-MS), or combinations thereof.

**[00211]** Another method to analyze glycan structure and composition is electrophoresis, including, e.g., capillary electrophoresis (CE), CE-MS, gel electrophoresis, agarose gel electrophoresis, acrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting using antibodies that recognize specific glycan structures, or combinations thereof. For example, the structure of an N-glycan can be determined by two dimensional sugar chain mapping (see, e.g., Anal. Biochem., 171, 73 (1988); Biochemical Experimentation Methods 23-Methods for Studying Glycoprotein Sugar Chains (Japan Scientific Societies Press) edited by Reiko Takahashi (1989)). Two dimensional sugar chain mapping is a method for deducing the structure of a saccharide chain by plotting the retention time or elution position of the saccharide chain by reverse phase chromatography as the X axis, and the retention time or elution position of the saccharide chain by normal phase chromatography as the Y axis, respectively, and comparing them with such results of known sugar chains. The structure deduced by two dimensional sugar chain mapping can be confirmed by mass spectrometry.

**[00212]** Another method to analyze glycan structure and composition is nuclear magnetic resonance (NMR), including, e.g., one-dimensional NMR (1D-NMR), two-dimensional NMR (2D-NMR), correlation spectroscopy magnetic-angle spinning NMR (COSY-NMR), total correlated spectroscopy NMR (TOCSY-NMR), heteronuclear single-quantum

coherence NMR (HSQC-NMR), heteronuclear multiple quantum coherence (HMQC-NMR), rotational nuclear overhauser effect spectroscopy NMR (ROESY-NMR), nuclear overhauser effect spectroscopy (NOESY-NMR), or combinations thereof.

**[00213]** Saccharide composition of a glycan can also be analyzed by fluorescence labeling. For example, acid-hydrolyzed glycans can be labeled with 2-aminopyridine and then analyzed by HPLC.

**[00214]** Immunological methods (e.g., antibody staining, lectin staining) may also be used to determine the structures of N-glycan. For example, lectin molecules can bind to the carbohydrate moieties of glycoproteins. Therefore, a lectin that binds to a specific N-glycan can be used to identify the presence and quantity of such glycoforms in a composition (e.g., by determining the amount of glycan-bound lectin using a secondary antibody). Examples of lectins that can be used for identifying the glycan structure of an antibody, or a Fc-fusion protein, include, e.g., WGA (wheat-germ agglutinin derived from *T. vulgaris*), ConA (concanavalin A derived from *C. ensiformis*), RIC (a toxin derived from *R. communis*), L-PHA (leucoagglutinin derived from *P. vulgaris*), LCA (lentil agglutinin derived from *L. culinaris*), PSA (pea lectin derived from *P. sativum*), AAL (Aleuria aurantia lectin), ACL (Amaranthus caudatus lectin), BPL (Bauhinia purpurea lectin), DSL (Datura stramonium lectin), DBA (Dolichos biflorus agglutinin), EBL (elderberry bark lectin), ECL (Erythrina cristagalli lectin), EEL (Euonymus europaeus lectin), GNL (Galanthus nivalis lectin), GSL (Griffonia simplicifolia lectin), HPA (Helix pomatia agglutinin), HHL (Hippeastrum hybrid lectin), Jacalin, LTL (Lotus tetragonolobus lectin), LEL (Lycopersicon esculentum lectin), MAL (Maackia amurensis lectin), MPL (Maclura pomifera lectin), NPL (Narcissus pseudonarcissus lectin), PNA (peanut agglutinin), E-PHA (Phaseolus vulgaris erythroagglutinin), PTL (Psophocarpus tetragonolobus lectin), RCA (Ricinus communis agglutinin), STL (Solanum tuberosum lectin), SJA (Sophora japonica agglutinin), SBA (soybean agglutinin), UEA (Ulex europaeus agglutinin), VVL (Vicia villosa lectin) and WFA (Wisteria floribunda agglutinin).

**[00215]** For example, a lectin that specifically recognizes a complex N-glycan in which a fucose residue is linked to the N-acetylglucosamine in the reducing end of the N-glycan may be used. Exemplary lectins include, e.g., Lens culinaris lectin LCA (lentil agglutinin derived from *Lens culinaris*), pea lectin PSA (pea lectin derived from *Pisum sativum*), broad

bean lectin VFA (agglutinin derived from *Vicia faba*) and Aleuria aurantia lectin AAL (lectin derived from *Aleuria aurantia*).

**[00216]** Another method to analyze glycan structure and composition is by capillary electrophoresis (CE), which is described e.g., in Szabo et al., *Electrophoresis*, 2010 April; 31(8): 1389–1395. doi:10.1002/elps.201000037.

**[00217]** Techniques described herein may be combined with one or more other technologies for the detection, analysis, and or isolation of glycans or glycoproteins. For example, any combination of NMR, mass spectrometry, liquid chromatography, 2-dimensional chromatography, SDS-PAGE, antibody staining, lectin staining, monosaccharide quantitation, capillary electrophoresis, fluorophore-assisted carbohydrate electrophoresis (FACE), micellar electrokinetic chromatography (MEKC), exoglycosidase or endoglycosidase treatments may be used. See, e.g., Anumula, *Anal. Biochem.* 350(1): 1, 2006; Klein et al., *Anal. Biochem.*, 179:162, 1989; Townsend, R.R. *Carbohydrate Analysis, High Performance Liquid Chromatography and Capillary Electrophoresis*, Ed. Z. El Rassi, pp 181-209, 1995. For example, Qian et al. (*Analytical Biochemistry* 364 (2007) 8-18) discloses a method for determining the structures of glycans using orthogonal matrix-assisted laser desorption/ionization hybrid quadrupole-quadrupole time-of-flight mass spectrometry (oMALDI Qq-TOF MS) and tandem mass spectrometry (MS/MS) in combination with exoglycosidase digestion. The N-linked glycans are released by treatment with N-glycanase F, reductively aminated with anthranilic acid, and fractionated by normal phase high-performance liquid chromatography (NP-HPLC). The fluorescent-labeled oligosaccharide pool and fractions are then analyzed by oMALDI Qq-TOF MS and MS/MS in negative ion mode. Each fraction is further digested with an array of exoglycosidase mixtures, and subsequent MALDI TOF MS analysis of the resulting products yields information about structural features of the glycan.

**[00218]** One exemplary saccharide composition analyzer is BioLC, manufactured by Dionex, which analyzes saccharide composition by HPAEC-PAD (high performance anion-exchange chromatography-pulsed amperometric detection).

**[00219]** Phosphorylated glycans (e.g., glycans that comprise a mannose-6-phosphate) can be detected, e.g., by fluorophore-assisted carbohydrate electrophoresis (FACE). See, e.g.,

Gao et al., Methods in Enzymology, vol. 415, Chapter 1, pp 3-19 (2006). Alternatively, Immobilized Metal Affinity Chromatography (IMAC) may be used. IMAC is a technique for separating proteins based on affinity differences for immobilized metal ions, and can be used to separate glycans based on differences in phosphorylation. *See, e.g.*, U.S. Patent Application Publication No. 2010/0279306.

## **2. Analysis of the activity of lysosomal glycoproteins**

**[00220]** The biological activity of the glycoprotein compositions described herein may be assessed using any art known method. Such biological activities include, e.g., bioavailability, pharmacokinetics, pharmacodynamics, enzymatic activity etc. Additionally, therapeutic activity of a glycoprotein may be assessed (e.g., efficacy of a lysosomal protein in decreasing severity or symptom of a disease or condition, or in delaying appearance of a symptom of a disease or condition).

**[00221]** Methods of analyzing bioavailability, pharmacokinetics, and pharmacodynamics of glycoprotein therapeutics have been described. *See, e.g.*, Weiner et al., /. Pharm. Biomed. Anal. 15(5):571-9, 1997; Srinivas et al., /. Pharm. Sci. 85(1):1-4, 1996; and Srinivas et al., Pharm. Res. 14(7):911-6, 1997. Assays for measuring ADCC activity mediated by therapeutic antibodies are also known. *See, e.g.*, Schnueriger A. et al., Mol. Immunol. (2011) 48(12-13):1512-7; Cancer Immunology Immunotherapy, 36, 373 (1993); Cancer Research, 5A, 1511 (1994). As would be understood to one of skill in the art, the particular biological activity or therapeutic activity that can be tested will vary depending on the particular glycoprotein or glycan structure.

**[00222]** The potential adverse activity or toxicity (e.g., propensity to cause hypertension, immunogenicity/allergic reactions, thrombotic events, seizures, or other adverse events) of glycoprotein preparations can be analyzed by any available method. For example, immunogenicity of a glycoprotein composition can be assessed, e.g., by determining in vitro by immunoassay (e.g., using an antibody that binds to a recognized immunogenic epitope), or by in vivo administration to determine whether the composition elicits an antibody response in a subject.

## **5. PHARMACEUTICAL COMPOSITIONS, METHODS OF ADMINISTRATION, AND KITS**

## A. Pharmaceutical Compositions

[00223] In one aspect, the invention relates to pharmaceutical compositions comprising the lysosomal glycoproteins described herein.

[00224] The pharmaceutical compositions usually one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in Gennaro (2000) Remington: The Science and Practice of Pharmacy (20th edition). Examples of such carriers or additives include water, a pharmaceutical acceptable organic solvent, collagen, polyvinyl alcohol, polyvinylpyrrolidone, a carboxyvinyl polymer, carboxymethylcellulose sodium, polyacrylic sodium, sodium alginate, water-soluble dextran, carboxymethyl starch sodium, pectin, methyl cellulose, ethyl cellulose, xanthan gum, gum Arabic, casein, gelatin, agar, diglycerin, glycerin, propylene glycol, polyethylene glycol, Vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, a pharmaceutically acceptable surfactant and the like. Formulation of the pharmaceutical composition will vary according to the route of administration selected.

[00225] Optionally, the glycoprotein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. Any suitable lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of activity loss and that use levels may have to be adjusted to compensate.

[00226] A variety of aqueous carriers can be used to formulate suitable pharmaceutical compositions for administration, such as plain water (*e.g.* w.f.i.) or a buffer *e.g.* a phosphate buffer, a Tris buffer, a borate buffer, a succinate buffer, a histidine buffer, or a citrate buffer. Buffer salts will typically be included in the 5-20mM range.

