Title: PROGRANULIN (PGRN) AND ITS DERIVATIVES FOR DIAGNOSIS AND TREATMENT OF LYSOSOMAL STORAGE DISEASES

Abstract: The present invention provides composition and methods for diagnosis and treatment of lysosomal storage diseases and their diagnosis and treatment, including Gaucher’s Disease and Tay-Sachs disease, and particularly which utilize progranulin (PGRN), or active PGRN peptides, including atstatrin. The invention also provides animal models of lysosomal storage diseases, including Gaucher’s Disease and Tay-Sachs disease, based on or including PGRN mutations including PGRN null mutants and PGRN gene knock outs.
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PROGRANULIN (PGRN) AND ITS DERIVATIVES FOR DIAGNOSIS AND TREATMENT OF LYSOSOMAL STORAGE DISEASES

FIELD OF THE INVENTION

[0001] The present invention relates generally to lysosomal storage diseases and their diagnosis and treatment, including Gaucher’s Disease, and particularly to diagnostic and therapeutic aspects thereof which utilize progranulin (PGRN), or active PGRN peptides, including atstrin.

BACKGROUND OF THE INVENTION

[0002] Progranulin (PGRN) is a multifunctional growth factor, also known as PC-cell-derived growth factor (PCDGF), acrogranin, Granulin/epithelin precursor (GEP), proepithelin (PEPI), or GP80, and was first purified as a growth factor from conditioned tissue culture media (Wright WE et al (1989) Cell 56(4):607-617; Zhou J et al (1993) J Biol Chem 268(15):10863-10869). PGRN is a 593-amino-acid secreted glycoprotein with an apparent molecular weight of 88 kDa. PGRN contains seven and a half repeats of a cysteine-rich motif (CX5–6CX3CCX8CCX6CCXDX2HCCPX4CX5–6C) (SEQ ID NO:1) in the order P-G-B-A-C-D-E, where A-G are full repeats and P is the half motif (FIGURE 24). Notably, PGRN (GEP) undergoes proteolytic processing with the liberation of small, 6-kDa repeat units known as granulins (or epithelins), which retain biological activity (Davidson B et al (2004) Cancer 100(10):2139-2147)). These peptides are active in cell growth assays and may be related to inflammation (Zanocco-Marani, T et al (1999) Cancer Res 59(20):5331-5340; Lu R and Serrero G (2000) Proc Natl Acad Sci USA 97(8):3993-3998).

[0003] PGRN has multiple physiological and pathological functions in development, would healing, anti-inflammation, neuron system disorders, as well as cancer. PGRN (GEP) is abundantly expressed in rapidly cycling epithelial cells, in cells of the immune system, and in neurons (Baba T et al (1993) Mol Reprod Dev 34(3):233-243; Daniel R et al (2000) Histochem Cytochem 48(7):999-1009). High levels of GEP expression are also found in several human cancers and contribute to tumorigenesis in diverse cancers, including breast cancer, clear cell renal carcinoma, invasive ovarian carcinoma, glioblastoma, adipocytic teratoma, and multiple

Several PGRN-associated partners have been reported and found to affect PGRN action in various processes. One example is the secretory leukocyte protease inibitor (SLPI). Elastase digests PGRN exclusively in the intergranulin linkers with the generation of granulin peptides. SLPI blocks this proteolysis either by directly binding to elastase or by sequestering granulin peptides from the enzyme (Zhu J et al (2002) Cell 111(6):867-878). PGRN was also found to bind to Sortilin and mediate neurite growth (Hu F et al (2010) Neuron 68:654-667).

Recently, PGRN and PGRN peptides, particularly including the peptide denoted atsttrin, were identified as modulators of TNF/TNFR activity and signaling, and demonstrated to inhibit or block TNF-mediated signaling or response, including TNF-a-induced inflammatory arthritis (Tang W et al (2011) Science 332:478-484; WO 2010120374). Atsttrin is a PGRN-derived engineered protein (Antagonist of TNF/TNFR Signaling via Targeting TNF Receptors), comprising combinations of half units of PGRN units A, C and F in combination with linker units P3, P4 and P5 (US Patent 8,362,218; WO 2010120374). Atsttrin provides a PGRN-derived active peptide having overlapping activity and capability with the full length PGRN molecule. US Patent 8,362,218 and PCT publication WO 2010120374 describe PGRN-derived peptides comprising a combination of half units of progranin/granulin units, wherein at least one half unit is ½ F, and linker units, particularly at least two linker units. The amino acid sequence of PGRN, and PGRN-derived peptides, including atsttrin, are depicted in FIGURES 49 and 50.

Lysosomal storage diseases

Lysosomes are subcellular organelles responsible for the physiologic turnover of cell constituents. They contain catabolic enzymes, which require a low pH environment in order to function optimally. Lysosomal storage diseases (LSD) describe a heterogeneous group of dozens
of rare inherited disorders characterized by the accumulation of undigested or partially digested macromolecules, which ultimately results in cellular dysfunction and clinical abnormalities. LSDs result from gene mutations in one or more of lysosomal enzymes, resulting in accumulation of the enzyme substrates in lysosomes. Organomegaly, connective-tissue and ocular pathology, and central nervous system dysfunction may result. Classically, lysosomal storage diseases encompassed enzyme deficiencies of the lysosomal hydrolases. More recently, the concept of lysosomal storage disease has been expanded to include deficiencies or defects in proteins necessary for the normal post-translational modification of lysosomal enzymes, activator proteins, or proteins important for proper intracellular trafficking between the lysosome and other intracellular compartments.

[0007] Over 50 lysosomal storage diseases have been described. The age of onset and clinical manifestations may vary widely among patients with a given lysosomal storage disease, and significant phenotypic heterogeneity between family members carrying identical mutations has been reported. Lysosomal storage diseases are generally classified by the accumulated substrate and include the sphingolipidoses, oligosaccharidoses, mucolipidoses, mucopolysaccharidoses (MPSs), lipoprotein storage disorders, lysosomal transport defects, neuronal ceroid lipofuscinoses and others. FIGURE 1 depicts pathways for glycosphingolipids and indicates the altered metabolic enzymes associated with different lysosomal storage diseases.

[0008] The most common of the LSDs is Gaucher’s Disease, which involves dysfunctional metabolism of sphingolipids and results from hereditary deficiency of the enzyme glucocerebrosidase. Glucocerebrosidase enzyme acts on the fatty acid glucosylceramide and when the enzyme is defective, glucosylceramide accumulates particularly in white blood cells, most often macrophages. Over 300 unique mutations of the glucocerebrosidase encoding gene GBA1 have been identified in Gaucher’s Disease (Beutler E and Grabowski GA (2001) Gaucher Disease. in The Metabolic and Molecular Basis of Inherited Disease CR Scriver et al eds. McGraw Hill, NY pp3635-3668; Grabowski GA (2008) Lancet 372(9645): 1263-1271; Zhao et al (2003) Clin Genet 64(l):57-64). Glucosylceramide can collect in the spleen, liver, kidneys, lungs, brain and bone marrow.

[0009] Gaucher’s Disease (GD) falls into three subtypes, with varying pathology and severity. Type I (or non-neuropathic type) is the most common form of the disease, with an incidence of 1 in 50,000 live births of Ashkenazi Jewish heritage. Type I patients have hepatosplenomegaly. The brain is generally not affected pathologically, and depending on disease onset and severity, type I patients may live well into adulthood. Many patients have a
mild form of the disease or may not show any symptoms. Type I is associated genetically with a GBA1 gene mutation N370S homozygote. Type II (or acute infantile neuropathic Gaucher's disease), begins within 6 months of birth and has an incidence rate of approximately 1 in 100,000 live births. Type II patients have an enlarged liver and spleen, extensive and progressive brain damage, eye movement disorders, spasticity, seizures, limb rigidity, and a poor ability to suck and swallow. Type II patients suffer from serious convulsions, hypertonia, mental retardation and apnea. Affected children usually die by age 2. Type II GD is associated with GBA1 mutation alleles including GBA1 mutation L444P. Type III GD, a chronic neuropathic form, can begin at any time in childhood or even in adulthood, and occurs in approximately 1 in 100,000 live births. It is characterized by slowly progressive but milder neurologic symptoms compared to the acute or type II GD. Major symptoms include an enlarged spleen and/or liver, seizures, poor coordination, skeletal irregularities, eye movement disorders, blood disorders including anemia and respiratory problems. Type III patients suffer from muscle twitches known as myoclonus, convulsions, dementia and ocular muscle apraxia. Patients often live into their early teen years and adulthood. The genetics and any specific GBA1 mutations associated with Type III GD are not clear.

[00010] Diagnostic indicators for Gaucher's Disease include increased alkaline phosphatase (ALP), angiotensin-converting enzyme (ACE) and immunoglobulin levels. Alternatively or in addition, cell analysis showing "crinkled paper" cytoplasm and glycolipid-laden macrophages, which are also called "Gaucher's cells" are cellular hallmarks of GD. Mutations in the GBA1 gene are also evaluated, particularly those known to be associated with the disease and Types as noted above. GBA1 mutational analysis can be valuable particularly in families at risk of GD due to family history or that are carriers of GBA1 mutations.

[00011] Therapy for LSDs includes enzyme replacement therapy to replace the disease mutant enzyme. Enzyme replacement therapy (ERT) and substrate reduction therapy (SRT) may be applicable for peripheral manifestations in patients with Gaucher disease types I and III, Fabry disease, mucopolysaccharidosis I (Hurler, Hurler-Scheie, and Scheie syndromes), mucopolysaccharidosis II (Hunter syndrome), mucopolysaccharidosis VI (Maroteaux-Lamy syndrome), and Pompe disease. Efforts are underway to develop enzyme replacement options for several other disorders. TABLE 1 provides ERTs being evaluated or approved for treatment of certain LSDs. Exemplary therapies, including ERT, for Gaucher's Disease are listed in TABLE 2. Thus far, ERT has been largely unsuccessful in improving central nervous system manifestations of the lysosomal storage diseases, possibly due to difficulty in penetrating the
blood-brain barrier. This has led to active clinical trials evaluating the safety and efficacy of intrathecal enzyme delivery in several lysosomal storage diseases. Also, immune response to enzyme replacement therepay proteins has been reported and can have adverse effects and alter the safety and efficacy of ERT (Brooks DA (1999) Molec Genet Metab 68(2):268-275).

**TABLE 1**

*Enzyme Replacement Therapy (ERT) for Lysosomal Storage Diseases (LSD)*

<table>
<thead>
<tr>
<th>Disease</th>
<th>Enzyme replaced</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaucher, type 1 and type 3</td>
<td>Glucocerebrosidase</td>
<td>Genzyme</td>
<td>approved EU/US (1991)</td>
</tr>
<tr>
<td>Fabry</td>
<td>α-galactosidase A</td>
<td>Genzyme</td>
<td>approved EU (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>approved EU (2001)</td>
</tr>
<tr>
<td>Pompe</td>
<td>α-glucosidase</td>
<td>Genzyme</td>
<td>phase III clinical trial</td>
</tr>
<tr>
<td>MPS II (Hunter)</td>
<td>α-L-iduronate sulfatase</td>
<td>Transkaryotic Therapies</td>
<td>phase III clinical trial</td>
</tr>
<tr>
<td>Niemann-Pick B</td>
<td>acid sphingomylinase</td>
<td>Genzyme</td>
<td>preclinical</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>arylsulfatase A</td>
<td>Zymenex</td>
<td>preclinical</td>
</tr>
<tr>
<td>α-Mannosidosis</td>
<td>1183α-mannosidase</td>
<td>Zymenex</td>
<td>preclinical</td>
</tr>
</tbody>
</table>

**TABLE 2**

*Therapies including ERT in Gaucher Diseases*

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism</th>
<th>Manufacturer</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imiglucerase (ERT)</td>
<td>Rh GBA1</td>
<td>Genzyme Corporation</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Velaglucerase alfa (ERT)</td>
<td>Rh GBA1</td>
<td>Shire plc</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Taliglucerase alpha (ERT)</td>
<td>Plant-derived GBA1</td>
<td>Protalix and Pfizer</td>
<td>FDA approved</td>
</tr>
<tr>
<td>Miglusta (SRT)</td>
<td>Inhibits glucosylceramide synthase</td>
<td>Actelion</td>
<td>Under development</td>
</tr>
<tr>
<td>Isofagomine tartrate (PCT)</td>
<td>Chaperoning, facilitates GBA folding and trafficking</td>
<td>Amicus Therapeutics</td>
<td>Under development</td>
</tr>
</tbody>
</table>

[00012] Therefore, in view of the aforementioned deficiencies attendant with prior art methods of evaluating, ameliorating and treating lysosomal storage diseases, including Gaucher’s Disease, it should be apparent that there still exists a need in the art for alternative therapies, additional agents, and improved and more correlative diagnostics for lysosomal storage diseases, including Gaucher’s Disease. The present invention provides novel activity, use and application of
progranulin (PGRN) and peptide derivatives thereof including asttrin, including in diagnosis, amelioration, and treatment of lysosomal storage diseases, including Gaucher’s Disease.

[00013] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[00014] In accordance with the present invention, it has been discovered that mutations in the gene encoding progranulin (PGRN), including the absence of PGRN by gene knockout, leads to Gaucher’s disease, a genetic disease previously known to be only caused by or associated with glycocerebrosidase enzyme gene (GBA1) mutations. Thus, PGRN and its encoding gene provides a novel gene associated with and capable of generating GD in an animal, in addition to or instead of the glycocerebrosidase GBA1 gene. The examples and studies provided herein demonstrate that PGRN knockout (KO) (null mutant) mice develop Gaucher’s disease, including the classical pathological appearance of Gaucher cells, which is diagnostic of lysosome storage disorders under the electronic microscope. Lipid analysis of PGRN KO mice shows glycocerebrosidase enzyme substrate glucosylceramide, denoted β-GlcCer, accumulation in macrophages.

[00015] The examples and studies provided herein demonstrate that PGRN binds glycocerebrosidase (GBA1), and the delivery of the GBA1 enzyme to the lysosome is impaired in PGRN KO mice. A clinical drug used to treat Gaucher’s disease imuglucerase rescues the Gaucher’s disease phenotype in PGRN KO mice.

[00016] Also, 15% Gaucher’s disease patients have a significantly reduced level of PGRN protein and over 70% of GD patients have a PGRN gene mutation. These findings reveal that PGRN has an important function in the lysosome and trafficking of proteins to the lysosome, and that mutation of the PGRN gene is associated with lysisosomal storage disease (LSD). PGRN, or PGRN-derived peptides including asttrin, provide novel protein therapeutics for prophylaxis and treatment of LSDs, including Gaucher’s Disease.

[00017] The invention provides PGRN and PGRN peptides, particularly including the peptide(s) denoted asttrin, as modulators of lysosomal storage disease and of lysosomal trafficking. In particular, the invention provides PGRN and PGRN peptides, including asttrin, as facilitators of lysosomal enzyme trafficking to the lysosome. In a particular embodiment, the present invention relates to all members of the herein disclosed family of PGRN peptides and of
atsttrin, which are capable of facilitating enzyme delivery to the lysosome, and/or binding or complexing with lysosomal enzymes such as glycocerebrosidase (GBA), or with sortilin and/or HSP70. The family of peptides includes fragments or portions, including mixed portions of PGRN sequence and half units, particularly comprising one or more granulin unit and one or more linker unit of PGRN. In one aspect the peptide comprises two or more half units of granulin units and one or more linker unit of PGRN. In a particular aspect of the invention, the PGRN peptide comprises the peptide atsttrin, comprising combinations of half units of granulin units A, C and F in combination with linker units P3, P4 and P5. In a particular aspect, the GEP peptide comprises a combination of half units of granulin units, wherein at least one half unit is $\frac{1}{2}F$, and linker units, particularly at least two linker units. In a further particular aspect atsttrin has the amino acid sequence set out in FIGURE 50 (SEQ ID NO: 4) and comprises granulin units and linker units $\frac{1}{2}F$-P3-P4-$\frac{1}{2}A$-P5-$\frac{1}{2}$ C, including as set out herein.