[00227] The pharmaceutical compositions are preferably sterile, and may be sterilized by conventional sterilization techniques.

[00228] The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, and tonicity adjusting agents and the like, for example, sodium acetate, sodium

chloride, potassium chloride, calcium chloride, sodium lactate and the like. Preferably, the pharmaceutical compositions of the invention may have a pH between 5.0 and 9.5, *e.g.* between 6.0 and 8.0.

[00229] Pharmaceutical compositions of the invention may include sodium salts (*e.g.* sodium chloride) to give tonicity. A concentration of  $10 \pm 2$  mg/ml NaCl is typical *e.g.* about 9 mg/ml.

[00230] Pharmaceutical compositions of the invention may have an osmolarity of between 200 mOsm/kg and 400 mOsm/kg, *e.g.* between 240-360 mOsm/kg, or between 290-310 mOsm/kg.

## **B. Methods of Administration**

[00231] In another aspect, the invention provide a method for treating Pompe disease (also known as acid  $\alpha$ -glucosidase deficiency, acid maltase deficiency, glycogen storage disease type II, glycogenosis II, and lysosomal  $\alpha$ -glucosidase deficiency), comprising administering to a subject in need thereof a therapeutically effectively amount of an acid  $\alpha$ -glucosidase as described herein. The subject is preferably human.

[00232] A therapeutically effectively amount of an acid  $\alpha$ -glucosidase is administered that such that symptoms of Pompe disease are ameliorated, or the onset of symptoms is delayed. A therapeutically effective amount will, for example, be sufficient to treat, prevent, reduce the severity, delay the onset, and/or reduce the risk of occurrence of one or more symptoms associated with acid  $\alpha$ -glucosidase deficiency.

[00233] The therapeutic efficacy of the administered acid  $\alpha$ -glucosidase may be determined by biochemical (*see, e.g.*, Zhu et al., J. Biol. Chem. 279: 50336-50341 (2004)) or histological observation of reduced lysosomal glycogen accumulation in, *e.g.*, cardiac myocytes, skeletal myocytes, or skin fibroblasts. Acid  $\alpha$ -glucosidase activity may also be assayed in, *e.g.*, a muscle biopsy sample, in cultured skin fibroblasts, in lymphocytes, and in dried blood spots. Dried blood spot assays are described in *e.g.*, Umpathysivam et al., Clin. Chem. 47:1378-1383 (2001) and Li et al., Clin. Chem. 50:1785-1796 (2004). The therapeutic efficacy of the administered acid  $\alpha$ -glucosidase may also be assessed by, *e.g.*, serum levels of creatinine kinase,

gains in motor function (*e.g.*, as assessed by the Alberta Infant Motor Scale), changes in left ventricular mass index as measured by echocardiogram, and cardiac electrical activity, as measured by electrocardiogram. Administration of acid  $\alpha$ -glucosidase as described herein may result in a reduction in one or more symptoms of Pompe disease such as cardiomegaly, cardiomyopathy, daytime somnolence, exertional dyspnea, failure to thrive, feeding difficulties, floppiness, gait abnormalities, headaches, hypotonia, organomegaly (*e.g.*, enlargement of heart, tongue, liver), lordosis, loss of balance, lower back pain, morning headaches, muscle weakness, respiratory insufficiency, scapular winging, scoliosis, reduced deep tendon reflexes, sleep apnea, susceptibility to respiratory infections, and vomiting.

[00234] In another aspect, the invention provide a method for treating Fabry disease comprising administering to a subject in need thereof a therapeutically effectively amount of an  $\alpha$ -galactosidase A as described herein. The subject is preferably human.

[00235] A therapeutically effectively amount of an  $\alpha$ -galactosidase A is administered that such that symptoms of Fabry disease are ameliorated, or the onset of symptoms is delayed. A therapeutically effective amount will, for example, be sufficient to treat, prevent, reduce the severity, delay the onset, and/or reduce the risk of occurrence of one or more symptoms associated with  $\alpha$ -galactosidase A deficiency.

[00236] Fabry disease is also known as Fabry's disease, Anderson-Fabry disease, angiokeratoma corporis diffusum and alpha-galactosidase A deficiency. Symptoms for Fabry disease usually begin during childhood or adolescence and include burning sensations in the hands that gets worse with exercise and hot weather and small, non-cancerous, raised reddish-purple blemishes on the skin. Some boys will also have eye manifestations, especially cloudiness of the cornea. Lipid storage may lead to impaired arterial circulation and increased risk of heart attack or stroke. The heart may also become enlarged and the kidneys may become progressively involved. Other signs include decreased sweating, fever, and gastrointestinal difficulties.

[00237] Fabry disease is indicated when associated symptoms are present, and can be diagnosed by a blood test to measure the level of alpha-galactosidase activity. Chromosomal analysis of the GLA gene may also be used, as many mutations which cause the disease have

been noted. Kidney biopsy may also be suggestive of Fabry Disease if excessive lipid buildup is noted.

[00238] In another aspect, the invention provide a method for treating alpha 1-antitrypsin deficiency, comprising administering to a subject in need thereof a therapeutically effectively amount of an alpha 1-antitrypsin as described herein. The subject is preferably human.

[00239] A therapeutically effectively amount of an alpha 1-antitrypsin is administered that such that symptoms of alpha 1-antitrypsin deficiency are ameliorated, or the onset of symptoms is delayed. A therapeutically effective amount will, for example, be sufficient to treat, prevent, reduce the severity, delay the onset, and/or reduce the risk of occurrence of one or more symptoms associated with alpha 1-antitrypsin deficiency.

[00240] In another aspect, the invention provide a method for treating C1-inhibitor deficiency (Types I, II, or III), comprising administering to a subject in need thereof a therapeutically effectively amount of a C1-inhibitor as described herein. The subject is preferably human.

[00241] A therapeutically effectively amount of a C1-inhibitor is administered that such that symptoms of C1-inhibitor deficiency are ameliorated, or the onset of symptoms is delayed. A therapeutically effective amount will, for example, be sufficient to treat, prevent, reduce the severity, delay the onset, and/or reduce the risk of occurrence of one or more symptoms associated with C1-inhibitor deficiency.

[00242] In another aspect, the invention provide a method for treating sepsis, vascular leak syndrome, acute myocardial infarction, pancreatitis, or thermal injury, comprising administering to a subject in need thereof a therapeutically effectively amount of a C1-inhibitor as described herein. The subject is preferably human.

[00243] In another aspect, the invention provide a method for reducing or preventing the adverse effect of xenotransplantation, comprising administering to a subject in need thereof a therapeutically effectively amount of a C1-inhibitor as described herein. The subject is preferably human.

[00244] The C1-inhibitors described herein can also be used to treat other diseases in which classical pathway complement activity (activated C1 component) and/or contact system (factor XIIa, kallikrein, factor XIa) activity contributes to undesired immune or inflammatory responses. Such diseases include myocardial infarction (WO 95/06479); acquired systemic inflammatory responses among which severe sepsis, septic shock, ARDS (Adult Respiratory Distress Syndrome), multiple organ failure and preeclampsia (WO 92/22320); capillary leakage syndrome and circulatory failure in cases of severe burns, polytraumata, operations with extracorporeal circulation (EP 0586909), therapeutic cytokine (e.g. IL2) infusion, acute graft versus host disease after allogeneic (or autologic) bone marrow transplantation. Other indications may be disorders in which excess classical route complement and/or contact activation, and/or C1 inhibitor consumption or (relative) functional C1-inhibitor deficiency has been implicated in the pathophysiology, such as meningitis, rheumatoid arthritis, hyper acute graft rejection after allo- and xeno-transplantation and pancreatitis.

[00245] In another aspect, the invention provide a method for treating Gaucher's disease (types 1, 2, or 3), comprising administering to a subject in need thereof a therapeutically effectively amount of a glucocerebrosidase as described herein. The subject is preferably human.

[00246] Subjects in need of treatment of Gaucher's disease include those that demonstrate pre-symptomatic phases of the disease, as well as those that demonstrate various symptoms of Gaucher's disease. Typically, the pre-symptomatic patient can be diagnosed with Gaucher's disease by genetic analysis known to the skilled artisan.

[00247] Gaucher's disease is a heterogeneous disease, and has been subdivided into three different types on the basis of age of onset, clinical signs and involvement of neurological symptoms. Type 1 (adult type, chronic, non-neuronopathic; MIM# 230800) is the most common form and is characterized by hematological abnormalities with hypersplenism, bone lesions, skin pigmentation, pingueculae (brown spots of Gaucher cells at corneoscleral limbus) and the lack of central nervous system involvement. It is heterogeneous in its clinical features (Beutler and Grabowski, 1995, Gaucher Disease, in Scriver et al., (eds.), The Metabolic and Molecular Bases of Inherited Diseases. Seven ed. McGraw Hill, Vol. II, pp. 2641-2663). Type 2 (infantile, acute neuronopathic; MIM# 230900) is a rare and lethal form of the disease. It is

characterized by early appearance of visceral signs, enlargement of the abdomen from hepatosplenomegaly and central nervous system involvement such as retroflexion of the head, strabismus, dysphagia, choking spells, and hypertonicity. Type 3 juvenile, subacute neuronopathic; MIM #321000) is characterized by early onset of visceral impairment (e.g., hepatosplenomegaly) and a later appearance of central nervous system symptoms.

**[00248]** A therapeutically effectively amount of a glucocerebrosidase is administered that such that symptoms of Gaucher's disease are ameliorated, or the onset of symptoms is delayed. A therapeutically effective amount will, for example, be sufficient to treat, prevent, reduce the severity, delay the onset, and/or reduce the risk of occurrence of one or more symptoms associated with glucocerebrosidase deficiency.

**[00249]** Subjects in need of treatment of various lysosomal storage diseases described herein include those that demonstrate pre-symptomatic phases of the disease, as well as those that demonstrate various symptoms of LSD (which can be diagnosed e.g., by genetic analysis known to the skilled artisan).

**[00250]** The compositions described herein may be administered to a subject orally, topically, transdermally, parenterally, by inhalation spray, vaginally, rectally, or by intracranial injection. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. Administration by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary injection and or surgical implantation at a particular site is contemplated as well. In certain embodiments, injection, especially intravenous, is preferred.

**[00251]** The amounts of a glycoprotein in a given dosage will vary according to the size of the individual to whom the therapy is being administered as well as the characteristics of the disorder being treated. In exemplary treatments, it may be necessary to administer about 1 mg/day, about 5 mg/day, about 10 mg/day, about 20 mg/day, about 50 mg/day, about 75 mg/day, about 100 mg/day, about 150 mg/day, about 200 mg/day, about 250 mg/day, about 400 mg/day, about 500 mg/day, about 800 mg/day, about 1000 mg/day, about 1600 mg/day or about 2000 mg/day. The doses may also be administered based on weight of the patient, at a dose of 0.01 to 50 mg/kg. The glycoprotein may be administered in a dose range of 0.015 to 30 mg/kg,

such as in a dose of about 0.015, about 0.05, about 0.15, about 0.5, about 1.5, about 5, about 15 or about 30 mg/kg.

[00252] Dosage can be by a single dose schedule or a multiple dose schedule. Multiple doses will typically be administered at least 1 week apart (*e.g.*, about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, etc.).

[00253] Standard dose-response studies, first in animal models and then in clinical testing, can reveal optimal dosages for particular diseases and patient populations.

[00254] The glycoprotein compositions described herein may be administered in combination with a second therapeutic agent. For example, for cancer treatment, a chemotherapeutic agent may be used as the second agent. For treatment of autoimmune diseases, non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, glucocorticoids, disease-modifying antirheumatic drugs (DMARDs), may be used as the second agent. Examples of such therapeutic agents can be found, *e.g.*, in WO 2008/156713.

### 3. Kits

[00255] In another aspect, the invention provides kits that comprise the lysosomal glycoprotein compositions described herein packaged in a manner that facilitates their use for therapy. For example, such a kit includes a lysosomal glycoprotein as described herein, packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the composition in practicing the method. The kit can further comprise another container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. Preferably, the composition is packaged in a unit dosage form. The kit may further include a device suitable for administering the composition according to a specific route of administration or for practicing a screening assay. Preferably, the kit contains a label that describes use of the composition.

[00256] In another aspect, the invention provides kits for testing the effect of a RNA effector molecule or a series of RNA effector molecules on the production of a lysosomal glycoprotein by the host cell, where the kits comprise a substrate having one or more assay

surfaces suitable for culturing cells under conditions that allow production of the glycoprotein. In some embodiments, the exterior of the substrate comprises wells, indentations, demarcations, or the like at positions corresponding to the assay surfaces. In some embodiments, the wells, indentations, demarcations, or the like retain fluid, such as cell culture media, over the assay surfaces.

**[00257]** In some embodiments, the assay surfaces on the substrate are sterile and are suitable for culturing host cells under conditions representative of the culture conditions during large-scale (e.g., industrial scale) production of the glycoprotein. Advantageously, kits provided herein offer a rapid, cost-effective means for testing a wide-range of agents and/or conditions on the production of the glycoprotein, allowing the cell culture conditions to be established prior to full-scale production of the glycoprotein.

**[00258]** In some embodiments, one or more assay surfaces of the substrate comprise a concentrated test agent, such as a RNA effector molecule, such that the addition of suitable media to the assay surfaces results in a desired concentration of the RNA effector molecule surrounding the assay surface. In some embodiments, the RNA effector molecules may be printed or ingrained onto the assay surface, or provided in a lyophilized form, e.g., within wells, such that the effector molecules can be reconstituted upon addition of an appropriate amount of media. In some embodiments, the RNA effector molecules are reconstituted by plating cells onto assay surfaces of the substrate.

**[00259]** In some embodiments, kits provided herein further comprise cell culture media suitable for culturing a cell under conditions allowing for the production of the glycoprotein of interest. The media can be in a ready to use form or can be concentrated (e.g., as a stock solution), lyophilized, or provided in another reconstitutable form.

**[00260]** In further embodiments, kits provided herein further comprise one or more reagents suitable for detecting production of the lysosomal glycoprotein by the cell, cell culture, or tissue culture. In further embodiments, the reagent(s) are suitable for detecting a property of the cell, such as maximum cell density, cell viability, or the like, which is indicative of production of the desired glycoprotein. In some embodiments, the reagent(s) are suitable for

detecting the glycoprotein or a property thereof, such as the *in vitro* or *in vivo* biological activity, homogeneity, or structure of the glycoprotein.

**[00261]** In some embodiments, one or more assay surfaces of the substrate further comprise a carrier for which facilitates uptake of RNA effector molecules by cells. Carriers for RNA effector molecules are known in the art and are described herein. For example, in some embodiments, the carrier is a lipid formulation such as Lipofectamine™ transfection reagent (Invitrogen; Carlsbad, CA) or a related formulation. Examples of such carrier formulations are described herein. In some embodiments, the reagent that facilitates RNA effector molecule uptake comprises a charged lipid, an emulsion, a liposome, a cationic or non-cationic lipid, an anionic lipid, a transfection reagent or a penetration enhancer as described throughout the application herein. In particular embodiments, the reagent that facilitates RNA effector molecule uptake comprises a charged lipid as described in U.S. Application Ser. No. 61/267,419, filed on December 7, 2009.

**[00262]** In some embodiments, one or more assay surfaces of the substrate comprise a RNA effector molecule or series of RNA effector molecules and a carrier, each in concentrated form, such that plating test cells onto the assay surface(s) results in a concentration the RNA effector molecule(s) and the carrier effective for facilitating uptake of the RNA effector molecule(s) by the cells and modulation of the expression of one or more genes targeted by the RNA effector molecules.

**[00263]** In some embodiments, the substrate further comprises a matrix which facilitates 3-dimensional cell growth and/or production of the glycoprotein by the cells. In further embodiments, the matrix facilitates anchorage-dependent growth of cells. Non-limiting examples of matrix materials suitable for use with various kits described herein include agar, agarose, methylcellulose, alginate hydrogel (e.g., 5% alginate + 5% collagen type I), chitosan, hydroactive hydrocolloid polymer gels, polyvinyl alcohol-hydrogel (PVA-H), polylactide-co-glycolide (PLGA), collagen vitrigel, PHEMA (poly(2-hydroxylmethacrylate)) hydrogels, PVP/PEO hydrogels, BD PuraMatrix™ hydrogels, and copolymers of 2-methacryloyloxyethyl phosphorylcholine (MPC).

[00264] In some embodiments, the substrate comprises a microarray plate, a biochip, or the like which allows for the high-throughput, automated testing of a range of test agents, conditions, and/or combinations thereof on the production of a glycoprotein by cultured cells. For example, the substrate may comprise a 2-dimensional microarray plate or biochip having m columns and n rows of assay surfaces (e.g., residing within wells) which allow for the testing of m x n combinations of test agents and/or conditions (e.g., on a 24-, 96- or 384-well microarray plate). The microarray substrates are preferably designed such that all necessary positive and negative controls can be carried out in parallel with testing of the agents and/or conditions.

[00265] In some embodiments, kits provided herein allow for the selection or optimization of at least one factor for enhancing production of the biological product. For example, the kits may allow for the selection of a RNA effector molecule from among a series of candidate RNA effector molecules, or for the selection of a concentration or concentration range from a wider range of concentrations of a given RNA effector molecule. In some embodiments, the kits allow for selection of one or more RNA effector molecules from a series of candidate RNA effector molecules directed against a common target gene. In further embodiments, the kits allow for selection of one or more RNA effector molecules from a series of candidate RNA effector molecules directed against two or more functionally related target genes or two or more target genes of a common host cell glycosylation pathway.

[00266] In another aspect, the invention provides kits that comprise one or more container that independently contain one or more RNA effector molecules and one or more suitable host cells.

[00267] The specification is most thoroughly understood in light of the teachings of the references cited within the specification. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan readily recognizes that many other embodiments are encompassed by the invention. All publications, patents, and sequences cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. The citation of any references herein is not an admission that such references are prior art to the present invention.

[00268] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following embodiments.

## Appendix I: Sequences of Exemplary Lysosomal Proteins

### 1. Alglucosidase alfa (SEQ ID NO: 1)

```

1 eeevvisins lgqlyfehlq ilhkgraake neeeasvdtS qengedlgw eekfgkfvdI
61 kangpssigl dfslhgfehl ygipqhaesh qlkntgdgda yrlynldvyg yqiydkmgiy
121 gsvpyllahk lgrtigifwl nasetlvein tepaveytlT qmgpvaakqk vrsrthvhwm
181 sesgiidvfl ltgptpsdvf kqyshltgtq ampplfslgy hqcrwnyede qdvkavdagf
241 dehdiipydam wldiehtegk ryftwdknrf pnpkrmgell rskkrklvvi sdphikidpd
301 ysvyvkakdq gffvknqege dfegvcwpgl ssyldftnpk vrewysslfa fpvyqgstDi
361 lflwndmnep svfrgpeqtm qknaihhgnw ehrelhniyg fyhqmataeg likrskgker
421 pfvltrsffa gsqkygavwt gdntaewsnl kisipmltl sitgisfcga diggfignpe
481 tellvrwyga gayqpffrgh atmntkrrep wlfgeehtrl ireaireryg llpywyslfi
541 hahvasqpvm rplwvefpde lktfdmedey mlgsallvhp vtepkattvd vflpgsnevW
601 ydyktfahwe ggctvkipva ldtipvfqrg gsvipikttv gkstgwmtes syglrvalst
661 kgssvgelyl ddghsfqylh qkqflhrkfs fcssvlinsf adqrghypsk cvvekilvlG
721 frkepssvtt hssdgkdqpv aftycaKtsi lsleklslni atdwevrii