[00018] It is an object of the present invention to provide pharmaceutical compositions for use in therapeutic methods which comprise or are based upon the PGRN peptides and/or atsttrin. It is an object of the present invention to provide pharmaceutical compositions for use in therapeutic methods which comprise or are based upon the PGRN peptides and/or atsttrin, including comprising the peptide sequences set out in any of SEQ ID NOs: 2, 3 and 4-9, particularly comprising SEQ ID NO: 2, 3, 4. The pharmaceutical compositions include combinations of one or more PGRN peptides and/or atsttrin which are capable of facilitating enzyme delivery to the lysosome, and/or binding or complexing with lysosomal enzymes such as glycocerebrosidase (GBA), or with sortilin and/or HSP70, and/or capable of reducing lysosomal substrate accumulation, such as $\beta$-GlcCer, in the lysosome or macrophage. The pharmaceutical compositions include combinations of one or more PGRN peptides and/or atsttrin having activity as provided herein and one or more lysosomal enzyme or lysosomal substrate reducing agent. Lysosomal enzymes or lysosomal substrate reducing agents include and may be selected from one or more of glucocerebrosidase, a-galactosidase, $\beta$-galactosidase, $\beta$-hexosaminidase and sphingomyelinase. The pharmaceutical compositions include combinations of one or more PGRN peptides and/or atsttrin having GBA binding activity and one or more of Imiglucerase, Velaglucerase alfa, Taliglucerase alpha, Miglusta and Isofagomine tartrate.

[00019] Thus, the invention provides a composition for treatment or alleviation of a lysosomal storage disease comprising isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50, including as set out in any of SEQ ID NOS: 2, 3 and 4-9. The composition
may further comprising an enzyme replacement therapy agent or substrate reduction therapy agent for a lysosomal storage disease, including one or more of glucocerebrosidase, α-galactosidase, β-galactosidase, β-hexosaminidase and sphingomyelinase. In one such aspect, the invention provides a composition comprising PGRN or aststtrin in combination with glucocerebrosidase for treatment or alleviation of Gaucher’s Disease. In an aspect, compositions of the invention may further comprise one or more molecular chaperone or lysosomal delivery protein, including HSP70 and/or sortilin. Compositions of the invention include pharmaceutical compositions further comprising a pharmaceutically acceptable carrier, vehicle, diluent or excipient.

[00020] In a further embodiment, the present invention relates to certain therapeutic methods which would be based upon the activity of PGRN, PGRN peptides and/or aststtrin, or active fragments thereof, in facilitating enzyme delivery to the lysosome, and/or binding or complexing with lysosomal enzymes such as glucocerebrosidase (GBA), or with sortilin and/or HSP70, and/or being capable of reducing lysosomal substrate accumulation, such as β-GlcCer, in the lysosome or macrophage.

[00021] Thus, the present invention provides methods for facilitating lysosomal delivery of a protein or enzyme in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including aststtrin. In an aspect thereof said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50, including comprising or as set out in any of SEQ ID NOS: 2, 3 and 4-9. In an aspect of the invention, a method is provided for facilitating delivery of glucocerebrosidase (GBA) in a patient with Gaucher’s Disease comprising administering to said patient isolated PGRN, or active fragments thereof including aststtrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50, including comprising or as set out in any of SEQ ID NOS: 2, 3 and 4-9.

[00022] The invention provides methods for treating or alleviating a lysosomal storage disease in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including aststtrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50. The invention provides methods for treating or alleviating a lysosomal storage disease in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including aststtrin, wherein said PGRN or active fragment comprises an amino acid sequence including comprising or as set out in any of SEQ ID NOS: 2, 3 and 4-9. In an aspect of these methods, the method comprises additionally administering one or more
lysosomal enzyme which is reduced, absent, mutated or altered in the lysosomal storage disease. The lysosomal enzyme may be selected from one or more of a glucocerebrosidase, α-galactosidase, β-galactosidase, β-hexosaminidase and sphingomyelinase.

[00023] The lysosomal storage disease of the methods of the invention may be selected from Gaucher's Disease (GD), Tay-Sachs disease, Fabry disease, Farber disease, Sandhoff disease, G\textsubscript{M1} gangliosidosis, Krabbe disease, Niemann-Pick Disease (Type A, Type B, Type C), Pompe disease, mucolipidosis Type II (Hunter syndrome), mucolipidosis Type IIIA, infantile free sialic acid storage disease (ISSD), lysosomal acid lipase deficiency, Juvenile Hexosaminidase A deficiency, Wollman disease and Salla disease. In an aspect, the lysosomal storage disease of the methods of the invention may be selected from Gaucher's disease (GD), Tay-sachs disease (TSD), mucolipidosis (ML), mucopolysaccharidosis (MPS), metachromatic leukodystrophy (MLD), Farber disease (FD) and Krabbe disease (KD). In one aspect, the lysosomal storage disease of the methods of the invention may be selected from Gaucher's disease (GD) including GD Type I, II or III, Tay-Sachs disease (TSD), mucolipidosis (ML) including ML III, mucopolysaccharidosis (MPS) including MPS II, III, VI, metachromatic leukodystrophy (MLD), Farber disease (FD) and Krabbe disease (KD). In a particular preferred aspect of the methods of the invention, the lysosomal storage disease (LSD) is Gaucher's Disease (GD). In an aspect of the methods of the invention, the method comprise additionally administering the lysosomal enzyme glucocerebrosidase (GBA) or an active fragment or recombinant form thereof for treating or alleviating Gaucher's Disease. In a particular preferred aspect of the methods of the invention, the lysosomal storage disease (LSD) is Tay-Sachs disease.

[00024] The present invention provides methods and assays for diagnosing or evaluating lysosomal storage disease in an animal comprising determining the expression or activity of PGRN or detecting one or more mutation in the genomic DNA or gene encoding PGRN in said animal.

[00025] Any such methods or assays may comprise additionally determining the expression or activity of one or more lysosomal enzyme or detecting one or more mutation in the genomic DNA or gene encoding one or more lysosomal enzyme in said animal. In a particular aspect, a method or assay is provided for diagnosing or evaluating Gaucher's disease in an animal. In this aspect, the method or assay may comprise additionally determining the expression or activity of GBA or detecting one or more mutation in the genomic DNA or gene encoding GBA in said animal.
In accordance with the present invention, it has now been recognized and determined that lysosomal storage disease patients may and indeed often carry PGRN gene or protein mutations. Particularly prevalent PGRN gene variations include four SNP sites rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A. Diagnostic methods and assays of the invention include particularly wherein one or more of the PGRN mutations provided herein are assessed or determined. Methods, assays and kits are provided wherein one or more PGRN mutation selected from rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A is determined.

Particular such methods or kits, are wherein one or more PGRN mutation selected from rs4792937, rs850713, rs78403836, rs5848 is determined. In an aspect of the invention, the 4 PGRN SNP sites are determined by Taqman genotyping methods. In one such aspect, the methods or kits utilize exemplary primers including: rs4792937 forward primer, 5'-TGTCCCTGGAAACC ATCCCT-3' (SEQ ID NO: 11), reverse primer 5'-CTCCCCAAAGCGATTCTCCT A-3' (SEQ ID NO: 12), and Taqman tag sequence 5'-TCAGTAGCTACA[T/C]TTGTA-3'(SEQ ID NO: 13); rs850713 forward primer 5'-CCTTCCCTAGTGGGCTGGTA-3' (SEQ ID NO: 14), reverse primer 5'-AGTGCAACCTGTCTTCAACGC-3' (SEQ ID NO: 15), and Taqman tag sequence 5'-AGGTACAAATCTGGGAGATGGGG[G/A]TATGTGGGAGGGAAGTG GG GCCAGAG-3' (SEQ ID NO: 16); rs78403836 forward primer 5'-CTGTCCCTCTCCCATGGCTAC-3' (SEQ ID NO: 17), reverse primer 5'-GC GGACCTGT AAGCATGAAT-3' (SEQ ID NO: 18), and Taqman tag sequence 5'-AGGAAGAC[G/G]TATGTGGGAGGGAAGTG GG GCCAGAG-3' (SEQ ID NO: 20), reverse primer 5'-CACAGGGGTACCAATGTGGT-3' (SEQ ID NO: 21), and Taqman tag sequence TCTGCTCAGGCACTGGCCTATTCTCGACCTC[T/C]CCCTAACCAATTCTCCCTGGACCC (SEQ ID NO: 22). Point mutations of p.C315S, p.E316Q, and p.P365A may be amplified by recognized methods, including PCR, and may utilize exemplary primers including the forward primer 5'-G GTGGTGTGAAGCGGTACCCT-3' (SEQ ID NO: 23), reverse primer 5'-ACCTGCCCAGGCCAAGATGC-3' (SEQ ID NO: 24), followed by sequencing.

The invention includes kits for diagnosing or evaluating lysosomal storage disease in an animal by detecting the presence or activity and amount of PGRN comprising: (a) a predetermined amount of a detectably labelled specific binding partner of or antibody directed against PGRN; (b) other reagents; and (c) directions for use of said kit.
In an aspect, the invention includes kits for diagnosing or evaluating lysosomal storage disease in an animal by detecting the presence of a PGRN mutation in said animal comprising: (a) one or more nucleic acid probe or primer specific for or directed against the PGRN gene or encoding DNA; (b) other reagents; and (c) directions for use of said kit. An aspect of the kits is provided wherein one or more nucleic acid primer or probe is specific for or suitable for detection or determination of one or more of PGRN mutations rs4792937, rs850713, rs78403836, rs5848, and point mutations, p.C315S, p.E316Q, and p.P365A.

A kit of the invention may further comprise a detectably labelled specific binding partner of or antibody directed against GBA or one or more nucleic acid probe or primer specific for or directed against the GBA gene or encoding DNA.

In an assay, diagnostic method or kit of the invention, a control quantity of the PGRN, PGRN peptides, atstrin, GBA, or antibodies thereto, or the like may be prepared and labeled with an enzyme, a specific binding partner and/or a radioactive element, and may then be introduced into a cellular sample. After the labeled material or its binding partner(s) has had an opportunity to react with sites within the sample, the resulting mass may be examined by known techniques, which may vary with the nature of the label attached. In the instance where a radioactive label, such as the isotopes $^{3}$H, $^{14}$C, $^{32}$P, $^{35}$S, $^{36}$Cl, $^{51}$Cr, $^{57}$Co, $^{58}$Co, $^{59}$Fe, $^{90}$Y, $^{125}$I, $^{131}$I, and $^{186}$Re are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

The present invention provides an animal model for Gaucher’s Disease wherein the animal comprises altered PGRN, wherein PGRN is null, absent or mutated and lysosomal substrate β-GlcCer is increased in macrophages. The animal model may additionally comprise a GBA mutation associated with Gaucher’s Disease or wherein GBA is null or absent.

The invention includes an assay system for screening of potential drugs or compounds effective to modulate lysosomal enzyme trafficking and/or lysosomal substrate accumulation by mimicking the activity of PGRN or the PGRN peptides. This aspect includes assays to screen for additional active PGRN fragments, granulin/linker unit combinations, derivatives, variants and amino acid modifications effective to modulate lysosomal enzyme trafficking and/or lysosomal substrate accumulation in a like manner to PGRN and atstrin peptide. In one instance, the test drug or compound is administered to a cellular sample with GBA, to determine the effect of the...
test drug or compound upon β-GlcCer accumulation, by comparison with a control, including wherein the control is PGRN, active PGRN peptide(s), atstrin.

[00034] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[00035] FIGURE 1 depicts pathways for glycosphingolipids implicated in lysosomal storage diseases (LSD). Glycosphingolipid metabolism is a process mediated by multiple enzymes. Enzyme insufficiency causes accumulation of the corresponding substrate in lysosomes. Gaucher’s disease, the most common LSD, is caused by mutation of glucocerebrosidase (GBA). Mutation of GBA leads to the accumulation of the GBA substrate, β-glucosylceramide (β-GlcCer), in macrophages.

[00036] FIGURE 2A-2D. OVA-challenged PGRN KO mice develop Gaucher disease. WT and PGRN KO mice received LP. injection of OVA at Day 1 and 15, followed by intranasal challenge of 1% OVA at Day 29 for three times a week for four weeks. (A) H&E staining shows giant Gaucher cells in lung of both male and female PGRN KO mice, especially after OVA treatment. (n=10, 5 male and 5 female for each group). (B) Quantification of Gaucher cells in WT and PGRN KO mice, control and OVA challenged. (C) PAS staining of lung from WT and PGRN KO mice, control and OVA challenged. The results show the accumulation of glycolipid in Gaucher cells in PGRN KO mice. (D) Flow cytometry of cells isolated from bronchial alveolar lavage from WT and PGRN KO mice, control and OVA challenged. There is a subpopulation of giant macrophages in PGRN KO mice after OVA treatment, as evidenced by CD11b +FSC high.

[00037] FIGURE 3. Macrophage from PGRN KO mice is much larger than that in WT mice, and lysosome become tubular-like shape instead of regular round shape, assayed by transmission electronic microscope (EM) (upper and lower left: 2650 X; upper right: 11500 X, lower right: 7100 X).

[00038] FIGURE 4. β-GlcCer accumulates in PGRN KO mice. Lung tissue from WT and PGRN KO mice, with or without OVA challenge, was lysed and 1mg of protein of each sample was used for lipid composition analysis. The levels of β-GlcCer (pmol/mg protein) with different carbon chain lengths are graphed, as indicated.
FIGURE 5A-5D. (A) Imiglucerase alleviates lipid accumulation in PGRN KO mice. OVA-challenged PGRN KO mice were treated with imiglucerase (60u/kg) once a week starting at the week of first intranasal challenge with 1% OVA. H&E and PAS staining of lung tissues (n=6 for each group). (B) Imiglucerase treatment reduces β-GlcCer accumulation in OVA-challenged PGRN KO mice. Lung tissue from OVA-unchallenged (Ctrl), OVA-challenged PGRN KO mice treated with vehicle (OVA) or Imiglucerase (Imig,), was processed and analyzed as described in Figure 4. (C) Quantification of Gaucher cells in PGRN KO mice with or without imiglucerase treatment. (D) Sizes of the liver and spleen of PGRN KO mice induced by OVA challenge, with and without imiglucerase treatment. Both liver and spleen were significantly reduced following imiglucerase treatment. One-way ANOVA tests was used to compare means among groups (* p<0.05; ** p<0.01, two sided).

FIGURE 6A-6C. Aged PGRN null mice develop Gaucher’s disease spontaneously. 1 year-old WT and PGRN KO mice without any challenge were sacrificed and lung, spleen, liver, and bone marrow were collected for histology. (A) Aged PGRN KO mice develop hepatosplenomegaly. Liver and spleen weight divided by total animal body weight is graphed for WT and KO mice (n=8 per group). (B) Histology of lung, spleen and bone marrow. Gaucher cells were found in lung, spleen, and bone marrow in PGRN KO mice, but not in WT mice. (C) PAS staining of bone marrow shows glycolipid storage in Gaucher cells.

FIGURE 7A-7B. (A) Histology of bone marrow in WT and PGRN KO mice, BM were replaced by fat tissues in PGRN KO, but not in WT mice. (B) Gaucher cells under EM. Typical tubular-like lysosomes were founded in lung and bone marrow in PGRN KO mice. Non-paired student T-test were used to compare liver size and spleen size between WT and PGRN KO mice (* p<0.05).