```

### 2. Imiglucerase (SEQ ID NO: 2) *Arg495His Glucocerebrosidase*

```

ARPCIPKSEFGYSSVVCVCNATYCDSEDPPTFPALGTFSRYESTRSGRRMELSMGPIQANH
TGTGLLLTLQPEQKFQKVKGFGGAMTDAAALNILALSPPAQNLLLSYFSEEGIGYNIIR
VPMASCDSEFIRTYTYADTPDDFQLHNFSLPEEDTKLKIPLIHRALQLAQRPVSLLASPWT
SPTWLKTNGAVNGKGSILKGQPGDIYHQTWARYFVKFLDAYAEHKLQFWAVTAENEPSAGL
LSGYPFQCLGFTPEHQRFIARDLGPTLANSTHHNVRLMLDDQRLLLPHWAKVVLTDPE
AAKYVHGIHAVHWYLDLFLAPAKATLGETHRLFPNTMLFASEACVGSKEWEQSVRLGSWDRG
MQYSHSIITNLLYHVVGWTDWNLALNPEGGPNNWRNFVDSPIIVDITKDTFYKQPMFYHL
GHFSKFIPEGSQRVGLVASQKNDLDAVALMHPDGSVAVVVVLNRSSKDVPLTIKDPVGFLL
ETISPGYSIHTYLWHRQ

```

### 3. Alglucerase (SEQ ID NO: 3)

```

ARPCIPKSEFGYSSVVCVCNATYCDSEDPPTFPALGTFSRYESTRSGRRMELSMGPIQANH
TGTGLLLTLQPEQKFQKVKGFGGAMTDAAALNILALSPPAQNLLLSYFSEEGIGYNIIR
VPMASCDSEFIRTYTYADTPDDFQLHNFSLPEEDTKLKIPLIHRALQLAQRPVSLLASPWT
SPTWLKTNGAVNGKGSILKGQPGDIYHQTWARYFVKFLDAYAEHKLQFWAVTAENEPSAGL
LSGYPFQCLGFTPEHQRFIARDLGPTLANSTHHNVRLMLDDQRLLLPHWAKVVLTDPE
AAKYVHGIHAVHWYLDLFLAPAKATLGETHRLFPNTMLFASEACVGSKEWEQSVRLGSWDRG
MQYSHSIITNLLYHVVGWTDWNLALNPEGGPNNWRNFVDSPIIVDITKDTFYKQPMFYHL
GHFSKFIPEGSQRVGLVASQKNDLDAVALMHPDGSVAVVVVLNRSSKDVPLTIKDPVGFLL
ETISPGYSIHTYLWRRQ

```

### 4. $\alpha$ 1-antitrypsin (SEQ ID NO: 4)

```

EDPQGDAQAQKTDTSHHQDHPTFNKITPNLAEFAFSLYRQLAHQSNSTNIFFSFVSIATA
FAMLSLGTAKADTHDEILEGLNFNLTETPEAQIHEGFQELLRTLNQPDSQLQLTTGNGFLFL
SEGLKLVDKFLQDVKKLYHSEAFVTNFGDTEEAQQINDYVEKGTQGGKIVDLVKELDRDT
VFALVNYIFFKKGWERPFQVVDTEEDFHVDQVTTVKVPMKRLGMFNIQHCKKLSSWVL
LMKYLGNATAIFFLPDEGKLQHLENELTHDIITKLENEEDRRSASLHLPKLSITGTYDLK

```

SVLGQLGITKVFNSGADLSGVTEEAPLKLSKAVHKAVLTIDEKGTAAAGAMFLEAIPMSI  
PPEVKFNKPEVFLMIEQNTKSPLFMGKVVNPTQK

## 5. Human C1-inhibitor (SEQ ID NO: 5)

1 masrltlltl lllllagdra ssnpnatsss sqdpeslqdr gegkvattvi skmlfvepil  
61 evsslpttns ttnsatkita nttdepttqp ttepttqpti qptqpttqlp tdsptqpttg  
121 sfcpgpvtlc sdleshstea vlgdalvdfs lklyhafsam kkvetnmafs pfsiaslltq  
181 vllgagentk tnlesilsyp kdftcvhqal kgfttkgvts vsqifhspdl airdtfvns  
241 rtlysssprv lsnsdanle lintwvaknt nnkisrlls lpsdtrlvll naiylsakwk  
301 ttfdpkktrm epfhfknsvi kvpmnnskey pvahfidqtl kakvgqlqls hnlsilvilp  
361 qnlkhrledm egalpsvfk aimeklemsk fqptlltlpr ikvttsqdm1 simeklefffd  
421 fsydlncgl tedpdlqvsa mqhgtvlelt etgveaaaaas aisvartllv fevqqpflfv  
481 lwdqqhkfpv fmgrvydpra

## 6. Agalsidase (SEQ ID NO: 408)

LDNGLARTPTMGWLHWERFCNLDQEEPDSCISEKLFMEMAELMVSEGWKDAGYEYLCI  
DDCWMAQRDSEGRQLQADPQRFPHGIRQLANYVHSKGLKLGİYADVGNKTCAGFPGSFGY  
YDIDAQTFADWGVDDLKFDGCYCDSENLDGYKHMSLALNRTGRSIVYSCEWPLYMWPF  
QKPNYTEIRQYCNHWRNFADIDDSWKSISILDWTSFNQERIVDVAGPGGWNDPDMLVIG  
NFGLSWNQVQTQMALWAIMAAPLFMSNDRHISPOAKALLQDKDVIAINQDPLGKQGYQL  
RQGDNFEVWERPLSGLAWAVAMINRQEIGGPRSYTIAVASLGKGVACNPACFITQLLPVK  
RKLGEYEWTSRLRSHINPTGTVLLQLENTMQMSLKDLL

## Appendix II: nucleotide sequences of exemplary target genes from Chinese Hamster

SEQ ID NO: 6: acid phosphatase 5, tartrate resistant (Acp5)

[illegible]

SEQ ID NO: 7: mannose-6-phosphate receptor, cation dependent

[illegible]

AACCCCGCAACGTGCCTGCTGCCTATCGTGGAGTTGGGGATGACCAGCTGGGGGAAGAGT  
 CAGAAGAAAGGGATGACCATTGCTACCAATGTGATTGCACCTTACTGTCCAGCCAGCTC  
 TTTATTCCCCACACCAAAGCATACACAGCCAGACTTCTCAAGCCGCCTCTTCTCATCTCT  
 CACCTTGCCGTTAATACTCTTGCTTCCAGTTGGCTTTTGATTTGACCTAAACTGCCTC  
 CCTTGTCTCATATTGTTTCTCTCTGCACAAGTACAGTGGAAAGTAGACATAGGGTCTTC  
 GGGGCTGACTCTTGTCGCGTCAGGAAGAGCAAGGACCGCTAAATGGGCAGAAAGCCACGA  
 GCCCTGTCTAAATATTTTCAACAAGTTGAGACACTGGATTTCTTTAATTAATAAAAAAAGA  
 TTACTTGTACCTTAATTGCTTCTGTTGTCTCTGCAGTGCTTACCTGGCTGTAACCCAAA  
 TACTCCCATTTGCCTCGAGCCTGGAGTGAGTCACTGAAATGCCCTTGTATGGGATTAGG  
 GCAGGGAAAAAATGTGAAATTTCCAGCTGCAGGATTCTAAGGCACCACCTAAGGTGTT  
 TTCCGATCTTTCTGTGACTTGATAGCTAGAATTGGGCTCCAAGCTGTGGTTTTAAAGCCA  
 TATGTAGCCCAATGTCTAATCAAGCAGTCTACCTAAAGGAAATAGACCTGTGTTCTT  
 GAGGCCCCAGAGCTTGTGTGCAGGAAAAGCTGGTAAACCTGCTCTTGCTCATTTTGCTCT  
 ACTAGTACTTCTCAACTTGTTGCACTTTTCTCACAAGTCTGTAGTTTGGCTTTTATA  
 TTTCTCCCAATCTTATAGAAAGGGAATGGAAGCAGAAGTGGACATGGGTCTGCAGTTCTC  
 TGCTCCGGGTAGGGAAGACTGCTGCTCCTGTTCTCCTGCTGGCTCAGGGAAGTGCTTA  
 CCCACGTACCTATGAGAAAAGGGTTTAGCCTCTCCCACTTCTTCTGTTTAGGCCATGTG  
 CCAGAATCACAGGCTGATCTTCTGTAATAGTGAACCTCCTAGGCCAGTAAGTAACAACAC  
 ATCTGAACACAGGAGATGTGGAAGAGCAGGGCCATTTGTACCTTTAGAGAAGGACATG  
 AAATGATGTCACTTCTGACTTCTCCGTACCCTTGTCAATGGTAGTGGGATTTTTGTTTCT  
 TTCTATACAAGGAAATTAATGTTATTTTTTAAAAAGGCTTCTTTTTTGTGTCATAGTTC  
 CCCGTGTTTTAAATAAAAAAAAAATATACCACTTTTGTACTAAAAACAAAATAATTAATAA  
 CAAAAAACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

SEQ ID NO: 409: mannose-6-phosphate receptor, cation independent (Gen\_Bank ACCESSION  
EGW05739, *Cricetulus griseus*)

1 mplvoslyvh plplligdsl lrsssrslle fnttttdcpps dsqhrqtqtsi tflcgtlgt  
 61 pefvttatdcv hyfewrttaa ckdkdifikadk evpcyafddk lkkhdlnpli klsggylvdd  
 121 sdpetselfin vordidslrd pstqlracpa gtaacllkgd qafdvgrpke glklvskdrl  
 181 vlsyvkeeae kpdfonghsp avtitfvcps erregtipkl tassncryev ewiteyachr  
 241 dylesetcs1 sseqhdiaid lsplaqgggs syvsdgreyt fylnvcgnat vplcnlkesa  
 301 vcqvkkadst qvkiagrhn qtlrysdgdl tliysggdec ssgfqrmsvi nfeckntagn  
 361 dgrgepvftg evdotyfftw dtryacvkek edllcgvsdg kkrydlsvla rhseseknwe  
 421 avdgdgaese kkyffinvch rvlqegkars cpedaavcav dkngsknlkg fvssptsekq  
 481 yiqlsysdgd dcgsdkkitt nitlvckpgd lesapvlrta gpdgcfyefe whtaaacvls  
 541 ktegenctvf daqagfsfdl spltkkngay kvgtekydfy invcgvsvsd lcqpnsgacq  
 601 vakrgeedns tynfrwytsy acpeepolecv vtdpsmmeqy dlsslkseg rsggnwyame  
 661 nsrehvtrrk yyinvcrpln pvpgcdryaa acqmryense gslaetvsls nlgvaktgvp  
 721 veesgslle yvngsactts dgrltystr ihlvogrsl nthpiftyw ecvvsflwnt  
 781 eaacpiqtit dtdqacsird pnsqfvfnls plnrsqgyvv pgigknfvfn icgampecgt  
 841 iagnpafgce agtqtelesl kperpvgmek slqlstegfl tltkygssps dkgtafiiirf  
 901 icnddiypgt akflhqdds trgirntffe fetalacvps rvdccvtdpa gneydlsals  
 961 magkpwtavd tsvdgkkrsf ylsvctplpy ipgchgsavg scmvlpaedr glnlgvvgis  
 1021 pqadangs1s ilyvngdkcg nqrfsstriif ecaqtlgspv fqfvkdceym fvwrtveacp  
 1081 vvreegdnoc vkdprhgnfy dlkplalsdt iitageytyy frvcgklssn vchvrdgskv  
 1141 vsscqqekkg qgfqkvagll tqkltyengl lkmnytggt chqvyrstt iyfycdrstq  
 1201 kpvflrettd csylfewrtq yacppfnvte cstqdgagns idlsslrsys dnweavtrtg  
 1261 atehylinic kslspragg epcppeaavc lldgskplnl gkvrdgppwt ggvtvlkyvd  
 1321 gdlepdqirk rstiirftos esqvssrplf isavqdceyt fswptpaacp vknsthddcq

1381 vtnpstghlf dlsslsgkag vtvseyseql vymciogene ncgagvgacf ggtrisvgka  
 1441 skrlsyvdqv lqlvyeeegsl cpsksglryk svisfvcrpe agptnrpmli sldkqtctlf  
 1501 fswhtplace qvtectvrng ssiidlspli hrtggyeayd esedgtsdtt pdfyinicqp  
 1561 lnpmhgvpcp agaavckvpv ngppidigrv tgppifnpva nevylnfess tpcladkhmn  
 1621 ytsliafhck rgvsmgtpkl irtndcdfvf ewetpivcpd evktqgcsvt deqllysfnl  
 1681 tslststfka rlpsiargsd

Table 8

| Target gene | SenseSeq 5' to 3'    | SEQ ID No. | AntisSeq 5' to 3'    | SEQ ID No. |
|-------------|----------------------|------------|----------------------|------------|
| Acp5        | CCGCCAAGAUGGAUUCAUG  | 8          | CAUGAAUCCAUCUUGGCCGG | 9          |
|             | GCUCCAAAGAAAUGAGUAU  | 10         | AUACUCAUUUCUUUGGAGC  | 11         |
|             | GGCCAAUGCUAAAAGAAAUC | 12         | GAUUUCUUUAGCAUUGGCC  | 13         |
|             | GCAUGAUGCCAAAGACAAG  | 14         | CUUGUCUUUGGCAUCAUGC  | 15         |
|             | GCAAUGUCUCAGCACAAAU  | 16         | AUUUGUGCUGAGACAUUGC  | 17         |
|             | CUGGAAAAUACUCUUCUAA  | 18         | UUGAAGAGUGAUUUUCCAG  | 19         |
|             | CCGCAAUUCCCCUGGUAC   | 20         | GUACCAGGGGAUAUUGCGG  | 21         |
|             | GUGGCCAAUGCUAAAAGAAA | 22         | UUUCUUUAGCAUUGGCCAC  | 23         |
|             | AGAAAUGAGUAUCACCUAU  | 24         | AUAGGUGAUACUCAUUCU   | 25         |
|             | CUUCAAAAUCCACGGACA   | 26         | UGUCCGUGGAAUUUUGAAG  | 27         |
|             | GCACUUACAGCCACUGUUG  | 28         | CAACAGUGGCUGUAAGUGC  | 29         |
|             | GGAAAAUACUCUUCUAGA   | 30         | UCUUGAAGAGUGAUUUUCC  | 31         |
|             | GCUGGGCCUACAAAUCAUG  | 32         | CAUGAUUUGUAGGCCCAGC  | 33         |
|             | CUAUCGUUUGCACUUCAAA  | 34         | UUUGAAGUGCAAACGAUAG  | 35         |
|             | CGUUUGCACUUCAAAAUUC  | 36         | GAAUUUUGAAGUGCAAACG  | 37         |
|             | GGAUGUGUUCUCUGACCGU  | 38         | ACGGUCAGAGAACAACAUCC | 39         |
|             | GGAAGUGGCCAAUGCUAAA  | 40         | UUUAGCAUUGGCCACUUC   | 41         |
|             | GUCCAUUGCUGAACACGGA  | 42         | UCCGUGUUCAGCAAUGGAC  | 43         |
|             | CCAUCUGGUCAUUGCUGA   | 44         | UCAGCAAUGGACCAGAUGG  | 45         |
|             | CUACCUGCGCUUUCACUUA  | 46         | AUAGUGAAAGCGCAGGUAG  | 47         |
|             | GGUCACUGCCUACCUUUGU  | 48         | ACAAAGGUAGGCAGUGACC  | 49         |
|             | GCCCGAAGUCAGCUGUCCU  | 50         | AGGACAGCUGACUUCGGGC  | 51         |
|             | GGGGUCACUGCCUACCUUU  | 52         | AAAGGUAGGCAGUGACCCC  | 53         |
|             | GCCAAGGAAGACUACAUUU  | 54         | AAAUGUAGUCUUCUUGGC   | 55         |
|             | UGCUGGGCCUACAAAUCAU  | 56         | AUGAUUUGUAGGCCCAGCA  | 57         |
|             | UAUCGUUUGCACUUCAAAA  | 58         | UUUUGAAGUGCAAACGAUA  | 59         |
|             | CAAAUUGCCUACUCCAAGA  | 60         | UCUUGGAGUAGGCAAUUUG  | 61         |
|             | GCUGUGUGGCAACUCUGAU  | 62         | AUCAGAGUUGCCACACAGC  | 63         |
|             | CCCUCUGUGCGACACCAAC  | 64         | GUUGGUGUCGCACAGAGGG  | 65         |
|             | CGUGAUGCUGUGUGGCAAC  | 66         | GUUGCCACACAGCAUCACG  | 67         |
|             | CCUGGGUGGCUUCACAUUA  | 68         | AUAUGUGAAGCCACCAGG   | 69         |
|             | CGCCAAGAUGGAUUCAUGG  | 70         | CCAUGAAUCCAUCUUGGCC  | 71         |

|                      |     |                     |     |
|----------------------|-----|---------------------|-----|
| CAGCUGUCCUGGCUCAAAA  | 72  | UUUUGAGCCAGGACAGCUG | 73  |
| GCUGCUGGAAAAUCACUCU  | 74  | AGAGUGAUUUUCCAGCAGC | 75  |
| GGGAAGUGGCCAAUGCUAA  | 76  | UUAGCAUUGGCCACUUCCC | 77  |
| CAAAGACAAGAGGUUCCAG  | 78  | CUGGAACCUCUUGUCUUUG | 79  |
| UGCUAAGAAAUCGCCAGA   | 80  | UCUGGCGAUUUCUUJAGCA | 81  |
| CUCCCUGGGUGGCUUCACA  | 82  | UGUGAAGCCACCCAGGGAG | 83  |
| AAAAUUCCACGGACAAACA  | 84  | UGUUUGUCCGUGGAAUUUU | 85  |
| UGGGUGGCUUCACAUUGU   | 86  | ACAUUGUGAAGCCACCCA  | 87  |
| GCUGGAAAAUCACUCUUCA  | 88  | UGAAGAGUGAUUUUCCAGC | 89  |
| AGACAACUUCUACUUCACU  | 90  | AGUGAAGUAGAAGUUGUCU | 91  |
| CCAAAAUGCCCCGAGACAU  | 92  | AUGUCUCGGGGCAUUUUGG | 93  |
| ACCACAACUUGCAGUAUCU  | 94  | AGAUACUGCAAGUUGUGGU | 95  |
| UCGUUUGCACUUCAAAAUU  | 96  | AAUUUUGAAGUGCAAACGA | 97  |
| GGAGACAACUUCUACUUCA  | 98  | UGAAGUAGAAGUUGUCUCC | 99  |
| AAUUCCACGGACAAACACA  | 100 | UGUGUUUGUCCGUGGAAUU | 101 |
| CUUUGUGGACAUGACCACA  | 102 | UGUGGUCAUGUCCACAAAG | 103 |
| GUGCAUGAUGCCAAAGACA  | 104 | UGUCUUUGGCAUCAUGCAC | 105 |
| ACUUCUACUUCACUGGAGU  | 106 | ACUCCAGUGAAGUAGAAGU | 107 |
| AGUGGCCAAUGCUAAAGAA  | 108 | UUCUUUAGCAUUGGCCACU | 109 |
| AAGAAAUGAGUAUCACCUA  | 110 | UAGGUGAUACUCAUUFUUU | 111 |
| CUGCUGGAAAAUCACUCUU  | 112 | AAGAGUGAUUUUCCAGCAG | 113 |
| CCUGGUCAAGCACUUACAG  | 114 | CUGUAAGUGCUUGACCAGG | 115 |
| GCUGCUGGGCCUACAAAUC  | 116 | GAUUUGUAGGCCCAGCAGC | 117 |
| CAACUUCUACUUCACUGGA  | 118 | UCCAGUGAAGUAGAAGUUG | 119 |
| GACAAGAGGUUCCAGGAGA  | 120 | UCUCCUGGAACCUCUUGUC | 121 |
| GGCCUACAAAUCAUGUCAC  | 122 | GUGACAUGAUUUGUAGGCC | 123 |
| CCAUGACCACCUUGGCAAU  | 124 | AUUGCCAAGGUGGUCAUGG | 125 |
| CCAAUGGCUACCUGCGCUU  | 126 | AAGCGCAGGUAGCCAUUGG | 127 |
| CUACUUCACUGGAGUGCAU  | 128 | AUGCACUCCAGUGAAGUAG | 129 |
| CUUCCCAAGCCCUACUAAU  | 130 | AUAGUAAGGGCUUGGGAAG | 131 |
| GGCAAUGUCUCAGCACAAA  | 132 | UUUGUGCUGAGACAUUGCC | 133 |
| CAAUGUCUCAGCACAAAUU  | 134 | AAUUUGUGCUGAGACAUUG | 135 |
| CACUUCAAAAUCCACGGA   | 136 | UCCGUGGAAUUUUGAAGUG | 137 |
| GGAGUGGCCCCGAAGUCAGC | 138 | GCUGACUUCGGGCCACUCC | 139 |
| UGUGGAGAUAAAGCUCCAAA | 140 | UUUGGAGCUUAUCUCCACA | 141 |
| CGUGCAGAUUAUGGGUGCU  | 142 | AGCACCCAUAUUCUGCACG | 143 |
| GUAUCUUCAGGAUGAGAAU  | 144 | AUUCUCAUCCUGAAGAUAC | 145 |
| CCCUUACUAUCGUUUGCAC  | 146 | GUGCAAACGAUAGUAAGGG | 147 |
| GAGUAUCACCUAUGUGGAA  | 148 | UUCACAUAGGUGAUACUC  | 149 |
| CCUGGUACGUGCUGGCUGG  | 150 | CCAGCCAGCACGUACCAGG | 151 |
| CUGGGAGACAACUUCUACU  | 152 | AGUAGAAGUUGUCUCCAG  | 153 |
| UUCAAAAUCCACGGACAA   | 154 | UUGUCCGUGGAAUUUGAA  | 155 |