FIGURE 8A-8C. rPGRN rescues the GD phenotype in vitro and in vivo. (A) β-GlcCer is accumulated in PGRN-deficient BMDM. BMDM from WT and PGRN KO mice were treated with lipid mixture at 5 and 5C^g/ml for 10 days. Accumulation of β-GlcCer was detected by immunofluorescence staining with specific antibodies against β-GlcCer and visualized by confocal microscope. (B) rPGRN prevents β-GlcCer accumulation in PGRN KO BMDM in dose-dependent manner. BMDM from PGRN KO mice were treated with lipid mixture in the presence of various amounts of PGRN, as indicated, or imiglucerase (Imig, 160mU/ml, serving as a positive control) for 10 days. β-GlcCer was stained by immunofluorescence and a representative image presented. (C) Quantification of β-GlcCer accumulation. Ten individual
images, as shown in panel B, were selected and fluorescence intensities measured by Image J software. Data are presented as mean values obtained from three independent experiments.

FIGURE 9. rPGRN prevents GBA aggregation and β-GlcCer accumulation in the fibroblasts from GD patients. Fibroblasts from type II GD patients were treated with lipid mixture in the presence or absence of rPGRN for 2 days, and levels of GBA (Green) and β-GlcCer (Red) were measured by immunofluorescence staining with their specific antibodies, the nuclei stained with DAPI, and images captured by co-focal microscope.

FIGURE 10A-IOC. (A) rPGRN prevents GD development in OVA-challenged PGRN null mice. PGRN KO mice were challenged by OVA, and treated with PBS or rPGRN (4mg/kg) once a week starting at the week of first intranasal challenge with OVA (n=6 per group). H&E staining of lung tissues reveals that rPGRN dramatically decreased Gaucher cells formation. (B) Quantification of Gaucher cells number from untreated, OVA-treated, and OVA+PGRN treated PGRN KO mice. (C) Quantification of Gaucher cell size from untreated, OVA-treated, and OVA+PGRN treated PGRN KO mice. One-way ANOVA was used to compare means among multiple groups (* p<0.05; ** p<0.01; two sided).

FIGURE 11A-1C. PGRN is required to deliver GBA and LIMP2 to lysosomes. (A) GBA enzyme activities are unchanged in PGRN KO vs. WT mice. Lung tissues from WT and PGRN KO mice after either PBS or OVA challenge were lysed, and GBA activity measured by cleavage of its substrate 4 MUGP. (B) GBA protein levels are not decreased, in fact are slightly increased, in KO vs. WT mice after PBS and OVA challenge. Lung tissues were lysed and the level of GBA was measured by Western blotting. (C) Distribution of GBA, but not GLA, is altered in PGRN KO macrophage. Paraffin-embedded lung slides were stained with GBA or GLA antibody by immunohistochemistry. The aggregation of GBA in PGRN KO macrophages is indicated with an arrow.

FIGURE 12A-12C. (A) Distribution of GBA is altered accompanied with β-GlcCer accumulation in PGRN KO mice. Frozen sections of lung tissue from OVA-challenged WT and PGRN KO mice were stained with GBA and β-GlcCer antibodies by immunofluorescence. The aggregation of GBA is indicated with an arrow. (B) GBA is aggregated in the cytoplasm in PGRN null macrophage, assayed by immunogold labeling of lung tissue. GBA is expressed in lysosome, indicated by an arrow, in WT macrophage (left panel 53,000 X), while GBA is absent in tubular-like lysosome, and GBA is aggregated in the macrophage of PGRN KO mice (right panel, 25,000 X). A aggregation region of denser immunogold labeling is circled with a dashed line. (C) Lysosomal GBA is undetectable in PGRN deficient macrophages, assayed with activity-
based probe MDW933. BMDMs from WT and PGRN KO mice prestimulated with lipid mixture was labelled with 50nM MDW933 for 2 hours, followed by fixation and DAPI staining, and the images were taken under confocal microscope.

[00047] FIGURE 13A-13D. (A) Expression of LIMP2 and LAMP2 in macrophages from WT and PGRN KO mice. Paraffin-embedded lung slides from WT and PGRN KO mice were stained with LIMP2 and LAMP2 by immunohistochemistry. Aggregation of LIMP2 in PGRN KO macrophages was indicated with an arrow. (B) LIMP2 is not detectable in lysosome, and aggregated in the cytoplasm in PGRN null macrophage, assayed by immunogold labeling of lung tissue. LIMP2 is detectable in the lysosome, indicated with an arrow, of WT macrophage (left panel, 53,000X), whereas it is aggregated in the cytoplasm of PGRN-null macrophage and not observed in the tubular-like lysosome (right panel 31,000X). A aggregation region of denser immunogold labelling is circled with a dashed line. (C) Aggregated GBA and aggregated LIMP2 co-localize in PGRN KO macrophages. Frozen sections of lung tissues of WT and PGRN KO mice were stained with GBA and LIMP2 antibodies, the distribution of GBA and LIMP2 was assayed by immunofluorescence staining and imaged by confocal microscope. The aggregated region is indicated with an arrow. (D) GBA binds to LIMP2 in the absence of PGRN in vivo, assayed by co-immunoprecipitation (Co-IP). Lung tissue from both WT and PGRN KO mice were lysed and protein complexes were immunoprecipitated with anti-GBA antibody and detected with anti-LIMP2 antibody.

[00048] FIGURE 14A-14C. Molecular pathway by which PGRN mediates lysosomal delivery of GBA. (A) PGRN binds to GBA in vivo, assayed by co-immunoprecipitation (Co-IP). Lung tissue from WT mice were lysed and protein complexes were immunoprecipitated with anti-GBA antibody and detected with anti-PGRN antibody. (B) PGRN directly binds to GBA in vitro, assayed by solid phase binding assay. Various amounts of PGRN were coated, and biotin-labeled BSA and GBA were added, followed by HRP-labeled Streptavidin and its substrates. (C) PGRN binds to GBA with a high affinity K_D of 0.71nM, assayed by surface plasmon resonance (SPR) with COOH1 chips.

[00049] FIGURE 15A-15D. (A) The scheme of the method used to identify potential molecules involved in PGRN-mediated delivery of GBA to lysosomes, i.e. PGRN-dependent GBA associated proteins. Immunoprecipitation was performed with GBA antibody from both WT and PGRN KO mice, followed by high-sensitivity mass spectrum. (B) Summary of the hits isolated from both WT and PGRN KO mice and the identification of HSP70-mediated trafficking as a PGRN-dependent GBA-associated pathway. (C) Binding of GBA to HSP70 is PGRN-
dependent. Immunoprecipitation was conducted with anti-GBA antibody in WT and PGRN KO mice, and probed with HSP70 antibody. (D) rPGRN restores the interaction between GBA and HSP70 in PGRN deficient mice in vivo. Lung tissue lysate prepared from OVA-unchallenged (Ctrl), OVA-challenged PGRN KO mice treated with or without rPGRN was immunoprecipitated with anti-GBA antibody, and the presence of HSP70 in immunoprecipitated complex probed with HSP70 antibody.

FIGURE 16A-16D. (A) Suppression of PGRN and HSP70 via a siRNA approach markedly reduces the lysosomal GBA in macrophage. RAW264.7 macrophages were transfected with siRNA specifically against PGRN or HSP70, and the cells pre-stimulated with lipid mixture were labelled with MDW933 probe for 2 hours, and the expression levels of PGRN and HSP70 measured by immunofluorescence staining. The cell transfected with corresponding siRNA is indicated with an arrow. (B) Binding of LIMP2 to HSP70 is also PGRN-dependent. Immunoprecipitation was conducted with anti-HSP70 antibody in WT and PGRN KO mice, and probed with LIMP2 antibody. (C) Binding of GBA, but not GLA, to Sortilin is PGRN-dependent. Immunoprecipitation was performed with anti-Sortilin antibody in WT and PGRN KO mice, and detected with either GBA or GLA antibody. (D) Binding of HSP70 to Sortilin is also PGRN-dependent. Immunoprecipitation was performed with anti-HSP70 antibody in WT and PGRN KO mice, and detected with Sortilin antibody.

FIGURE 17 depicts a proposed model explaining the role of PGRN in mediating the lysosomal delivery of GBA/LIMP2 through HSP70 chaperone pathway. Co stands for Co-chaperones.

FIGURE 18A-18B. Transmission electronic microscope assays of lung tissues from OVA-challenged WT and PGRN KO mice. (A) Colored and enlarged image of left bottom panel of Figure 3. Tubular-like lysosomes and mitochondria are shown in purple and orange, respectively (Thanks to Chris Petzold and Kristen Dancel at NYU Medical School OCS Microscopy Core for creating this colored image from the original black and white electronic microscope image). (B) Transformation of lysosome in PGRN null macrophages. 1, Lysosomes show elongated profiles associated with accumulation of material storage (19,500 X); 2, Lysosomes became curved-shape with both high dense and low dense material in the lysosomes (19,500 X); 3, Lysosome eventually became tubular-like structures with both high dense and low dense material storage (19,500 X); 4, Low dense material were eventually replaced with high dense material with intact membrane structure (110,000 X).
FIGURE 19A-19B. (A) Mitochondria and Endoplasmic reticulum were normal in PGRN null macrophages. Lung tissues from OVA-challenged WT and PGRN KO mice were examined under transmission electronic microscope. Mitochondria (Mit.) from WT (11500x) and PGRN KO (31000x), and endoplasmic reticulum (ER) from WT (4400x) and PGRN KO macrophages (7100 X) appeared normal. (B) Type 1 and 2 pneumocytes were normal in PGRN null mice. Lung tissues from OVA-challenged WT and PGRN KO mice were examined under transmission electronic microscope. Both type 1 and 2 pneumocytes from WT and PGRN KO mice appeared normal (all the images were amplified 3400x, except type 1 pneumocytes from PGRN KO was amplified at 2650x).

FIGURE 20A-20C. Lipid composition analyses in the tissues and plasma of PGRN KO mice and GD patients. The levels of diacylglycerol (DAG) (A), sphingomyelin (SM) (B), and ceramide (C) are not increased in the lung tissues from OVA-challenged PGRN KO mice compared to OVA-challenged WT mice. Levels of DAG, SM, and ceramide in wild type and PGRN KO animals with and without ovalbumin (OVA) challenge were measured, and levels of DAG, SM, and ceramide are indicated as pmol per mg protein.

FIGURE 21A-21D. Levels of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) in the fibroblasts and plasma of healthy control (Ctrl) and GD patients. The levels of GlcCer are significantly increased in GD fibroblasts (A) but not in GD plasma (B), whereas the levels of GlcSph are increased in GD plasma (D) but not in GD fibroblasts (C).

FIGURE 22A-22C. (A) The levels of GlcCer are not elevated in plasma of OVA-challenged PGRN KO mice compare to the OVA-challenged WT mice. (B-C) The levels of GlcSph are elevated in both lung tissues (B) and plasma (C) in OVA-challenged PGRN KO mice compare to the OVA-challenged WT mice.

FIGURE 23A-23C. The levels of GlcCer in plasma (A), lung (B) and spleen (C) are increased in aging of PGRN KO mice. 3 month-, 9 month-, and 15month-old PGRN KO mice (n=6 for each group) were sacrificed, and plasma, lung and spleen were used to measured β-GlcCer. Independent student-T test and one-way ANOVA were used for statistical analysis (*p<0.05; **p<0.01; two-sided).

FIGURE 24A-24H. Aged PGRN KO mice develop osteopenia in long bone. 1 year-old WT and PGRN KO mice were sacrificed and two femurs of each mice were dissected, one was for bone histology and the other one was for micro-CT. (A) Longitudinal sections of WT and PGRN KO femurs stained with H&E. Trabecular bone thickness and connectivity are decreased in the PGRN KO femur. Scale bars represent 0.5 mm. (B) Representative images of three-
dimensional micro-CT reconstructions of femur from aged WT and PGRN KO femurs. Histograms of 3-D trabecular structure parameters in the secondary spongiosa of the proximal femurs of WT and KO mice: BV/TV = bone volume/total volume (C); TbN = trabecular number (D); Tb.Th = trabecular thickness (E); Tb.Sp = trabecular separation (F). Cortical thickness (Crt.Th) (G). (H) Whole-body bone mineral density (BMD) as assessed by dual x-ray absorptiometry scanning.

FIGURE 25A-25C. Aged PGRN KO mice develop osteopenia in spine. Lumbar spine from aged WT and PGRN KO mice were dissected for micro-CT analysis. (A) Images of L4 vertebrae from WT and PGRN KO mice shows reduced bone mineral density. Bone volume (B) and thickness of trabecular bone (C) were significantly decreased in PGRN KO mice. Data are expressed as means ± S.D. (n=8 per group); Independent student-T test and one-way ANOVA were used for statistical analysis (*P<0.05; **P<0.01; ***P<0.001 versus the WT group; two-sided)

FIGURE 26. Giant macrophages were found in PGRN-deficient BMDM treated with lipid. BMDM were isolated and differentiated from WT and PGRN KO mice, and the cells were treated with lipid mixture at 5 and 5C^g/ml for 10 days. H&E staining of cultured BMDM. Giant BMDM are present in PGRN KO mice after lipid treatment.

FIGURE 27. PGRN reversed lipid-induced morphology changes of PGRN null BMDM. BMDM from PGRN KO mice were isolated and differentiated in vitro for 5 days. Then BMDM were treated with 50 µg/ml lipid mixture alone or with different doses of recombinant PGRN (0.1, 0.4 µg/ml) for 10 days, the cell culture medium were replenished every three days. The cell morphology was observed under phase-contrast microscope.

FIGURE 28. Distribution of Sortilin is normal in PGRN null macrophages. PGRN KO mice were challenged with OVA, and lung were fixed and processed for transmission electronic microscope. Expressions of Sortilin were stained by Sortilin antibody, followed by gold-labeled secondary antibody. The distribution of Sortilin is normal in Gaucher cells in PGRN KO mice, mainly expressed in tubular-like lysosomes (upper panel), and some expressed on the cell surface to mediate endocytosis (lower panel).

FIGURE 29. rPGRN was efficiently taken-up through endocytosis in BMDMs and blocking known PGRN's signal pathways, including ERK, PI3K and mTOR, did not affect rPGRN's effect on β-GlcCer clearance. Endocytosis of rPGRN in PGRN null BMDMs. BMDMs from PGRN KO mice were differentiated for 5 days as described in Materials and Methods. rPGRN were added in the culture medium at final concentration of 5µg/ml for different time
points, as indicated. The cells were fixed immediately at the end of each time points. The uptake and distribution of rPGRN were examined by immunofluorescence staining with PGRN antibody and antibodies against different molecules, as indicated. The images in the 1st row shows the endocytosis of rPGRN alone, and the images in the 2nd, the 3rd, the 4th and the 5th row reveal the co-localizations between uptaked rPGRN and EEA1, GBA, LIMP2, and LAMP2, respectively.

[00064] FIGURE 30. rPGRN’s effect on β-GlcCer clearance is independent of ERK, PI3K and mTOR signaling pathways. WT and PGRN KO BMDMs were stimulated lipid mixture (5 μg/ml) for 2 days, in the presence or absence of 0.4 μg/ml rPGRN protein with or without kinase inhibitors of ERK, PI3K and mTOR pathways, as indicated. The cells were labeled with MDW933 for 2 hours, then the cells were fixed and mounted with DAPI-medium. The MDW933-labelled lysosomal GBA was visualized by the intensity of green fluorescence under confocal microscope, and the accumulation of β-GlcCer was stained with β-GlcCer antibody (red) by immunofluorescence staining.

[00065] FIGURE 31. Co-localization of GBA and PGRN in the intracellular trafficking compartments in macrophages. BMDMs from WT mice were fixed and the expressions of PGRN and GBA, as well as the markers for the trafficking compartments were detected with respective specific primary antibodies, followed by corresponding secondary antibodies labeled with different fluorescence dyes. PGRN was stained with sheep anti-mouse PGRN primary antibody and secondary antibody labeled with Alexa-488 (a-f), GBA was stained with rabbit anti-mouse primary antibody, followed by Cy3-labeled secondary antibody (g-1), and the markers for the trafficking compartments (Calregulin, GM130, TGN38, EEA1, LIMP2, and LAMP2) were stained with corresponding mouse-originated primary antibodies, and followed by Alexa-647 labeled secondary antibodies (m-r). Panels s-x are the merged images of 4-color staining. The nucleus was stained with DAPI (blue).