|                      |     |                      |     |
|----------------------|-----|----------------------|-----|
| AAGGAAGACUACAUUUUGG  | 156 | CCAAAAUGUAGUCUUCCUU  | 157 |
| GCCACUGUUGGCCACAUAC  | 158 | GUAUGUGGCCAACAGUGGC  | 159 |
| AAUGUCUCAGCACAAAUUG  | 160 | CAAUUUGUGCUGAGACAUU  | 161 |
| CCUACAAAUCAUGUCACUC  | 162 | GAGUGACAUGAUUUUGUAGG | 163 |
| GUCUCAGCACAAAUUGCCU  | 164 | AGGCAAUUUGUGCUGAGAC  | 165 |
| GACCUUUGAGGAUGUGUUC  | 166 | GAACACAUCCUCAAGGUC   | 167 |
| CCAAGAUGGAUUAUGGGU   | 168 | ACCCAUGAAUCCAUCUUGG  | 169 |
| GCAACUCUGAUGACUUCGU  | 170 | ACGAAGUCAUCAGAGUUGC  | 171 |
| CUCCCCACCCUGAGAUUUGU | 172 | ACAAAUCUCAGGGUGGGAG  | 173 |
| AAAUACUCUUAAGACCA    | 174 | UGGUCUUGAAGAGUGAUUU  | 175 |
| CAAACACAACUGUGGCCAU  | 176 | AUGGCCACAGUUGUGUUUG  | 177 |
| CUGCUGGGCCUACAAAUCA  | 178 | UGAUUUGUAGGCCCAGCAG  | 179 |
| CCCAUCUGGUCCAUFUGCUG | 180 | CAGCAAUGGACCAGAUGGG  | 181 |
| AAUCACUCUUAAGACCAG   | 182 | CUGGUCUUGAAGAGUGAUU  | 183 |
| CCCAAUGGCUACCGCGCU   | 184 | AGCGCAGGUAGCCAUGGG   | 185 |
| UGGCAAUGUCUCAGCACAA  | 186 | UUGUGCUGAGACAUUGCCA  | 187 |
| AACUCUGAUGACUUCGUCA  | 188 | UGACGAAGUCAUCAGAGUU  | 189 |
| CCUUAUAUCGUUUGCACU   | 190 | AGUGCAAACGAUAGUAAGG  | 191 |
| GCUAAAGAAAUCGCCAGAA  | 192 | UUCUGGCGAUUUCUUUAGC  | 193 |
| GCCUGGUCAAGCACUUACA  | 194 | UGUAAGUGCUUGACCAGGC  | 195 |
| GGUACGUGCUGGCUGGGAA  | 196 | UUCCCAGCCAGCACGUACC  | 197 |
| GACAACUUCUACUUCACUG  | 198 | CAGUGAAGUAGAAGUUGUC  | 199 |
| GAAAAUCACUCUUAAGAC   | 200 | GUCUUGAAGAGUGAUUUUC  | 201 |
| CCUGGGAGACAACUUCUAC  | 202 | GUAGAAGUUGUCUCCCAGG  | 203 |
| GGGUCACUGCCUACCUUUG  | 204 | CAAAGGUAGGCAGUGACCC  | 205 |
| CUUACUAUCGUUUGCACUU  | 206 | AAGUGCAAACGAUAGUAAG  | 207 |

Table 9

| Target gene | SenseSeq 5' to 3'   | SEQ ID No. | AntisSeq 5' to 3'   | SEQ ID No. |
|-------------|---------------------|------------|---------------------|------------|
| M6pr (CD)   | CAAUCUUAUAGAAAGGGAA | 208        | UUCCCUUUCUUAUAGAUUG | 209        |
|             | CGUUAUACUCUUGCUUUC  | 210        | GAAAGCAAGAGUAUUAACG | 211        |
|             | CUACCCUAAAGGAAAUAGA | 212        | UCUAUUUCCUUUAGGGUAG | 213        |
|             | CUGUCUAAAUUUUUCACA  | 214        | UGUGAAAAUAAUUAGACAG | 215        |
|             | GGUUGCUGUCUACAUAUU  | 216        | AAUAAUGUAGACAGCAACC | 217        |
|             | GCCCAAUUGUCUAAUCAAG | 218        | CUUGAUUAGACAUUUGGGC | 219        |
|             | CCACGUACCUAUGAGAAAA | 220        | UUUUCUCAUAGGUACGUGG | 221        |
|             | GCUCUAUCUUACUGGUCAU | 222        | AUGACCAGUAAGAUAGAGC | 223        |
|             | CCCUUUGCUCAUAUUGUUU | 224        | AAACAAUAUGAGCAAAGGG | 225        |
|             | CCACUUUUGUACUAAAACA | 226        | UGUUUUAGUACAAAAGUGG | 227        |

|                      |     |                      |     |
|----------------------|-----|----------------------|-----|
| CGGCCACUGUUUAAACAAAA | 228 | UUUUGUUAAACAGUGGCCG  | 229 |
| AGUCCUACCCUAAAGGAAA  | 230 | UUUCCUUUAGGGUAGGACU  | 231 |
| GGCCAGGGCUUAGAUACAU  | 232 | AUGUAUCUAAGCCCUGGCC  | 233 |
| CCGUUAAUACUCUUGCUUU  | 234 | AAAGCAAGAGUAUUAACGG  | 235 |
| GAUACAUACAGCUAUAGAU  | 236 | AUCUAUAGCUGUAUGUAUC  | 237 |
| UACCACUUUUGUACUAAAA  | 238 | UUUUAGUACAAAAGUGGUA  | 239 |
| GGCUGUAACCCAAAACUC   | 240 | GAGUAUUUGGGUUACAGCC  | 241 |
| AGCCCAAAGUCUAAUCAA   | 242 | UUGAUUAGACAUUUGGGCU  | 243 |
| CGCUAAAUGGGCAGAAAGC  | 244 | GCUUUCUGCCCAUUUAGCG  | 245 |
| CAGCUAUAGAUUCAGAGUA  | 246 | UACUCUGAAUCUAUAGCUG  | 247 |
| ACUUGUACCUUAAUUGCUU  | 248 | AAGCAAUUAAGGUACAAGU  | 249 |
| GAACAAAGUCCAAGAUUGU  | 250 | ACAAUCUUGGACUUUGUUC  | 251 |
| CCUCCCUUUGCUCAUUAUG  | 252 | CAAUAUGAGCAAAGGGAGG  | 253 |
| AUACCACUUUUGUACUAAA  | 254 | UUUAGUACAAAAGUGGUUAU | 255 |
| GUACUUCUCAACUUGUGUU  | 256 | AACACAAGUUGAGAAGUAC  | 257 |
| GCCAUUUGUUACCUUUAGA  | 258 | UCUAAAGGUAAACAAAUGGC | 259 |
| GACUGAACUGUUACUAUUG  | 260 | CAAUAGUACAGUUCAGUC   | 261 |
| CAGUCCUACCCUAAAGGAA  | 262 | UUCCUUUAGGGUAGGACUG  | 263 |
| CCUGGUAGGAGACAAUGAU  | 264 | AUCAUUGUCUCCUACCAGG  | 265 |
| GGAAAAGGCAGGGCCAUUU  | 266 | AAAUGGCCCGCCUUUUCC   | 267 |
| GCAUAGUUCCTCGUGUUUU  | 268 | AAAACACGGGGAACUAUGC  | 269 |
| CUAUCUUACUGGUCAUAUU  | 270 | AAUAUGACCAGUAAGAUAG  | 271 |
| GAAUCAACGAGACUCACAU  | 272 | AUGUGAGUCUCGUUGAUUC  | 273 |
| GGCUUAGAUACAUCAGCU   | 274 | AGCUGUAUGUAUCUAAGCC  | 275 |
| GUAAUUGGAUCAUGCUGAU  | 276 | AUCAGCAUGAUCCAAUUAAC | 277 |
| ACGGCCACUGUUUAAACAAA | 278 | UUUGUUAAACAGUGGCCGU  | 279 |
| GGAAAGUAGACAUAGGGUC  | 280 | GACCCUAGUCUACUUUCC   | 281 |
| CCUUGCCGUUAAUACUCUU  | 282 | AAGAGUAUUAACGGCAAGG  | 283 |
| CUGGUAGGAGACAAUGAUA  | 284 | UAUCAUUGUCUCCUACCAG  | 285 |
| CACAUUAGCGGGCAAUUUU  | 286 | AAAAUUGCCCGCUAAUGUG  | 287 |
| GUUACUAUUGCUAGUCCUG  | 288 | CAGGACUAGCAAUAGUAAC  | 289 |
| AGAUUACUUGUACCUJAAU  | 290 | AUUAAGGUACAAGUAAUCU  | 291 |
| GCACUUUUCUCACAAGUCU  | 292 | AGACUUGUGAGAAAAGUGC  | 293 |
| UGACCUGGUAGGAGACAAU  | 294 | AUUGUCUCCUACCAGGUCA  | 295 |
| GCUAGAAUUGGGCUCCAAG  | 296 | CUUGGAGCCCAAUUCUAGC  | 297 |
| CUAUGAGAAAAGGGUUUAG  | 298 | CUAAACCCUUUUCUCAUAG  | 299 |
| GGAAGUAAUUGGAUCAUGC  | 300 | GCAUGAUCCAAUUAUUCUCC | 301 |
| GUGUGGGCUCUAUCUUACU  | 302 | AGUAAGAUAGAGCCACAC   | 303 |
| UCCCUUUGCUCAUUAUGUU  | 304 | AACAAUAUGAGCAAAGGGA  | 305 |
| GGGUCUUCGGGGCUGACUC  | 306 | GAGUCAGCCCCGAAGACCC  | 307 |
| UAGCCCAAAGUCUAAUCA   | 308 | UGAUUAGACAUUUGGGCUA  | 309 |
| CCAAUCUUUAUAGAAAGGGA | 310 | UCCCUUUCUAUAAGAUUGG  | 311 |