[00066] FIGURE 32. Effects of pH on the binding of GBA to PGRN. (a) The direct interactions between GBA and PGRN at various pH, as indicated, were detected by SPR at SensiQ Technologies Inc. by using COOHV1 chip. (b) Effect of Assay pH on Kinetic Binding Values.

[00067] FIGURE 33A-33B. The effects of rPGRN on the diseased lysosomes in fibroblasts from various kinds of LSDs. Fibroblasts from healthy control and different LSDs were treated rPGRN protein (0.4 μg/ml), with or without lipid stimulation. The lysosomes were visualized by
Lysotracker (A). (B) PGRN significantly corrects altered lysosomes in type I and II GD, Tay-Sachs disease, Farber disease, and Mucolipidosis type III with or without lipid stimulation.

FIGURE 34A-34B. PGRN normalizes diseased lysosomes in type III GD, type III and VI mucopolysaccharidosis (MPS) only in the presence of lipid stimulation.

FIGURE 35A-35B. PGRN fails to revert lysosomes in Niemann-Pick disease type B (NPD B), Fabry disease, and type IV Mucolipidosis (ML) with or without lipid stimulation. Ten cells were randomly selected from every sample, and the fluorescence intensity of each cells were quantified by Image J software. Data are presented as mean values obtained from three independent experiments. The significance was tested by using one-way ANOVA (* p<0.05, ** p<0.01, two sided).

FIGURE 36A-36C. GD patients have decreased serum level of PGRN and GRN variants were identified in GD patients. (A) Serum levels of PGRN is significantly lower in GD patients. Serum levels of PGRN from 115 GD patients, 44 healthy controls from general population (GP), and 55 healthy control from Ashkenazi Jews (AJ) were measured by ELISA. GD patients have significant lower levels of PGRN (96.65 ± 53.45 ng/ml) compared to healthy controls of GP (164.99 ±34.16 ng/ml), and healthy controls of AJ (150.64 ±33.9 ng/ml). The significance was tested by using one-way ANOVA, (p<0.0001). (B) Summary of GRN gene variants in 40 GD patients. Genomic DNA of 40 GD patients were used to amplify a 9-kb GRN gene DNA fragment covering 1-kb promoter region and 8-kb full-length GRN gene by high-fidelity PCR. DNA sequencing was performed by PacBio RS II Sequencing System at Genomic facility at Yale University. 4 SNP sites and 3 point mutations were identified in GD patients. (C) A diagram showing the positions of GRN variants identified in GRN gene.

FIGURE 37. HexA, but not HexB were aggregated in PGRN KO mice after OVA challenge. Lung tissues from WT and PGRN KO mice after OVA challenge were stained with HexA and HexB by immunohistochemistry. The results show that HexA, but not HexB is aggregated in PGRN KO mice.

FIGURE 38. Aged PGRN KO mice have elevated expression of GM2 in brain. Brain tissue from aged WT and PGRN KO mice were stained with GM2 antibody by immunohistochemistry.

FIGURE 39 depicts that PGRN and Atsttrin, the PGRN-derived engineered protein, rescue β-GlcCer accumulation in PGRN KO macrophages. BMDM from PGRN KO mice were administered 50μg/ml lipid for 10 days, with or without various amounts of PGRN or Atsttrin, as indicated (100 ng/ml and 400 ng/ml PGRN or Atsttrin). Under light microscopy (LM), BMDM
become mess-like after lipid treatment, and this morphological change was partially rescued by both PGRN and Atstrin in dose-dependent manner (upper panel). β-GlcCer is accumulated with lipid treatment, and the accumulation is blocked by addition of recombinant PGRN or Atstrin in a dose-dependent manner.

[00074] FIGURE 40 shows that PGRN and Atstrin rescue β-GlcCer accumulation in fibroblasts from GD patients. Fibroblasts were isolated from Type II GD patients and treated with 50 µg/ml lipid mixture for 2 days. Expression of GBA and β-GlcCer were measured by immunofluorescence staining, and images were acquired by co-focal microscope. The lipid treatment significantly induced GBA aggregation and β-GlcCer accumulation in GD fibroblasts. 400ng/ml PGRN treatment almost completely prevents the GBA aggregation and β-GlcCer storage. Atstrin show some effect but less than PGRN.

[00075] FIGURE 41 shows that Atstrin rescues β-GlcCer accumulation in a dosage-dependent manner in fibroblasts from GD patients treated with lipid. Fibroblasts from GD patients were treated with 50 µg/ml lipid mixture, together with various amounts of Atstrin, as indicated (0, 0.5, 5 and 50 µg/ml Atstrin). Immunofluorescence staining for β-GlcCer is shown. Atstrin rescues β-GlcCer accumulation in a dose-dependent manner, particularly effective at 50 µg/ml.

[00076] FIGURE 42. PGRN and Atstrin have therapeutic effect to treat Gaucher’s disease. 8 week-old PGRN KO mice were induced Gaucher’s disease by challenging with OVA as described previously. Some groups of mice were were LP injected with recombinant PGRN protein, BSA, and Atstrin (4 mg/kg/week) for four weeks (n=8 per group). (A)The histology changes of lung tissues were examined by H&E staining. (B) Quantifications of Gaucher cell numbers. (C) Quantification of Gaucher cell sizes. (** p<0.01)

[00077] FIGURE 43. LSD fibroblasts that can be treated by both PGRN and Atstrin. LSD fibroblasts were challenged with lipid alone or with recombinant PGRN or Atstrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red. Ten images for each sample were randomly taken under co-focal microscope, and lysosome storage were quantified based on fluorescence intensity by image J software. GDI, GDII: Gaucher’s disease I and II; TSD: Tay-sachs disease; MLIII: mucolipidosis III. (** p<0.01)

[00078] FIGURE 44. LSD fibroblasts that can be treated by both PGRN and Atstrin. LSD fibroblasts were challenged with lipid alone or with recombinant PGRN or Atstrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red. Ten images for each sample were randomly taken under co-focal microscope, and lysosome storage were quantified
based on fluorescence intensity by image J software. MPSII, MPSIII, MPSVI: Mucopolysaccharidosis Type II, III, VI; MLD: metachromatic leukodystrophy; FD: Farber disease. (** p<0.01)

[00079] FIGURE 45. PGRN, but not Atsttrin rescues lysosome storage in GD III after lipid challenge. Fibroblasts from GD III were challenged with lipid alone or with recombinant PGRN or Atsttrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red. Ten images for each sample were randomly taken under co-focal microscope, and lysosome storage were quantified based on fluorescence intensity by image J software. (** p<0.01)

[00080] FIGURE 46. LSD fibroblasts that both PGRN and Atsttrin show minimal effect to reduce lysosome storages. LSD fibroblasts were challenged with lipid alone or with recombinant PGRN or Atsttrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red. Ten images for each sample were randomly taken under co-focal microscope, and lysosome storage were quantified based on fluorescence intensity by image J software. ML: mucolipidosis; MPS: mucopolysaccharidosis NPD: Niemann-Pick disease.

[00081] FIGURE 47. PGRN and Atsttrin rescue lysosome storage in Krabbe Disease. Fibroblasts from Krabbe Disease were challenged with lipid alone or with recombinant PGRN or Atsttrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red, and ten images were taken under co-focal microscope.

[00082] FIGURE 48 depicts PGRN protein structure with the Granulin units denoted.

[00083] FIGURE 49A and 49B depict the amino acid sequence of (A) human PGRN (SEQ ID NO: 2) and (B) mouse PGRN (SEQ ID NO: 3). In (B), the granulin units GrnA, GrnC, GrnD and GrnE are underlined and indicated at each unit.

[00084] FIGURE 50 depicts the sequence of atsttrin peptide (½F+P3+P4+½A+P5+½C) (SEQ ID NO: 4), and sequences of various other PGRN peptides (SEQ ID NOS: 5-9).

[00085] FIGURE 51 provides the 8kb PGRN human gene nucleic acid sequence (SEQ ID NO: 10). PGRN mRNA starts at nucleotide 101.

DETAILED DESCRIPTION

[00086] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III
Therefore, if appearing herein, the following terms shall have the definitions set out below.

The terms "progranulin", "PGRN", "granulin-epithelin precursor", "GEP", "PC-cell-derived growth factor", "PCDGF", "proepithelin", "acrogranin", and "GP80" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and active fragments thereof and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 49, including as set out in SEQ ID NO: 2 and SEQ ID NO: 3, and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "progranulin", "PGRN", "granulin-epithelin precursor", "GEP", "PC-cell-derived growth factor", "PCDGF", "proepithelin", "acrogranin", and "GP80" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The terms "granulin(s)", "epithelins" or any of "Granulins A-E", "GrnA", "GrnB", "GrnC", "GrnD", "GrnE" refer to particular cysteine rich motifs, of approximately 6kDa in size, including comprising or having the sequence motif  \( CX_{5-6}CX_5CCX_5CCX_6CCX_6DX_2HCCPX_4CX_{5-6}C \) (SEQ ID NO: 1), which granulins can be released by proteolytic processing from the GEP polypeptide molecule. These granulin(s) may retain biological activity and be active in activity assays, including in cell growth assays, enzyme substrate accumulation assays, protein binding including GBA, sortilin and/or HSP70 binding, GBA and/or other lysosomal enzyme processing or delivery to the lysosome, and assessment for Gaucher type cells. The granulins can provide active fragments of PGRN. Exemplary granulin sequences include those proteins having the amino acid sequence data described herein and presented in FIGURE 49 or 50 or fragments thereof, and the profile of activities set forth herein. Accordingly, proteins displaying
substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "granulin(s)", "epithelins" or any of "Granulins A-E", "GrnA", "GrnB", "GrnC", "GrnD", "GrnE" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

[00090] The terms "Atsttrin", "Antagonist of TNF/TNFR Signaling via Targeting TNF Receptors ", "atsttrin peptide" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to peptides including single or multiple proteins, particularly which are derived from or fragments of PGRN and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 50 and also diagrammed in FIGURE 48, and as set out in SEQ ID NO: 4, and the profile of activities and capabilities described and set forth herein and provided in the Claims. Active PGRN peptides having activity in facilitating enzyme delivery to the lysosome, and/or binding or complexing with lysosomal enzymes such as glycoscerbrosidase (GBA), or with sortilin and/or HSP70, are included and provided herein. These active PGRN peptides may retain biological activity and be active in activity assays, including in cell growth assays, enzyme substrate accumulation assays, protein binding including GBA, sortilin and/or HSP70 binding, GBA and/or other lysosomal enzyme processing or delivery to the lysosome, and assessment for Gaucher type cells. The full length sequence of human PGRN and of mouse PGRN is provided in FIGURE 49A and 49B respectively (SEQ ID NO: 2 and 3). Thus, lysosomal enzyme binding peptides, including GBA-binding peptides, derived from PGRN sequences(s) or comprising PGRN sequence(s), particularly including Atsttrin or atsttrin derived sequences, and having activity in binding to and/or facilitating delivery of enzymes to the lysosome are encompassed herein. These atsttrin peptides include and encompass fragments, variants, and derivatives of the peptides. Accordingly, proteins displaying substantially equivalent activity, and which are modifications thereof, are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Corresponding mouse or other species or ortholog PGRN sequences to the human atsttrin and active PGRN peptide sequences are further contemplated. Also, the terms "Atsttrin", "Antagonist of TNF/TNFR Signaling via Targeting TNF Receptors ", "atsttrin peptide", are
intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

[00091] The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

**TABLE OF CORRESPONDENCE**

<table>
<thead>
<tr>
<th>SYMBOL</th>
<th>AMINO ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Tyr</td>
</tr>
<tr>
<td>G</td>
<td>Gly</td>
</tr>
<tr>
<td>F</td>
<td>Phe</td>
</tr>
<tr>
<td>M</td>
<td>Met</td>
</tr>
<tr>
<td>A</td>
<td>Ala</td>
</tr>
<tr>
<td>S</td>
<td>Ser</td>
</tr>
<tr>
<td>I</td>
<td>Ile</td>
</tr>
<tr>
<td>L</td>
<td>Leu</td>
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<tr>
<td>T</td>
<td>Thr</td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
</tr>
<tr>
<td>P</td>
<td>Pro</td>
</tr>
<tr>
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<td>Lys</td>
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<tr>
<td>H</td>
<td>His</td>
</tr>
<tr>
<td>Q</td>
<td>Gin</td>
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<tr>
<td>E</td>
<td>Glu</td>
</tr>
<tr>
<td>W</td>
<td>Trp</td>
</tr>
<tr>
<td>R</td>
<td>Arg</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
</tr>
<tr>
<td>N</td>
<td>Asn</td>
</tr>
<tr>
<td>C</td>
<td>Cys</td>
</tr>
</tbody>
</table>
It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.
A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3’ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3’ terminus by the transcription initiation site and extends upstream (5’ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease SI), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications,
depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

[000104] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[000105] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

[000106] A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

[000107] Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al, supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.
It should be appreciated that also within the scope of the present invention are DNA sequences encoding PGRN (including SEQ ID NO: 10), PGRN-derived peptide Atsttrin, or other PGRN peptide, including or comprising one or more granulin, which code for a peptide having the same amino acid sequence as PGRN or a PGRN peptide, including as set out in any of the Figures 49 or 50 and SEQ ID NOs: 2, 3 and 4-9 associated therewith, but which are degenerate to any such sequences. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

- Phenylalanine (Phe or F) UUU or UUC
- Leucine (Leu or L) UUA or UUG or CUU or CUA or CUG
- Isoleucine (Ile or I) AUU or AUC or AUA
- Methionine (Met or M) AUG
- Valine (Val or V) GUU or GUC of GUA or GUG
- Serine (Ser or S) UCU or UCC or UCA or UCG or AGU or AGC
- Proline (Pro or P) CCU or CCC or CCA or CCG
- Threonine (Thr or T) ACU or ACC or ACA or ACG
- Alanine (Ala or A) GCU or GCG or GCA or GCG
- Tyrosine (Tyr or Y) UAU or UAC
- Histidine (His or H) CAU or CAC
- Glutamine (Gin or Q) CAA or CAG
- Asparagine (Asn or N) AAU or AAC
- Lysine (Lys or K) AAA or AAG
- Aspartic Acid (Asp or D) GAU or GAC
- Glutamic Acid (Glu or E) GAA or GAG
- Cysteine (Cys or C) UGU or UGC
- Arginine (Arg or R) CGU or CGC or CGA or CGG or AGA or AGG
- Glycine (Gly or G) GGU or GGC or GGA or GGG
- Tryptophan (Trp or W) UGG
- Termination codon UAA (ochre) or UAG (amber) or UGA (opal)

It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

Mutations can be made in the PGRN, PGRN-derived peptide Atsttrin, or PGRN peptide(s) sequence(s) such that a particular codon is changed to a codon which codes for a
different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein.

[000110] The following is one example of various groupings of amino acids, based on their R groups: Amino acids with nonpolar R groups: Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine; Amino acids with uncharged polar R groups: Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine; Amino acids with charged polar R groups (negatively charged at pH 6.0): Aspartic acid, Glutamic acid; Basic amino acids (positively charged at pH 6.0): Lysine, Arginine, Histidine (at pH 6.0). Another grouping may be those amino acids with phenyl groups: Phenylalanine, Tryptophan, and Tyrosine. Another grouping may be according to molecular weight (i.e., size of R groups):

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>75</td>
</tr>
<tr>
<td>Alanine</td>
<td>89</td>
</tr>
<tr>
<td>Serine</td>
<td>105</td>
</tr>
<tr>
<td>Proline</td>
<td>115</td>
</tr>
<tr>
<td>Valine</td>
<td>117</td>
</tr>
<tr>
<td>Threonine</td>
<td>119</td>
</tr>
<tr>
<td>Cysteine</td>
<td>121</td>
</tr>
<tr>
<td>Leucine</td>
<td>131</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>131</td>
</tr>
<tr>
<td>Asparagine</td>
<td>132</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>133</td>
</tr>
<tr>
<td>Glutamine</td>
<td>146</td>
</tr>
</tbody>
</table>
Particularly preferred substitutions are:
- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gin for Asn such that a free NH₂ can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA.
sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[000116] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5xSSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined Tm with washes of higher stringency, if desired.

[000117] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "µg" mean microgram, "mg" means milligram, "ul" or "µl" mean microliter, "ml" means milliliter, "l" means liter.

[000118] An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567.

[000119] An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

[000120] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[000121] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')2 and F(v), which portions are preferred for use in the therapeutic methods described herein. Fab and F(ab')2
portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. Fab' antibody molecule portions are also well-known and are produced from F(ab') 2 portions followed by reduction of the disulfide bonds linking the two heavy chain portions with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

The phrase "pharmacologically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

The term "therapeutically effective amount" means that amount of a drug, compound, peptide, or pharmaceutical agent that will elicit the biological, physiological, clinical, or medical response of a subject that is being sought by a medical doctor or other clinician. The phrase "therapeutically effective amount" is used herein to include an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in the S phase activity of a target cellular mass, in the enlargement of an organ, in the accumulation of a substrate or protein, in a neurological deficit or impairment, or other feature of pathology such as for example, elevated blood pressure, fever or white cell count, enlargement of the spleen or liver as may attend its presence and activity.

The term "preventing" or "prevention" refers to a reduction in risk of acquiring or developing a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop) in a subject that may be exposed to a disease-causing agent, or predisposed to the disease in advance of disease onset.

The term "prophylaxis" is related to and encompassed in the term "prevention", and refers to a measure or procedure the purpose of which is to prevent, rather than to treat or cure a disease. Non-limiting examples of prophylactic measures may include the administration of vaccines; the administration of low molecular weight heparin to hospital patients at risk for
thrombosis due, for example, to immobilization; and the administration of an anti-malarial agent such as chloroquine, in advance of a visit to a geographical region where malaria is endemic or the risk of contracting malaria is high.

[000127] The term "solvette" means a physical association of a compound useful in this invention with one or more solvent molecules. This physical association includes hydrogen bonding. In certain instances the solvate will be capable of isolation, for example when one or more solvent molecules are incorporated in the crystal lattice of the crystalline solid. "Solvate" encompasses both solution-phase and isolable solvates. Representative solvates include hydrates, ethanolates and methanolates.

[000128] The term "subject" includes humans and other mammals.

[000129] The term "treating" or "treatment" of any disease or disorder refers, in one embodiment, to ameliorating the disease or disorder (i.e., arresting the disease or reducing the manifestation, extent or severity of at least one of the clinical symptoms thereof). In another embodiment 'treating' or 'treatment' refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, 'treating' or 'treatment' refers to modulating the disease or disorder, either physically, (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. In a further embodiment, 'treating' or 'treatment' relates to slowing the progression of the disease.

[000130] The term "lysosomal storage disease(s)" "LSD" refers to a heterogeneous group of diseases or disorders characterized by the accumulation of undigested or partially digested macromolecules, which ultimately results in cellular dysfunction and clinical abnormalities. LSDs result from gene mutations in one or more of lysosomal enzymes, resulting in accumulation of the enzyme substrates in lysosomes, ultimately leading in many instances to organomegaly, connective-tissue and ocular pathology, and central nervous system dysfunction. Lysosomal storage disease(s) include sphingolipidoses, gangliosidosis, mucopolysaccharidoses, glycoprotein storage diseases, mucolipidoses. The term includes, but is not limited to, exemplary diseases selected from Gaucher’s Disease (GD), Tay-Sachs disease, Fabry disease, Farber disease, Sandhoff disease, G_{MI} gangliosidosis, Krabbe disease, Niemann-Pick Disease (Type A, Type B, Type C), Pompe disease, mucolipidosis Type II (Hunter syndrome), mucolipidosis Type IIIA, infantile free sialic acid storage disease (ISSD), lysosomal acid lipase deficiency, Juvenile Hexosaminidase A deficiency, Wollman disease and Salla disease. In a particular aspect a preferred lysosomal storage disease is Gaucher’s Disease, including Type I, Type II and/or Type III Gaucher’s Disease.
The term "Gaucher's Disease", "GD", refers to the most common of the lysosomal storage diseases, Gaucher's Disease. Gaucher's disease involves dysfunctional metabolism of sphingolipids and classically results from hereditary deficiency of the enzyme glucocerebrosidase (GBA).

The present invention demonstrates that the protein Progranulin PGRN plays an important and critical role in the transport of lysosomal enzymes to the lysosome. As such, PGRN, and PGRN-derived active peptides, including atsttrin, have a therapeutic, prophylactic, and diagnostic use and application in lysosomal storage diseases and disorders. PGRN is demonstrated herein to bind to lysosomal enzymes, including particularly to galactocerebrosidase (GBA). PGRN and particularly PGRN/lysosomal enzyme complexes, such as PGRN/GBA complexes, bind to lysosomal/endoosomal trafficking and sorting proteins, including sortilin and HSP70. Lysosomal storage disease, including Gaucher's disease, develops in the absence of PGRN or with mutated PGRN, such as in PGRN knockout (KO) animals. Over 70% of GD patients also have mutations in PGRN. Thus, PGRN and PGRN-derived active peptides including atsttrin, are applicable for diagnosis, amelioration and therapy in lysosomal storage disease(s), including Gaucher's Disease.

The invention includes use and applications of PGRN, PGRN peptides, atsttrin, and/or active derivatives thereof for prevention, treatment or alleviation of lysosomal storage disease or disorders (LSD). The invention includes use and applications of PGRN, PGRN peptides, atsttrin, and/or derivatives thereof for prevention, treatment or alleviation of lysosomal storage diseases, including conditions, symptoms and clinical manifestations of accumulation of substrates and/or molecules in lysosomes. Lysosomal storage diseases include sphingolipidoses, gangliosidosis, mucopolysaccharidoses, glycoprotein storage diseases, mucolipidoses and exemplary diseases selected from Gaucher's Disease (GD), Tay-Sachs disease, Fabry disease, Farber disease, Sandhoff disease, G* M1 gangliosidosis, Krabbe disease, Niemann-Pick Disease (Type A, Type B, Type C), Pompe disease, mucolipidosis Type II (Hunter syndrome), mucolipidosis Type IIIA, infantile free sialic acid storage disease (ISSD), lysosomal acid lipase deficiency, Juvenile Hexosaminidase A deficiency, Wollman disease and Salla disease. The invention includes use and applications of GEP, GEP peptides, atsttrin, and/or derivatives thereof for prevention, treatment or alleviation of and/or for specific therapeutic intervention of lysosomal storage disorders by facilitating delivering of required or relevant lysosomal agents, enzymes and/or other molecules to the lysosome.
The possibilities both diagnostic and therapeutic that are raised by the existence of lysosomal protein/ enzyme binding peptides, including PGRN, PGRN peptides and/or atsttrin as described herein, derive from the fact that the peptides participate in direct and causal protein-protein interaction with lysosomal protein(s)/enzyme(s), such as glucocerebrosidase (GBA), and serve to initiate, facilitate, mediate or are required for the transport and/or trafficking of lysosomal protein(s)/enzyme(s), such as glucocerebrosidase (GBA), to the lysosome where they are required for activity to maintain the lysosomal compartment and overall proper and effective protein trafficking and degradation. Thus, the present invention contemplates pharmaceutical intervention in the trafficking and delivery of required enzymes/proteins in and to the lysosome and proper lysosomal function to modulate, alleviate, prevent or treat lysosomal storage diseases or disorders and any other conditions which are associated with altered or insufficient trafficking of proteins and enzymes to the lysosome or endosome.

The invention provides PGRN and PGRN peptides, particularly including the peptide(s) denoted atsttrin, as modulators of lysosomal storage disease and of lysosomal trafficking. In particular, the invention provides PGRN and PGRN peptides, including atsttrin, as facilitators of lysosomal enzyme trafficking to the lysosome. In a particular embodiment, the present invention relates to all members of the herein disclosed family of PGRN peptides and of atsttrin, which are capable of facilitating enzyme delivery to the lysosome, and/or binding or complexing with lysosomal enzymes such as glycocerebrosidase (GBA), or with sortilin and/or HSP70. The family of peptides includes fragments or portions, including mixed portions of PGRN sequence and half units, particularly comprising one or more granulin unit and one or more linker unit of PGRN. In one aspect the peptide comprises two or more half units of granulin units and one or more linker unit of PGRN. In a particular aspect of the invention, the PGRN peptide comprises the peptide atsttrin, comprising combinations of half units of granulin units A, C and F in combination with linker units P3, P4 and P5. In a particular aspect, the GEP peptide comprises a combination of half units of granulin units, wherein at least one half unit is ½ F, and linker units, particularly at least two linker units. In a further particular aspect atsttrin has the amino acid sequence set out in FIGURE 50 and SEQ ID NO: 4 and comprises granulin units and linker units ½F-P3-P4-½A-P5-½ C, including as set out herein.

It is an object of the present invention to provide pharmaceutical compositions for use in therapeutic methods which comprise or are based upon the PGRN peptides and/or atsttrin. The pharmaceutical compositions include combinations of one or more PGRN peptides and/or atsttrin which are capable of facilitating enzyme delivery to the lysosome, and/or binding or
complexing with lysosomal enzymes such as glycocerebrosidase (GBA), or with sortilin and/or HSP70, and/or capable of reducing lysosomal substrate accumulation, such as β-GleCer, in the lysosome or macrophage. The pharmaceutical compositions include combinations of one or more PGRN peptides and/or atsttrin having activity as provided herein and one or more lysosomal enzyme or lysosomal substrate reducing agent. Lysosomal enzymes or lysosomal substrate reducing agents include and may be selected from one or more of glucocerebrosidase, α-galactosidase, β-galactosidase, β-hexosaminidase and sphingomyelinase. The pharmaceutical compositions include combinations of one or more PGRN peptides and/or atsttrin having GBA binding activity and one or more of Imiglucerase, Velaglucerase alfa, Taliglucerase alpha, Miglusta and Isofagomine tartrate.

[000137] Thus, the invention provides a composition for treatment or alleviation of a lysosomal storage disease comprising isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50 and in SEQ ID NOS: 2, 3 or 4-9. In an aspect, the invention provides a composition for treatment or alleviation of a lysosomal storage disease comprising isolated PGRN, wherein said PGRN comprises an amino acid sequence having at least one amino acid substitution, deletion or addition in comparison to the sequence as set out in FIGURE 49 and in SEQ ID NOS: 2 and/or 3. Thus, in one aspect the PGRN of use in the invention has at least one amino acid difference versus wild type or natural human or mouse PGRN. In one such aspect, compositions are provided comprising PGRN, a PGRN peptide, or atsttrin in combination with glucocerebrosidase for treatment or alleviation of Gaucher’s Disease. In an aspect, compositions of the invention may further comprise one or more molecular chaperone or lysosomal delivery protein, including HSP70 and/or sortilin.

[000138] Compositions of the invention include pharmaceutical compositions further comprising a pharmaceutically acceptable carrier, vehicle, diluent or excipient. The PGRN, PGRN peptides and/or atsttrin as described herein, may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient experiencing an adverse medical condition associated with altered or ineffective lysosomal processing or lysosomal enzyme(s), particularly any of a lysosomal storage disease or associated condition, particularly Gaucher’s Disease. A variety of administrative techniques may be utilized, among them parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like. Average quantities of the PGRN, PGRN peptides and/or atsttrin as described herein or their subunits may vary and in particular
should be based upon the recommendations and prescription of a qualified physician or veterinarian.

[000139] The peptides and compositions of the invention include those PGRN peptides, including atsttrin, which are based on the human PGRN sequence, including as set out in FIGURES 49 and 50, including SEQ ID NOS: 2, 3 and 4-9, as well as variants thereof having one or more or a few or many substitutions, wherein the binding and activity profiles of the variant(s) are retained when compared to PGRN, PGRN peptide or the atsttrin peptide. In as much as PGRN peptides from various animals or mammals, including humans, are known, these sequences provide alternative amino acid sequences and variants of potential use in the compositions and methods of the invention, including by substitution of some of the atsttrin human peptide amino acids. Mouse GEP sequence is provided herein in FIGURE 49B (SEQ ID NO: 2). PGRN sequences for various animals are publicly known and disclosed and would be available for evaluation and assessment in the methods and compositions of the invention, and their corresponding and correlating amino acids suitable for evaluation and use as variants of the PGRN peptides herein. PGRN sequences are available and herein incorporated by reference as follows: rat (Genbank accession AAA16903.1, CAA44198.1), mouse (Genbank accession P28798.2, BAE35389.1, NP_032201.2), Sumatran orangutan (Genbank accession NP_00126689.1), crab-eating macaque (Genbank accession BAE01796.1), horse (Genbank accession XP_001489791.1), cattle (Genbank accession NP_001070482.1), rabbit (Genbank accession XP_002719228.1), pig (Genbank accession NP_001038043.1), chimpanzee (Genbank accession XP_511549.2) and opossum (Genbank accession XP_001374870.1).

[000140] Also, antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the production or activity of PGRN and/or PGRN peptides and/or their subunits may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring conditions associated with or resulting from altered PGRN, lysosomal storage diseases, Gaucher’s disease. For example, the PGRN, atsttrin or its subunits may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Likewise, small molecules that mimic or antagonize the activity(ies) of the PGRN of the invention, particularly those which demonstrate binding to lysosomal enzyme, such as binding to GBA, may be discovered or synthesized and may be used in diagnostic and/or therapeutic protocols.
The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against PGRN peptides can be screened for various properties; i.e., isotype, epitope, affinity, etc. Of particular interest are monoclonal antibodies that neutralize the activity of the PGRN or its subunits or that bind to GBA. Such monoclonals can be readily identified in binding or activity assays. Preferably, the anti-PGRN antibody used in the diagnostic methods of this invention is an affinity purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-PGRN antibody molecules used herein be in the form of Fab, Fab', F(ab')₂ or F(v) portions of whole antibody molecules.

The invention provides therapeutic methods based upon the activity of PGRN, PGRN peptides and/or atstrrin, or active fragments thereof, in facilitating enzyme delivery to the lysosome, and/or binding or complexing with lysosomal enzymes such as glycosphingolipid β-glucocerebrosidase (GBA), or with sortilin and/or HSP70, and/or being capable of reducing lysosomal substrate accumulation, such as β-GlcCer, in the lysosome or macrophage.

Methods are thus provided for facilitating lysosomal delivery of a protein or enzyme in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including atstrrin. In an aspect thereof said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50, including SEQ ID NOS: 2, 3 and 4-9. Methods include methods for treating or alleviating a lysosomal storage disease in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including atstrrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50. In an aspect of these methods, the method comprises additionally administering one or more lysosomal enzyme which is reduced, absent, mutated or altered in the lysosomal storage disease. The lysosomal enzyme may be selected from one or more of a glucocerebrosidase, α-galactosidase, β-galactosidase, β-hexosaminidase and sphingomyelinase. The lysosomal storage disease of the methods of the invention may be selected from Gaucher’s Disease (GD), Tay-Sachs disease, Fabry disease, Farber disease, Sandhoff disease, GM1 gangliosidosis, Krabbe disease, Niemann-Pick Disease (Type A, Type B, Type C), Pompe disease, mucolipidosis Type II (Hunter syndrome), mucolipidosis Type IIIA, infantile free sialic acid storage disease (ISSD), lysosomal acid lipase deficiency, Juvenile Hexosaminidase A deficiency, Wollman disease and Salla disease. In an aspect, the lysosomal storage disease of the
methods of the invention may be selected from Gaucher's disease (GD), Tay-sachs disease (TSD), mucolipidosis (ML), mucopolysaccharidosis (MPS), metachromatic leukodystrophy (MLD), Farber disease (FD) and Krabbe disease (KD). In one aspect, the lysosomal storage disease of the methods of the invention may be selected from Gaucher's disease (GD) including GD Type I, II or III, Tay-Sachs disease (TSD), mucolipidosis (ML) including ML III, mucopolysaccharidosis (MPS) including MPS II, III, VI, metachromatic leukodystrophy (MLD), Farber disease (FD) and Krabbe disease (KD). In a particular preferred aspect of the methods of the invention, the lysosomal storage disease (LSD) is Gaucher's Disease (GD). In a particular preferred aspect of the methods of the invention, the lysosomal storage disease (LSD) is Gaucher's Disease (GD). In an aspect of the methods of the invention, the method comprise additionally administering the lysosomal enzyme glucocerebrosidase (GBA) or an active fragment or recombinant form thereof for treating or alleviating Gaucher's Disease. In a particular preferred aspect of the methods of the invention, the lysosomal storage disease (LSD) is Tay-Sachs Disease (TSD).