|                      |     |                      |     |
|----------------------|-----|----------------------|-----|
| AUAUUUUCACAAGUUGAGA  | 312 | UCUCAACUUGUGAAAAUAU  | 313 |
| ACAUUAGCGGGCAAUUUUA  | 314 | UAAAAUUGCCCGCUAAUGU  | 315 |
| UAUAUUUCUCCCAAUCUUA  | 316 | UAAGAUGGGGAGAAUAUA   | 317 |
| CUGAAAUGCCCCUUGUAUG  | 318 | CAUACAAGGGGCAUUCAG   | 319 |
| CAAGCAGUCCUACCCUAAA  | 320 | UUUAGGGUAGGACUGCUUG  | 321 |
| CUCUCACCUUGCCGUUAAU  | 322 | AUUAACGGCAAGGUGAGAG  | 323 |
| CCAUGUGCCAGAAUCACAG  | 324 | CUGUGAUUCUGGCACAUGG  | 325 |
| GAUUACUUGUACCUUAAU   | 326 | AAUUAAGGUACAAGUAAUC  | 327 |
| GUCAAUGGUAGUGGGAUUU  | 328 | AAAUCCACUACCAUUGAC   | 329 |
| UAUCUUACUGGUCAUAUUU  | 330 | AAAUAUGACCAGUAAGAUA  | 331 |
| GUCUUACCCACGUACCUAU  | 332 | AUAGGUACGUGGGUAAGAC  | 333 |
| GUCUGUACCUCUUCGAGAU  | 334 | AUCUCGAAGAGGUACAGAC  | 335 |
| AAAGCCAUAUGUAGCCCAA  | 336 | UUGGGCUACAUAUGGCUUU  | 337 |
| GAGCAAGGACCGCUAAAUG  | 338 | CAUUUAGCGGUCCUUGCUC  | 339 |
| CAUUAGCGGGCAAUUUUA   | 340 | UAAAAUUGCCCGCUAAUG   | 341 |
| CUUGUCAAUGGUAGUGGGA  | 342 | UCCCACUACCAUUGACAAG  | 343 |
| CAAAGCAUACACAGCCAGA  | 344 | UCUGGCUGUGUAUGCUUUG  | 345 |
| GGCUGAUUUUCUGUAAUA   | 346 | UAUUACAGGAAGAUAGCC   | 347 |
| CCAUUUGCCUCGAGCCUGG  | 348 | CCAGGCUCGAGGCAAUGG   | 349 |
| UGCAGGAAAAGCUGGUAAA  | 350 | UUUACCAGCUUUUCCUGCA  | 351 |
| CUCUACUAGUACUUCUCAA  | 352 | UUGAGAAGUACUAGUAGAG  | 353 |
| GGAGGACUGAACUGUUACU  | 354 | AGUAACAGUUCAGUCCUCC  | 355 |
| AUACAGCUAUAGAUUCAGA  | 356 | UCUGAAUCUAUAGCUGUAU  | 357 |
| AGAUACAUAACAGCUAUAGA | 358 | UCUAUAGCUGUAUGUAUCU  | 359 |
| AUAUUUCUCCCAAUCUUAU  | 360 | AUAAGAUUGGGAGAAUAU   | 361 |
| GGGCCAGGGCUUAGAUACA  | 362 | UGUAUCUAAGCCCUGGCCC  | 363 |
| ACUGAAAUGCCCCUUGUAU  | 364 | AUACAAGGGGCAUUUCAGU  | 365 |
| CUCACAAGUCUGUAGUUUG  | 366 | CAAACUACAGACUUGUGAG  | 367 |
| AUUACUUGUACCUUAAUUG  | 368 | CAAUUAAGGUACAAGUAAU  | 369 |
| CUUGUAUGGGAUUAGGGCA  | 370 | UGCCCUAAUCCCAUACAAG  | 371 |
| UACCCACGUACCUAUGAGA  | 372 | UCUCAUAGGUACGUGGGUA  | 373 |
| UAUACCACUUUUGUACUAA  | 374 | UUAGUACAAAAGUGGUUAU  | 375 |
| UGGAGUGAGUCACUGAAAU  | 376 | AUUUCAGUGACUCACUCCA  | 377 |
| AACAACACAUCUGAACACA  | 378 | UGUGUUCAGAUUGUUGUU   | 379 |
| ACUCCUAGGCCAGUAAGUA  | 380 | UACUUAACUGGCCUAGGAGU | 381 |
| GCACAUUAGCGGGCAAUUU  | 382 | AAAUUGCCCGCUAAUGUGC  | 383 |
| CUCCCAAUCUUAUAGAAAG  | 384 | CUUUUUAUAAGAUUGGGAG  | 385 |
| CAUAGUUCUCCCGUGUUUUA | 386 | UAAAACACGGGGAACUAUG  | 387 |
| CGGGCAAUUUUAAACCCAGU | 388 | ACUGGGUUAAAAUUGCCCG  | 389 |
| CCAGGGCUUAGAUACAUAC  | 390 | GUAUGUAUCUAAGCCCUGG  | 391 |
| GUGCCUGCUGCCUAUCGUG  | 392 | CACGAUAGGCAGCAGGCAC  | 393 |
| CUUCGGGGCUGACUCUUGU  | 394 | ACAAGAGUCAGCCCCGAAG  | 395 |

|  |                     |     |                     |     |
|--|---------------------|-----|---------------------|-----|
|  | AGAAUCAACGAGACUCACA | 396 | UGUGAGUCUCGUUGAUUCU | 397 |
|  | GCCUCCCCUUUGCUCAUUU | 398 | AAUAUGAGCAAAGGGAGGC | 399 |
|  | GCUUUUGAUUUGACCCUAA | 400 | UUAGGGUCAAUCAAAGC   | 401 |
|  | CUUCCUUCUGUUUAGGCCA | 402 | UGGCCUAAACAGAAGGAAG | 403 |
|  | AAAAGUUUUGAGAGCACUG | 404 | CAGUGCUCUAAAACUUUU  | 405 |
|  | UUUCUUUCUAUACAAGGAA | 406 | UUCCUUGUAUAGAAAGAAA | 407 |

## CLAIMS

What is claimed is:

1. A method for producing a composition comprising a lysosomal protein, comprising:  
culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule,
  - (a) wherein said host cell:
    - i expresses the lysosomal protein; and
    - ii comprises a target gene that encodes Acid Phosphatase 5 (ACP5);
  - (b) wherein said RNA effector is substantially complementary to said target gene that encodes ACP5, and reduces or prevents the expression of said target gene; and
  - (c) wherein said host cell is cultured for a period of time sufficient for the production of said lysosomal protein.
2. The method of claim 1, wherein said lysosomal protein is not iduronate 2-sulfatase (IDS) or arylsulfatase A.
3. The method of claim 1 or 2, said RNA effector transiently reduces the expression of said target gene that encodes ACP5.
4. The method of any one of claims 1-3, wherein said lysosomal protein is an exogenous protein.
5. The method of any one of claims 1-4, wherein said host cell further comprises a second target gene that encodes a mannose-6-phosphate receptor (MPR), and wherein the method further comprises:  
  
culturing said large scale host cell culture in a medium that comprises an effective amount of a second RNA effector molecule, wherein said second RNA

effector is substantially complementary to said second target gene that encodes MPR, and wherein said RNA effector reduces or prevents the expression of said target gene that encodes MPR.

6. The method of claim 5, wherein said RNA effector transiently reduces the expression of said target gene that encodes said MPR.
7. The method of any one of claims 1-6, wherein said host cell further comprises a second target gene that encodes phosphomannomutase (PMM), and wherein the method further comprises:

culturing said large scale host cell culture in a medium that comprises an effective amount of a second RNA effector molecule, wherein said second RNA effector is substantially complementary to said second target gene that encodes PMM, and wherein said RNA effector reduces or prevents the expression of said target gene that encodes PMM.
8. The method of claim 7, said RNA effector transiently reduces the expression of said target gene that encodes PMM.
9. The method of any one of claims 1-8, further comprising harvesting said lysosomal protein from said large scale culture.
10. The method of any one of claims 1-9, further comprising isolating said lysosomal protein.
11. The method of any one of claims 1-10, wherein said lysosomal protein is secreted into the medium.
12. The method of any one of claims 1-11, wherein said RNA effector molecule is an siRNA.
13. The method of any one of claims 1-11, wherein said RNA effector molecule is a shRNA.

14. The method of any one of claims 1-11, wherein said RNA effector molecule is an antisense molecule.
15. The method of any one of claims 1-11, wherein said RNA effector molecule that is substantially complementary to a target gene that encodes ACP5 comprises a sequence selected from the group consisting of SEQ ID NOs: 8-207.
16. The method of any one of claims 5-11, wherein said RNA effector molecule that is substantially complementary to a target gene that encodes MPR comprises a sequence selected from the group consisting of SEQ ID NOs: 208-407.
17. A method for producing a composition comprising a lysosomal protein, comprising:  
culturing a large scale host cell culture in a medium that comprises an effective amount of a first RNA effector molecule and a second RNA effector molecule,
  - (a) wherein said host cell:
    - i expresses the lysosomal protein; and
    - ii comprises a first target gene that encodes Acid Phosphatase 5 (ACP5), and a second target gene that encodes a mannose-6-phosphate receptor (MPR);
  - (b) wherein said first RNA effector is substantially complementary to said first target gene that encodes ACP5, and reduces or prevents the expression of said target gene that encodes ACP5;
  - (c) wherein said second RNA effector is substantially complementary to said second target gene that encodes MPR5, and reduces or prevents the expression of said target gene that encodes MPR5; and
  - (d) wherein said host cell is cultured for a period of time sufficient for the production of said lysosomal protein.
18. The method of claim 17, wherein said first and second RNA effectors transiently reduce the expression of their respective target genes.