[000144] With regard to the lysosomal storage disease, Gaucher's disease, methods are provided for facilitating delivery of glucocerebrosidase (GBA) in a patient with Gaucher's Disease comprising administering to said patient isolated PGRN, or active fragments thereof including atsstrin. The PGRN or active fragment may particularly comprise an amino acid sequence as set out in any of FIGURES 49 or 50, including SEQ ID NOS: 2, 3 and 4-9. For Gaucher's Disease in humans, the human PGRN protein or a peptide thereof may particularly be utilized, and may optionally be combined with recombinant human glucocerebrosidase or GBA, such as imiglucerase.

[000145] Methods and assays of the invention include methods and assays for diagnosing or evaluating lysosomal storage disease in an animal comprising determining the expression or activity of PGRN or detecting one or more mutation in the genomic DNA or gene encoding PGRN in said animal. Any such methods or assays may comprise additionally determining the expression or activity of one or more lysosomal enzyme or detecting one or more mutation in the genomic DNA or gene encoding one or more lysosomal enzyme in said animal. A method or assay is provided for diagnosing or evaluating Gaucher's disease in an animal. In this aspect, the method or assay may comprise additionally determining the expression or activity of GBA or detecting one or more mutation in the genomic DNA or gene encoding GBA in said animal.

[000146] In accordance with the present invention, it has now been recognized and determined that lysosomal storage disease patients may and indeed often carry PGRN gene or protein
mutations. Particularly prevalent PGRN gene mutations include rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A. Diagnostic methods and assays of the invention include particularly wherein one or more of the PGRN mutations provided herein are assessed or determined. Methods, assays and kits are provided wherein one or more PGRN mutation selected from rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365 are determined.

Particular such methods or kits, are wherein one or more PGRN mutation selected from rs4792937, rs850713, rs78403836, rs5848 is determined. In an aspect, the 4 PGRN SNP sites are determined by Tagman genotyping methods including the following exemplary primers: rs4792937 forward primer, 5'-TGTCCCTGGAAA CCATCCTTC-3' (SEQ ID NO: 11), reverse primer 5'-CTCCCCAACGGATTCTCCTA-3' (SEQ ID NO: 12), and Taqman tag sequence 5'-TCAGTAGCTCACA[T/C][TGTAA-3' (SEQ ID NO: 13); rs850713 forward primer 5'-CCTTCCCTGAGTGGGCTGGTA-3' (SEQ ID NO: 14), reverse primer 5'-AGTGCCACCTGTCTCCACAG C-3' (SEQ ID NO: 15), and Taqman tag sequence 5'-AGGTACAAATCTGGGGGAGATGGG[A/G]TATGGGAGGGAAGTG GG GCCAGAG-3' (SEQ ID NO: 16); rs78403836 forward primer 5'-CTGTCCTCTCCATGGCTAC-3' (SEQ ID NO: 17), reverse primer 5'-GGAGCCTTGAAGGATGAAT-3' (SEQ ID NO: 18), and Tagman tag sequence 5'-AGGAAGAC [G/C]TGATTTT-3' (SEQ ID NO: 19); rs5848 forward primer 5'-CCAGGGGTAACAGTGTTTG-3' (SEQ ID NO: 20), reverse primer 5'-CACAGGGTCCACTGAAACG-3' (SEQ ID NO: 21), and Taqman tag sequence TCTGCTCAGGCTCCCTGCCTAGC ACCTC[C/T]CCCTAAACCAAATTCTCCCTGGACCC (SEQ ID NO: 22). In an aspect, point mutations of p.C315S, p.E316Q, and p.P365A are amplified by PCR, and the forward primer 5'-GTTGGGTGTAAGGGTACCCT-3' (SEQ ID NO: 23), reverse primer 5'-ACCTGGCAGGCGCAGATGC-3' (SEQ ID NO: 24), followed by sequencing.

Kits are provided herein for diagnosing or evaluating lysosomal storage disease in an animal by detecting the presence or activity and amount of PGRN comprising: (a) a predetermined amount of a detectably labelled specific binding partner of or antibody directed against PGRN; (b) other reagents; and (c) directions for use of said kit. Such kits include kits for diagnosing or evaluating lysosomal storage disease in an animal by detecting the presence of a PGRN mutation in said animal comprising: (a) one or more nucleic acid probe or primer specific for or directed against the PGRN gene or encoding DNA; (b) other reagents; and(c) directions for use of said kit. An aspect of the kits is provided wherein one or more nucleic acid primer or probe is specific for or suitable for detection or determination of one or more PRN
mutation selected from rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A.

[000149] A kit of the invention may further comprise a detectably labelled specific binding partner of or antibody directed against GBA or one or more nucleic acid probe or primer specific for or directed against the GBA gene or encoding DNA.

[000150] In an assay, diagnostic method or kit of the invention, a control quantity of the PGRN, PGRN peptides, atsttrin, GBA, or antibodies thereto, or the like may be prepared and labeled with an enzyme, a specific binding partner and/or a radioactive element, and may then be introduced into a cellular sample. After the labeled material or its binding partner(s) has had an opportunity to react with sites within the sample, the resulting mass may be examined by known techniques, which may vary with the nature of the label attached. In the instance where a radioactive label, such as the isotopes $^3$H, $^{14}$C, $^{32}$P, $^{35}$S, $^{36}$Cl, $^{51}$Cr, $^{57}$Co, $^{58}$Co, $^{59}$Fe, $^{90}$Y, $^{125}$I, $^{131}$I, and $^{186}$Re are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

[000151] As suggested earlier, the diagnostic method of the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of an antagonist to PGRN protein or a peptide thereof including atsttrin, such as an anti-PGRN antibody, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the antibody molecules used herein be in the form of Fab, Fab', F(ab')$_2$ or F(v) portions or whole antibody molecules. As previously discussed, patients capable of benefiting from this method include those suffering from a lysosomal storage disease or Gaucher's disease. Methods for isolating the antibody and inducing anti-PGRN antibodies and for determining and optimizing the ability of anti-PGRN antibodies to assist in the examination and evaluation of the target cells or of clinical samples are all well-known in the art.

[000152] The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more of PGRN, polypeptide analog thereof or fragment thereof such as atsttrin, as described herein as an active ingredient.

[000153] The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions
are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic PGRN polypeptide, analog such as atsttrin, or active fragment-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example, but may be administered via any suitable means including IM, IP, IV, orally, transdermally, etc. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and PGRN activity or PGRN-GBA binding capacity desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.001 to 1, 0.01 to 10, 0.1 to 20, 0.5 to 50, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and subsequent
administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

[000157] The present invention naturally contemplates several means for preparation of the PGRN, PGRN peptides and/or atsttrin of the present invention, including as illustrated herein and/or using known recombinant techniques, and the invention is accordingly intended to cover such synthetic preparations within its scope. The determination of the amino acid sequences disclosed herein facilitates the reproduction of the peptides by any of various synthetic methods or any known recombinant techniques, and accordingly, the invention extends to expression vectors comprising nucleic acid encoding the peptides of the present invention for expression in host systems by recombinant DNA techniques, and to the resulting transformed hosts.

[000158] The present invention also relates to a recombinant DNA molecule, recombinant nucleic acid, or cloned gene, or a degenerate variant thereof, preferably a nucleic acid molecule, in particular a recombinant DNA molecule or cloned gene, encoding the amino acid of one or more PGRN peptides shown in FIGURE 49 or 50 or variants thereof. In a particular embodiment, the recombinant DNA molecule, recombinant nucleic acid, or a degenerate variant thereof, preferably a nucleic acid molecule, encodes a PGRN peptide capable of binding GBA, facilitating lysosomal enzyme transport, and/or reducing lysosomal substrate such as β-GlcCer accumulation, which comprises one or more granulin unit and one or more linker unit of PGRN as depicted in FIGURE 48 and as set out in the sequence of PGRN, or PGRN peptide, such as atsttrin, for example as in FIGURE 48, 49 and 50, including comprising a sequence as set out in SEQ ID NOS: 2, 3 or 4-9. In a further particular embodiment, the recombinant DNA molecule, recombinant nucleic acid, or a degenerate variant thereof, preferably a nucleic acid molecule, encodes PGRN or a PGRN peptide atsttrin capable of binding GBA, facilitating lysosomal enzyme transport, and/or reducing lysosomal substrate such as β-GlcCer accumulation as set out in FIGURE 49 or 50 and comprising granulin units and linker units ½F-P3-P4-½A-P5-½C.

[000159] As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host. Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.
A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCRI, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage λ, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2µ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Any of a wide variety of expression control sequences —sequences that control the expression of a DNA sequence operatively linked to it —may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the lac system, the *trp* system, the TAC system, the TRC system, the LTR system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast a-mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture.

One skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention.

Synthetic DNA sequences allow convenient construction of genes which will express PGRN or atrstrin analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native PGRN genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis.
In assays and diagnostic kits of the invention, labels may be used. The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate. The PGRN or its binding partner(s) can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from \(^3\)H, \(^{14}\)C, \(^{32}\)P, \(^{35}\)S, \(^{36}\)Cl, \(^{51}\)Cr, \(^{57}\)Co, \(^{58}\)Co, \(^{59}\)Fe, \(^{90}\)Y, \(^{125}\)I, \(^{131}\)I, and \(^{186}\)Re. Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, B-glucuronidase, B-D-glucosidase, B-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase.

The generation of animal models for lysosomal storage diseases, including Gaucher’s Disease, particularly ones that recapitulate the clinical conditions has proven to be a challenge (Farfel-Becker T et al (2011) Dis Model Mech 4(6):746-752). For GD, many GBA knockouts and null mutations in animals have led to lethality or early death (Sun Y et al (2005) J Lipid Res 46:2102-2113). Animal models have been generated based on known GBA mutations, including L449P, N370S, V394L, D409H and D409V point mutations, which are associated with various common forms of GD, as well as chemically induced models, for example involving administration of a GlcCerase inhibitor (Farfel-Becker T et al (2011) Dis Model Mech 4(6):746-752).

Tay-Sachs disease naturally exists in Jacob sheep and the biochemical mechanism for the disease in Jacob Sheep is virtually identical to that in humans (Torres PA, et al (2010) Molecular Genetics and Metabolism 101 (4): 357-363). In Jacob sheep, diminished activity of hexosaminidase A resulting in increased concentrations of GM2 ganglioside in the affected animal sheep has been shown (Porter BF, et al (2011) Veterinary Pathology 48 (3): 807-813). The sheep HexA gene is identical in number of nucleotides and has 86% nucleotide identity to the human HexA gene. A missense mutation (G444R) was found in the HEXA cDNA of affected sheep, providing a single nucleotide change at the end of exon 11, resulting in that exon's
deletion (before translation) via splicing (Kolodny E, Horak F, Horak J (2011) ALBC Newsletter (Pittsboro, North Carolina, USA: American Livestock Breeds Conservancy). Jacob sheep provide an available animal model for Tay-Sachs disease, however, sheep are not as readily manipulated or housed as smaller animals or those with established recombinant methods protocols, such as mice or rats. Therefore, an alternative model for Tay-Sachs in mice, etc would be very beneficial.

[000168] The invention provides new and novel animal models for lysosomal storage diseases, including Gaucher’s disease. Animals with altered PGRN or PGRN knock out/null (KO) develop lysosomal storage disease, resembling clinical Gaucher’s disease. The animals accumulate the GBA substrate β-GlcCer in lysosomes, develop hepatosplenomegaly, which is a well-documented symptom of Gaucher’s disease, and Gaucher cells can be seen histologically. PGRN KO or null mutant animals may be treated with ovalbumin or other agent(s) to generate chronic inflammatory models which have GD and lysosomal storage disease phenotypes and mimic the disease. Thus, in an aspect of the invention, animal models for GD and/or other lysosomal storage diseases are provided. Exemplary animal models of the invention may be based on knockout or null mutations of PGRN, altered expression or conditional PGRN mutants, rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A. One or more PGRN alteration or mutation may be combined with one or more GBA mutation or alteration, or with a mutation in another lysosomal enzyme, including for example, one or more of an α-galactosidase, β-galactosidase, sphingomyelinase, to generate an animal model of GD or other lysosomal storage disease.

[000169] The invention also provides animal models for other lysosomal storage diseases, including Tay-Sachs disease, particularly wherein animals with altered PGRN or PGRN knock out/null animals show physiological or molecular aspects of the lysosomal storage disease. For instance, the examples provided herein demonstrate alterations in HexA and GM2 ganglioside with PGRN KO mice that are hallmarks of Tay-Sachs disease. Animals with altered PGRN or PGRN knock out/null animals, alone or combined with HexA gene mutations or HexA mutants, provide a novel alternative Tay-Sach’s disease model.

[000170] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.
EXAMPLE 1
Progranulin is Required for the Lysosomal Delivery of β-Glucocerebrosidase and its Deficiency Causes Gaucher Disease

[000171] Glycosphingolipid metabolism is a process mediated by multiple enzymes. Insufficiency of a metabolic enzyme causes accumulation of its corresponding substrate in the lysosome. Pathways for glycosphingolipid metabolism implicated in lysosome storage diseases (LSD) are depicted in FIGURE 1. Gaucher's disease, the most common LSD, is caused by mutation of glucocerebrosidase (GBA), which metabolizes β-glucosylceramide to ceramide, as shown in FIGURE 1. Mutation of GBA leads to the accumulation of the GBA substrate, β-glucosylceramide (β-GlcCer), in macrophages.

[000172] Gaucher disease (GD), the most common lysosomal storage disease, is typically caused by inherited deficiency of glucocerebrosidase (GBA). Herein we report that deficiency of progranulin (PGRN), a growth factor with a unique structure and multiple functions, also causes Gaucher-like disease unexpectedly. Both ovalbumin-challenged and aged PGRN-deficient mice exhibit signs of GD, and relevant tissues are infiltrated with "Gaucher cells", i.e., macrophages that show a characteristic "crinkled paper" cytoplasmic appearance resulting from the accumulation of glucosylceramide. Recombinant PGRN promotes glucosylceramide clearance in PGRN-deficient macrophages and prevents the GD development in PGRN-deficient mice. PGRN binds directly to GBA and is required for the delivery of GBA to lysosome. Unbiased Mass Spectrometry approaches identify heat shock protein 70 (HSP70), an evolutionarily conserved molecular chaperone, as a GBA-associated protein that mediates trafficking of GBA through PGRN as an indispensable adaptor. Collectively, these findings not only demonstrate that PGRN is a novel co-chaperone of HSP70-mediated folding and trafficking pathway and plays an essential role in the GBA lysosomal delivery, but they also provide a new paradigm to guide therapeutic interventions for various HSP70-mediated pathologies and lysosomal storage diseases, including GD.

[000173] Gaucher's disease (GD), the most common lysosomal storage disease (LSD), is caused by glucocerebrosidase (GBA) mutations that lead to the accumulation of glucosylceramide (β-GlcCer) in macrophages and other cell types. β-GlcCer storage transforms lysosome into tubular-like structure viewed by electronic microscopy, with the lipid-engorged macrophage (Gaucher cell) showing characteristic "wrinkled tissue paper" appearance under light microscopy. There are three types of GD based on its neurological complications (type I is
Progranulin (PGRN), also known as granulin epidermin precursor (GEP), PC-cell-derived growth factor (PCDGF), proepithelin, and acrogranin, contains seven-and-a-half repeats of a cysteine-rich motif (CX5-eCXsCCXsCCXeCCXeHCCPXs6C) (SEQ ID NO: 1) and forms a unique "beads-on-a-string" structure. PGRN is abundantly expressed in epithelial cells, in cells of the immune system, and in neurons. PGRN is known to play a critical role in a variety of physiologic and disease processes, including early embryogenesis, wound healing, and host defense. PGRN also functions as a neurotrophic factor and mutations in the PGRN gene (GRN) resulting in partial or complete loss of the PGRN protein cause frontotemporal dementia (FTD) and neuronal ceroid lipofuscinosis (NCL), respectively.

PGRN associates with some members in the TNF receptor superfamily, including TNFRI, TNFR2 and DR3, and possesses the ability to suppress inflammation in various kinds of conditions. Auto-antibodies against PGRN have been found in several autoimmune diseases, including rheumatoid arthritis, psoriatic arthritis, and inflammatory bowel disease, and such antibodies promoted a proinflammatory environment in a subgroup of patients. In an effort to determine whether PGRN also plays a role in chronic lung inflammation, we challenged PGRN deficient mice with ovalbumin (OVA), which led to the unanticipated discovery of PGRN as an indispensible GBA-associated factor. Here we report that PGRN is an essential co-chaperone for the lysosomal delivery of GBA through linking GBA/LIMP2 complex to heat shock protein 70 (HSP70), an evolutionarily highly conserved molecular chaperone that mediates the folding and trafficking of numerous proteins.

PGRN deficiency causes Gaucher-like diseases in both OVA-challenged and "aged" mice models

The findings that PGRN plays important anti-inflammatory and immune regulatory roles in various conditions, including inflammatory arthritis, prompted us to examine its involvements in the chronic lung inflammation. For this purpose, chronic lung inflammation was induced in 8-week old WT and PGRN knockout (KO) mice by intraperitoneal (IP) injection of OVA at Day 1 and 15, followed by intranasal challenge of 1% OVA beginning at Day 29 three times a week for four weeks. Surprisingly and remarkably, large numbers of "giant cells" were found in the lungs of PGRN KO mice, particularly after OVA treatment (FIGURE 2A). These
cells were engorged with materials with a "wrinkled tissue paper" appearance, which is the typical morphology of Gaucher cells. No such cell was found in WT mice, either with or without OVA challenge. A few Gaucher-like cells were identified in unchallenged PGRN KO mice, and the number significantly increased after OVA challenge (FIGURE 2A, 2B). Periodic acid-Schiff (PAS) staining showed accumulation of glycolipid material in Gaucher-like cells in PGRN KO mice (FIGURE 2C). Flow cytometry analysis of bronchoalveolar lavage identified a subpopulation of giant macrophages in PGRN KO mice after OVA treatment, evidenced by CD11b+FSC^{high}, as the value of forward scattered light (FSC) is proportional to cell size (FIGURE 2D).

[000177] The PGRN null macrophage displayed the classical tubular-like lysosomal appearance of Gaucher-like cells when examined by transmission electronic microscope (TEM). Macrophage from PGRN KO mice was much larger than those in WT mice, and the PGRN null lysosome became tubular-like instead of a regular round shape (FIGURE 3, FIGURE 18A). The transformation of lysosome from normal round to tubular-like structure was found along with material accumulation (FIGURE 18B). Other organelles, such as mitochondria and endoplasmic reticulum, and other types of cells, typel and 2 pneumocyte appeared normal (FIGURE 19A, 19B).

[000178] GD is caused by reduced GBA enzymatic activity that leads to the β-GlcCer accumulation. Lipid composition of lung lysates from WT and PGRN KO mice, with or without OVA challenge, was analyzed as reported previously. As shown in FIGURE 4, β-GlcCer showed increases in all chain-length species in both WT and PGRN KO mice with OVA vs. PBS challenge. Moreover, after OVA challenge all species of β-GlcCer were significantly higher in the PGRN KO vs. WT mice (FIGURE 4). Even untreated PGRN KO mice had a higher level of β-GlcCer than WT mice. The accumulation of β-GlcCer in PGRN KO mice was specific, because other lipid compositions, such as sphingomyelin, diacylglycerol (DAG), and ceramide, remained unchanged between WT and PGRN KO mice (FIGURE 20A-20C). Interestingly, plasma levels of beta-glucosylsphingosine was found to be significantly elevated in the PGRN deficient mice, although no significant increase was observed in the plasma levels of β-GlcCer (FIGURE 21A-21D and FIGURE 22A-22C).

[000179] Although GBA is transported to lysosome independently of the mannose-6-phosphate receptor (MPR) system, Imiglucerase, a macrophage-targeted, mannose-terminated human GBA for use in enzyme replacement therapy (ERT) for Gaucher's disease, is delivered to lysosome via an MPR-dependent pathway. PGRN KO mice were challenged with OVA, and
treated with PBS or imiglucerase injection (60 u/kg/week) at the beginning of the first week of intranasal challenge until to the end of the experiment. Following OVA challenge many Gaucher-like cells were present and almost occupied the whole alveolar space (FIGURE 5A, middle panel). Imiglucerase injection significantly decreased size and accumulation of PAS-positive material (FIGURE 5A, lower panels) as well as β-GlcCer storage (FIGURE 5B) in Gaucher-like cells. The numbers of Gaucher-like cells were comparable, however the ratio between size of the cytoplasm and nucleus significantly decreased with imiglucerase (FIGURE 5C), indicating the size of the Gaucher-like cells became smaller after imiglucerase treatment. The size of the enlarged liver and spleen of PGRN KO mice induced by OVA challenge was also significantly reduced following imiglucerase treatment (FIGURE 5D). The general physical activities in PGRN null mice challenged with OVA were improved by imiglucerase treatment. PGRN KO mice challenged with OVA had decreased physical activity because of compromised pulmonary function. Such mice were sick and did not like to move until touched by forceps (data not shown). OVA-challenged PGRN KO mice receiving imiglucerase therapy became normal and active (data not shown). Collectively, these data and the response of the PGRN KO mice to imiglucerase confirmed that OVA-challenged PGRN deficient mice developed the Gaucher’s disease phenotype.

Since the PGRN KO mice showed pulmonary Gaucher-like cells even in the absence of OVA challenge, we next sought to determine whether aged PGRN deficient mice developed Gaucher-like disease spontaneously. WT and PGRN KO mice were maintained for up to one year, and then 1 year-old WT and PGRN KO mice without any challenge were sacrificed. Lung, spleen, liver, femur, and spine were collected for histology and micro-CT analyses. Similar to GD patients, aged PGRN KO mice developed hepatosplenomegaly (FIGURE 6A), which is a most common symptom of Gaucher's disease. Histologically, Gaucher-like cells were found in lung, spleen, and bone marrow in aged PGRN KO mice, but not in age-matched WT mice (FIGURE 6B). PAS staining of bone marrow showed glycolipid storage in the PGRN null Gaucher-like cells (FIGURE 6C). β-GlcCer levels in tissues and plasma were increased in aging in PGRN deficient mice (FIGURE 23A-23C). The bone marrow was replaced by fat tissue in some aged PGRN KO mice (FIGURE 7A). When examined under TEM, the tubular-like lysosome was observed in PGRN-null Gaucher-like cells from PGRN KO lung and bone marrow (FIGURE 7B). In addition, aged PGRN deficient mice exhibited features of osteopenia in long bone (FIGURE 24A-24H) and vertebrae (FIGURE 25A-25C), which is also a well-documented
symptom of Gaucher’s disease. In conclusion, aged PGRN deficient mice developed Gaucher-like disease phenotype spontaneously.

**Recombinant PGRN prevents β-GlcCer accumulation in PGRN null macrophages and GD development in PGRN-deficient mice**

[000181] To evaluate whether recombinant PGRN (rPGRN) could rescue the GD-like phenotype seen in PGRN KO animals, we first developed an in vitro cell culture model to mimic β-GlcCer accumulation in macrophage in GD. Bone-marrow-derived-macrophage (BMDM) were isolated and differentiated from WT and PGRN KO mice as described previously. BMDM cells were treated with 5 and 50 µg/ml brain lysates which contains many types of lipids for 10 days. H&E staining showed that giant macrophages were present in PGRN KO BMDM, but not in WT BMDM (FIGURE 26). Immunofluorescence staining revealed that β-GlcCer was accumulated in PGRN KO BMDM in a dose-dependent manner after lipid mixture treatment. However BMDM from WT mice did not show an increased level of β-GlcCer (FIGURE 8A).

[000182] To test whether rPGRN, which was effectively taken-up through endocytosis and delivered to lysosome, could prevent β-GlcCer accumulation in PGRN KO BMDM, 0.1 and 0.4 µg/ml PGRN protein was added to the culture medium with lipid mixture (50 µg/ml) for 10 days, and the accumulation of β-GlcCer was measured by immunofluorescence staining. Under a light microscope, BMDM after lipid exposure looked enlarged and disorganized with highly refractile cytoplasmic punctae, and this morphological change was corrected by PGRN in a dose-dependent manner (FIGURE 27). β-GlcCer was accumulated with lipid treatment, and this accumulation was effectively blocked by addition of rPGRN and imiglucerase (serving as a positive control) (FIGURE 8B and 8C). In addition, blocking the known PGRN's signal pathways, including ERK, PI3K and mTOR, did not affect rPGRN's effect on β-GlcCer clearance (FIGURE 30), indicating that prevention of β-GlcCer accumulation by recombinant PGRN was mediated mainly by uptake through endocytosis, but not by activating cytosolic signaling pathways.

[000183] The finding that recombinant PGRN prevents GBA aggregation and β-GlcCer accumulation in PGRN KO BMDMs was further confirmed with fibroblasts from GD patients. Briefly, fibroblasts from type II GD patients were treated with 50 µg/ml lipid mixture with or without 0.4 µg/ml rPGRN. GBA became aggregated around nucleus accompanied with β-GlcCer accumulation following lipid treatment, and all these phenotypes were markedly inhibited by addition of rPGRN (FIGURE 9).
Next we examined whether rPGRN could also rescue the GD phenotype in vivo. PGRN KO mice challenged with OVA were LP injected with either PBS or rPGRN (4 mg/kg per week) from the first week of starting the intranasal challenge until to the end of the experiment. Histology of lung tissues showed infiltration with Gaucher-like cells induced by OVA challenge in PGRN KO mice, and rPGRN dramatically reversed the phenotype (FIGURE 10A). Unlike Imiglucerase treatment which reduced size without a significant effect on the number of Gaucher-like cells (FIGURE 5C, 5D), rPGRN significantly decreased both number and size of Gaucher-like cells (FIGURE 10B, IOC), indicating that PGRN inhibited both Gaucher-like cells formation and β-GlcCer accumulation.

GBA and its transport receptor LIMP2 are aggregated in the cytoplasm and absent in the lysosome of PGRN-deficient macrophage

We next sought to determine the mechanism underlying PGRN-null induced GD-like phenotype by evaluating GBA activity and expression. Accumulation of β-GlcCer in GD is caused by reduced GBA enzymatic activity or decreased GBA protein expression. To our surprise, both GBA enzymatic activities and protein expression were normal in PGRN KO mice, actually the GBA expression was slightly increased after OVA challenge in PGRN KO mice (FIGURE 11A, 11B). Although the protein level and activity of GBA were not decreased, immunohistochemistry staining of GBA revealed that GBA cellular distribution was dramatically altered. GBA was distributed in the cytoplasm of the macrophage in WT mice, while GBA expression was aggregated in the cytoplasm in PGRN KO mice (FIGURE 11C). In contrast, the cellular distribution of alpha-galactosidase A (GLA), a lysosomal enzyme that is primarily delivered to lysosome via a mannose-6-phosphate receptor-dependent pathway, was not affected in PGRN deficient cells (FIGURE 11C). Confocal staining with frozen sections of lung tissues confirmed that GBA was aggregated, accompanied with β-GlcCer accumulation in PGRN deficient tissues (FIGURE 12A). Immunogold labeling TEM also demonstrated that GBA was aggregated in the cytoplasm in Gaucher-like cells, and GBA was absent in the tubular-like lysosomes in PGRN null macrophages, while GBA was detectable in lysosomes in WT macrophages (FIGURE 12B). As a control for the immunogold labeling TEM, Sortilin was found mainly to be clustered close to the cell membrane, mediating endocytosis, and was present in the tubular-like lysosomes of Gaucher cells in PGRN KO mice (FIGURE 28). To further visualize the defect of GBA lysosomal appearance in PGRN KO macrophages, we employed the activity-based probe (ABP) MDW933, which can spontaneously cross membranes and allow sensitive
and specific labeling of active lysosomal GBA in living cells\textsuperscript{28,30}. This probe failed to detect GBA in PGRN deficient BMDMs, although it efficiently labelled lysosomal GBA in WT BMDMs (FIGURE 12C, FIGURE 30). In addition, recombinant PGRN rescued the lysosomal appearance of GBA in PGRN-deficient macrophages (FIGURE 30). Taken together, these results demonstrate that the delivery of GBA to the lysosome depends on the presence of PGRN.

Lysosomal integral membrane protein 2 (LIMP2), a lysosomal marker, was reported to function as a GBA-binding receptor that mediated the delivery of GBA to lysosomes\textsuperscript{31,32}. Interestingly, we found that lysosomal delivery of LIMP2 was also defective in PGRN-deficient macrophages (FIGURE 13A, 13B). However, cellular distribution of lysosomal associated membrane protein-2 (LAMP-2), another lysosomal marker, was not affected in PGRN null macrophages. Specifically, both LIMP2 and LAMP2 were distributed in the cytoplasm of WT macrophage; however the expression of LIMP2 was aggregated in the cytoplasm in PGRN null macrophages, while LAMP2 was distributed in PGRN KO macrophage (FIGURE 13A). The aggregation of LIMP2 was further confirmed with immunogold TEM staining (FIGURE 13B). In addition, both GBA and LIMP2 co-localized in the aggregate in PGRN KO macrophage (FIGURE 13C) and still bound to each other \textit{in vivo} (FIGURE 13D). Collectively, lysosomal delivery of both GBA and its receptor LIMP2 was defective in PGRN deficient mice. LIMP2 deficiency has been reported to cause increased secretion of GBA\textsuperscript{31}, however, no significant difference in GBA secretion was observed between wildtype and PGRN-deficient cells, although PGRN deletion, similar to LIMP2 deficiency\textsuperscript{31}, also led to the absence of GBA in lysosomes.

**PGRN is an essential co-chaperone of the HSP70 chaperone pathway that mediates GBA/LIMP2 lysosomal delivery**

The finding that PGRN was required to target GBA to lysosome prompted us to determine whether PGRN associated with GBA. In vivo interaction between PGRN and GBA was demonstrated by co-immunoprecipitation by using GBA antibody to immunoprecipitate the protein complex and probing with PGRN antibody (FIGURE 14A). We next determined whether PGRN directly binds to GBA using a solid-phase binding assay with recombinant PGRN and GBA. PGRN demonstrated dose-dependent binding and saturation to liquid-phase GBA (FIGURE 14B), whereas no direct interaction between PGRN and LIMP2 was detected (FIGURE 14B). The binding affinity between PGRN and GBA was then measured using surface plasmon resonance (SPR) with SensiQ Pioneer as described\textsuperscript{13,14}. The results demonstrated that PGRN binds to GBA with a very high affinity ($K_D = 0.71$ nM) (FIGURE 14C), higher than
PGRN’s affinity to Sortilin (K_D =3.67 nM) (not shown), a known PGRN-binding lysosomal receptor.