19. The method of claim 17 or 18, wherein said host cell further comprises a third target gene that encodes phosphomannomutase (PMM), and wherein the method further comprises:

culturing said large scale host cell culture in a medium that comprises an effective amount of a third RNA effector molecule, wherein said third RNA effector is substantially complementary to said third target gene that encodes PMM, and wherein said RNA effector reduces or prevents the expression of said target gene that encodes PMM.
20. The method of claim 19, said RNA effector transiently reduces the expression of said target gene that encodes PMM.
21. The method of any one of claims 17-20, further comprising harvesting said lysosomal protein from said large scale culture.
22. The method of any one of claims 17-21, further comprising isolating said lysosomal protein.
23. The method of any one of claims 17-22, wherein said lysosomal protein is secreted into the medium.
24. The method of any one of claims 17-23, wherein said RNA effector molecule is an siRNA.
25. The method of any one of claims 17-23, wherein said RNA effector molecule is a shRNA.
26. The method of any one of claims 17-23, wherein said RNA effector molecule is an antisense molecule.
27. The method of any one of claims 17-23, wherein said first RNA effector molecule that is substantially complementary to a target gene that encodes ACP5 comprises a sequence selected from the group consisting of SEQ ID NOs: 8-207.

28. The method of any one of claims 17-23, wherein said RNA effector molecule that is substantially complementary to a target gene that encodes MPR comprises a sequence selected from the group consisting of SEQ ID NOs: 208-407.
29. The method of any one of claims 17-24, wherein protein is a lysosomal protein.
30. The method of any one of claims 17-25, wherein said protein is an exogenous protein.
31. A method for producing a composition comprising a lysosomal protein, comprising: culturing a large scale host cell culture in a medium that comprises an effective amount of an RNA effector molecule,
- (a) wherein said host cell:
    - i expresses the lysosomal protein; and
    - ii comprises a target gene that encodes phosphomannomutase (PMM);
  - (b) wherein said RNA effector is substantially complementary to said target gene that encodes PMM, and reduces or prevents the expression of said target gene; and
  - (c) wherein said host cell is cultured for a period of time sufficient for the production of said lysosomal protein.
32. The method of claim 31, said RNA effector transiently reduces the expression of said target gene that encodes PMM.
33. The method of claim 31 or 32, further comprising harvesting said lysosomal protein from said large scale culture.
34. The method of any one of claims 31-33, further comprising isolating said lysosomal protein.

35. The method of any one of claims 31-34, wherein said lysosomal protein is secreted into the medium.
36. The method of any one of claims 31-35, wherein said RNA effector molecule is an siRNA.
37. The method of any one of claims 31-35, wherein said RNA effector molecule is a shRNA.
38. The method of any one of claims 31-35, wherein said RNA effector molecule is an antisense molecule.
39. A composition comprising a protein produced according to the method of any one of claims 1-38.
40. The composition of claim 39, wherein said lysosomal protein is selected from the group consisting of glucosidase, glucocerebrosidase, galactocerebrosidase, galactosidase, iduronidase, hexosaminidase, mannosidase, fucosidase, arylsulfatase, V-acetylgalactosamine-6-sulfate sulfatase, acetylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase,  $\alpha$ -glucosaminide-N-acetyltransferase, acetyl-CoA:  $\alpha$ -glucosaminide N-acetyltransferase,  $\beta$ -D-glucoronidase, hyaluronidase, mannosidase, neurominidase, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, sialidase and lipoprotein lipase.
41. The composition of claim 39, wherein said lysosomal protein is selected from the group consisting of glucocerebrosidase, idursulfase,  $\alpha$ -glucosidase, galsulfase, agalsidase beta, and laronidase.
42. The composition of claim 39, wherein said lysosomal protein is an acid  $\alpha$ -glucosidase.
43. The composition of claim 42, wherein said lysosomal protein comprising a sequence that is at least 90% identical to  $\alpha$ -glucosidase.

44. The composition of claim 43, wherein said lysosomal protein comprises SEQ ID NO: 1.
45. The composition of claim 39, wherein said lysosomal protein is an  $\alpha$ -galactosidase A.
46. The composition of claim 45, wherein said lysosomal protein comprising a sequence that is at least 90% identical to Agalsidase beta.
47. The composition of claim 46, wherein said lysosomal protein comprises SEQ ID NO: 408.
48. The composition of claim 39, wherein said lysosomal protein is a glucocerebrosidase.
49. The composition of claim 48, wherein said lysosomal protein comprising a sequence that is at least 90% identical to imiglucerase.
50. The composition of claim 49, wherein said lysosomal protein comprises SEQ ID NO: 2 or SEQ ID NO: 3.
51. The composition of claim 39, wherein said lysosomal protein is a protease inhibitor.
52. The composition of claim 51, wherein said lysosomal protein is a trypsin inhibitor.
53. The composition of claim 52, wherein said lysosomal protein comprising a sequence that is at least 90% identical to  $\alpha_1$ -antitrypsin.
54. The composition of claim 53, wherein said lysosomal protein comprises SEQ ID NO: 4.
55. The composition of claim 51, wherein said lysosomal protein is an esterase inhibitor.
56. The composition of claim 55, wherein said lysosomal protein comprising a sequence that is at least 90% identical to C1 Esterase Inhibitor.
57. The composition of claim 56, wherein said lysosomal protein comprises SEQ ID NO: 5.

58. A cell comprising an RNA effector molecule substantially complementary to a target gene encoding ACP5.
59. An RNA effector molecule substantially complementary to a target gene encoding ACP5.
60. The RNAi effector agent of claim 59, wherein the RNAi effector agent comprises a sequence selected from the group consisting of: SEQ ID NO: 8-207.
61. A cell comprising an RNA effector molecule substantially complementary to a target gene encoding a mannose-6-phosphate receptor (MPR).
62. An RNA effector molecule substantially complementary to a target gene encoding MPR.
63. The RNAi effector agent of claim 62, wherein the RNAi effector agent comprises a sequence selected from the group consisting of: SEQ ID NO: 208-407.

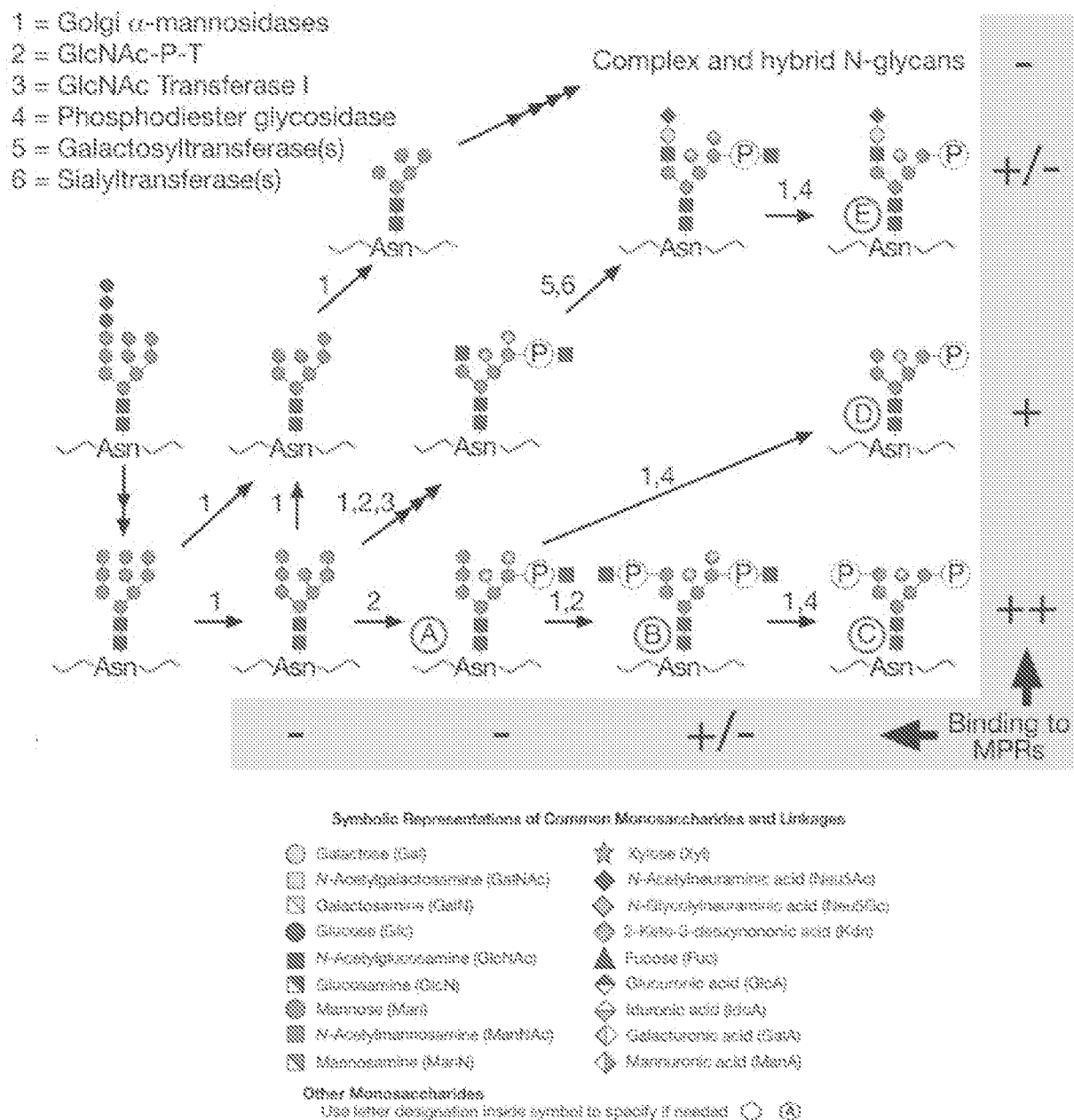


Figure 1