Four-color immunofluorescence staining revealed that GBA and PGRN co-localize in the intracellular traffic compartments of macrophages, including endoplasmic reticulum (ER), Golgi, and trans-Golgi network (TGN) (FIGURE 31). GBA/PGRN passes through intracellular compartments of varying pH en route from ER to lysosomes. The direct binding affinity of GBA to PGRN over a range of pH values was also tested using SensiQ Pioneer (FIGURE 32A). Similar to the interaction between GBA and LIMP2, binding of GBA to PGRN was favored at neutral pH, and there was a trend toward decreased affinity with decreasing pH (FIGURE 32B). Taken together, these results suggested that the probable points of GBA and PGRN association are prelysosomal, possibly beginning in compartments as early as the ER and Golgi apparatus.

We next sought to determine the molecular pathway by which PGRN regulates GBA. To isolate the molecules that are involved in PGRN regulation of GBA, immunoprecipitation was performed with GBA antibody from both WT and PGRN KO tissues, followed by high-sensitivity mass spectrometry (MS). Immunoprecipitation with GBA antibody pulled-down both PGRN-dependent and PGRN-independent GBA-associated proteins in WT tissues. When the same immunoprecipitation experiment was performed in PGRN KO tissues, only PGRN-independent GBA-associated proteins were immunoprecipitated. Hits from WT mice were subtracted by hits from PGRN KO, to yield PGRN-dependent GBA associated proteins, with the rationale that the molecules involved in PGRN-mediated GBA delivery would be among the hits only present in WT mice but not PGRN KO mice (FIGURE 15A). 134 hits in WT mice and 114 hits in PGRN KO mice were identified. 95 of them were common in both groups, and 39 proteins were found to be specific for WT mice, suggesting these proteins would be PGRN-dependent GBA-associated proteins (FIGURE 15B). Perlecan and Leukocyte elastase inhibitor, two known PGRN-binding proteins, were identified among the 39 hits, validating the technique. In addition, HSP70 and its co-chaperone protein TCP1, as well as cytoskeleton, vesicle-traffic related proteins, and an energy producing enzyme, were among the 39 hits.

**TABLE 3**

**GENES THAT MAY BE PGRN-DEPENDENT GBA ASSOCIATED PROTEINS**

| Perlecan (Heparan sulfate proteoglycan 2) | OS=Mus musculus | GN=Hspg2 | PE=4 | SV=1 | [B1BOC7_MOUSE] *1 |
| Moesin | OS=Mus musculus | GN=Msn | PE=1 | SV=3 | [MOES_MOUSE] |
| T-complex protein 1 subunit zeta | OS=Mus musculus | GN=Cct6a | PE=1 | SV=3 | [TCPZ_MOUSE] *2 |
| Heat shock 70 kDa protein 12B | OS=Mus musculus | GN=Hspal2b | PE=1 | SV=1 | [HS12B_MOUSE] *2 |
Uncharacterized protein OS=Mus musculus GN=Gm8991 PE=4 SV=1 - [E9Q7H5_MOUSE]
EH domain-containing protein 4 OS=Mus musculus GN=Ehd4 PE=1 SV=1 - [EHD4_MOUSE]
Polymeric immunoglobulin receptor OS=Mus musculus GN=Figr PE=1 SV=1 - [PIGR_MOUSE]
Leukocyte elastase inhibitor A OS=Mus musculus GN=Serpinbla PE=1 SV=1 - [ILEUA_MOUSE] *1
Uncharacterized protein (Fragment) OS=Mus musculus GN=Fus PE=4 SV=1 - [GJ3UXT7_MOUSE]
Alpha-1-antitrypsin 1-2 OS=Mus musculus GN=Serpinalb PE=1 SV=2 - [AIAT2_MOUSE]
Protein S100-A9 OS=Mus musculus GN=S100a9 PE=1 SV=3 - [S10A9_MOUSE]
Vinculin OS=Mus musculus GN=Vcl PE=1 SV=4 - [VTNC_MOUSE]
Clusterin OS=Mus musculus GN=Cul PE=1 SV=1 - [CLUS_MOUSE]
Putative ATP-dependent RNA helicase P10 OS=Mus musculus GN=DIPas1 PE=1 SV=1 - [DDX3L_MOUSE]
Nben-like protein 1 OS=Mus musculus GN=Faml29b PE=1 SV=2 - [NIBLI_MOUSE]
Serotransferrin OS=Mus musculus GN=Tf PE=1 SV=1 - [TRFE_MOUSE]
Annexin A11 OS=Mus musculus GN=Anxa11 PE=1 SV=2 - [ANX11_MOUSE]
Ankycorbin OS=Mus musculus GN=Anxcl PE=1 SV=1 - [RA114_MOUSE]
Ehd2 protein OS=Mus musculus GN=Ehd2 PE=2 SV=1 - [Q8R2X0_MOUSE]
Peroxiredoxin 1 (Fragment) OS=Mus musculus GN=Prdxl PE=4 SV=1 - [BIAXW5_MOUSE]
LIM and SH3 protein 1 (Fragment) OS=Mus musculus GN=Laspl PE=4 SV=1 - [A2A6G9_MOUSE]
Ribonuclease inhibitor OS=Mus musculus GN=Rubl PE=1 SV=1 - [PIJR_MOUSE]
Heterogeneous nuclear ribonucleoprotein U, isoform CRA_b OS=Mus musculus GN=Hnrnpu PE=4 SV=1 - [G3XA10_MOUSE]
Fibrinogen beta chain OS=Mus musculus GN=Fgb PE=2 SV=1 - [FIBB_MOUSE]
NK13 OS=Mus musculus GN=Serpinb6b PE=2 SV=2 - [O00804_MOUSE]
Chloride intracellular channel protein 4 OS=Mus musculus GN=Clic4 PE=1 SV=3 - [CLIC4_MOUSE]
Dimethylaniline monoxygenase [N-oxide-forming] 2 OS=Mus musculus GN=Fmo2 PE=1 SV=3 - [FM02_MOUSE]
Ceruloplasmin, isoform CRA_f OS=Mus musculus GN=Cp PE=4 SV=1 - [G3X9T8_MOUSE]
Myosin light polypeptide 6 OS=Mus musculus GN=Myl6 PE=1 SV=3 - [MYL6_MOUSE]
Fibrinogen, alpha polypeptide OS=Mus musculus GN=Fga PE=2 SV=1 - [Q99K47_MOUSE]
Alcohol dehydrogenase 1 OS=Mus musculus GN=Adhl PE=2 SV=2 - [ADHL_MOUSE]
Isoform 4 of Myosin-X Villa OS=Mus musculus GN=Myo18a - [MY18A_MOUSE]
Copine-3 OS=Mus musculus GN=Cpne3 PE=1 SV=2 - [CPNE3_MOUSE]
Pulmonary surfactant-associated protein D OS=Mus musculus GN=Sftpd PE=2 SV=1 - [SFTPD_MOUSE]
Actin-related protein 2/3 complex subunit IB OS=Mus musculus GN=Arpc1b PE=1 SV=4 - [ARCIB_MOUSE]
F-actin-capping protein subunit alpha-1 OS=Mus musculus GN=Capzal PE=1 SV=4 - [CAZAL_MOUSE]
Integrin beta OS=Mus musculus GN=Itgb2 PE=2 SV=1 - [Q542I8_MOUSE]
Fibrinogen gamma chain OS=Mus musculus GN=Fgg PE=2 SV=1 - [FIBG_MOUSE]
Long-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Mus musculus GN=Acadl PE=2 SV=2 - [ACADL_MOUSE]

Note: *1 known PGRN binding proteins, *2 HSP70 and its co-chaperones

[000190] These data were followed up with studies in HEK293EBAN cells stably transfected with an expression plasmid encoding His-tagged PGRN 13. Two proteins were co-purified with His-tagged PGRN, and MS analysis revealed that these were HSP70 and TCPl (not shown). To
confirm the MS data, we conducted immunoprecipitation using a GBA antibody in WT and PGRN KO tissues, and probed with an antibody against HSP70. HSP70 bound to GBA in WT mice after OVA challenge, and this interaction was undetectable in PGRN KO mice (FIGURE 15C). In addition, administration of rPGRN efficiently rescued the binding of GBA to HSP70 in PGRN KO mice (FIGURE 15D). We next examined whether HSP70 is required for the lysosomal delivery of GBA, and HSP70 was suppressed using an siRNA approach. Similar to knockdown of PGRN (serving as a control), suppression of HSP70 led to undetectable lysosomal GBA, assayed with MDW933 labeling in living cells (FIGURE 16A).

Previous reports that LIMP2 was the major GBA transport receptor, together with the finding that both GBA and LIMP2 were aggregated in PGRN deficient macrophages (FIGURE 13), led us to determine whether HSP70 also interacted with LIMP2. Similar to GBA, LIMP2 also associated with HSP70 in WT but not in PGRN KO tissues (FIGURE 16B). Sortilin was reported to be a receptor of PGRN and to mediate the delivery of PGRN to the endosome/lysosomal pathway in neurons. We thus determined whether Sortilin forms a ternary complex with LIMP2/GBA/PGRN/HSP70 through PGRN as a linker protein and facilitates the delivery of LIMP2/GBA/PGRN/HSP70 along the endosome/lysosomal pathway. Here we found that this was the case. The interaction between Sortilin and GBA was identified in WT lungs after OVA challenge, which was completely lost in PGRN KO lungs, while the interaction between Sortilin and GLA was not affected by deficiency of PGRN (FIGURE 16C). In addition, Sortilin very weakly associated with HSP70 in untreated WT lungs, and this interaction was markedly enhanced by OVA challenge (FIGURE 16D), while this interaction was also completely abolished in PGRN KO lungs (FIGURE 16D). Collectively, Sortilin associates with LIMP2/GBA/PGRN/HSP70 complex through PGRN as an indispensable adaptor.

The finding that PGRN acts as a co-chaperone of HSP70 pathway required for GBA folding and trafficking, together with the facts that (1) chaperone-based treatments aiming to enhance GBA trafficking have proven to be an effective alternative to ERT (enzyme replacement treatment) for GD, and (2) recombinant HSP70 has been shown to effectively correct altered lysosomal stability seen in Niemann-Pick disease (NPD), prompted us to examine whether PGRN would have therapeutic effects in GDs and other LSDs. Using the similar lysotracker approach, we examined the effects of rPGRN on fibroblasts from normal and 11 different patient fibroblasts of LSDs, including GDs. As expected, PGRN effectively reverted the altered lysosomes in fibroblasts from both Type I and II GD with or without lipid stimulation (FIGURE 33A, 33B). We were excited to observe that PGRN also remarkably normalized the altered
lysosomes in fibroblasts of Tay-Sachs disease, Farber disease, and Mucolipidosis III (FIGURE 33A, 33B). In line with these findings, the accumulation of GAG and M2 ganglioside was also observed in the tissues from aged PGRN deficient mice (data not shown). In the case of type III GD, Mucopolysacharidosis III and VI, PGRN demonstrated beneficial effects in the presence of lipid stimulation (FIGURE 34A, 34B). Although PGRN corrected the diseased lysosomes from LSDs aforementioned in the single patient disease sample fibroblasts we initially evaluated, significant improvements were not observed on fibroblasts in our samples from Niemann-Pick disease type B, Fabry disease and Mucolipidosis VI (FIGURE 35A, 35B). However, in view of the small sample size, further studies are planned with additional samples to evaluate PGRN effects in these latter diseases, as a conclusion of no significant effects cannot yet be made. Taken together, these results implicate PGRN, as a co-chaperone of HSP70 trafficking pathway, as involved in the lysosomal delivery of other lysosome enzymes in addition to GBA.

DISCUSSION

[000193] PGRN-deficient mice were reported to exhibit accelerated lipofuscinosis and ubiquitination. Here we report that PGRN null mice developed Gaucher-like disease, a finding that further supports the notion that PGRN may be a key regulator of lysosomes. Both OVA-challenged adult and aged PGRN null mice demonstrated Gaucher-like cells and a typical tubular-like lysosomal appearance, which therefore presents a novel mouse model that closely mimics the signs of human GD. This novel GD animal model not only helps us to better understand the pathogenesis of GD, but could also facilitate the testing and development of new drugs for treating GD. Neurons of the PGRN-deficient mice have been shown to accumulate lipofuscin and subunit c of mitochondrial ATP synthase (SCMAS) which are commonly regarded as neuronal ceroid lipofuscinosis (NCL) biochemical signatures. The findings that PGRN deficient mice display both GD and NCL indicate the overlapping features of the phenotypes. We also observed the accumulation of lipofuscin and SCMAS in the brain of our mice models. Thus, the discrepancy among PGRN deficient mice models reported is probably resulted from the differences in the cell populations studied (Macrophages in GD vs Neurons in NCL). The unexpected development of GD-like in PGRN deficient mice models led us to examine whether PGRN level was also associated with human patients with GD. Indeed, serum PGRN levels of GD patients were significantly lower than both general healthy controls and also Ashkenazi Jewish controls, and several GRN gene variants were also identified in GD patients through whole GRN gene sequencing (FIGURE 36).
HSP70 was isolated as one of numerous GBA-associated proteins dependent on the presence of PGRN in our unbiased screen. Interestingly, the associations of GBA with HSP70 as well as their involvements in GD were reported previously\textsuperscript{36,40,41}, although the nature of their association was unclear. In addition, the binding of PGRN to HSP70 was also recently reported\textsuperscript{42}. Our approach identified PGRN as an essential component of GBA/PGRN/HSP70 ternary complex that mediates lysosomal delivery of GBA. Importantly, PGRN, acting as an essential co-chaperone for linking GBA to the HSP70-mediated folding and trafficking pathway, effectively normalized the altered lysosomes in the fibroblasts from GD patients. In addition, chaperone-based treatment aiming to facilitate GBA folding and trafficking has proven to be a promising approach for treating GD\textsuperscript{36,43}. More importantly, current ERT is only effective for one LSD correspondingly, PGRN may be an alternative treatment for various kinds of LSDs in addition to GDs, as PGRN also significantly corrected diseased lysosomes of additional LSDs (FIGURES 33, 34, 35).

LIMP2, the known GBA transport receptor\textsuperscript{13,34,35}, was also aggregated in PGRN deficient cells, and GBA-LIMP2 ligand-receptor constituted a ternary complex with PGRN and HSP70 chaperone, which requires PGRN as an adaptor. In addition, Sortilin, a PGRN-binding lysosomal receptor known to participate in the delivery of several proteins to the lysosome\textsuperscript{26,27}, also associated with this ternary complex through direct binding to PGRN. A proposed model for explaining the role of PGRN in mediating the folding and lysosomal delivery of LSD enzymes, exemplified with GBA/LIMP2, through an HSP70 chaperone pathway is shown in FIGURE 17. GBA/LIMP2 associate with HSP70/co-chaperones in the ER/Golgi through PGRN as an essential adaptor, and transport simultaneously to lysosome. Other lysosomal receptors, such as Sortilin, may also facilitate the delivery of the complex to the endosome/lysosome. Additionally, the association with PGRN/HSP70 pathway may be also important for the folding of GBA and LIMP2. Collectively, PGRN acts as an indispensable component of GBA/LIMP2 lysosomal transport machinery via mediating the folding and trafficking of GBA/LIMP2 complex.

Although the mechanism by which OVA enhances the GD-like phenotype of PGRN KO macrophages is unclear, some known functions of PGRN may contribute to understand the unexpected observations in OVA-challenged PGRN KO mice. For instance, PGRN associates with TNF receptors and possesses the ability to suppress inflammation in various kinds of conditions\textsuperscript{7,11-16}, the lack of PGRN may thus lead to the abnormal response of macrophages to OVA-induced inflammation. In addition, inflammation is known to be involved in multiple sphingolipid LSDs and anti-inflammatory drugs have benefits in treating LSDs used alone or
combined with other treatments. Furthermore, PGRN was reported to associate with ER-stress related unfolded protein response, which was suggested to play a key role in cell death in GD. The loss of PGRN leads to the abnormal ER stress responses and the aggregation of various proteins, such as TDP-43 and GBA/LIMP2 (this example), which in turn induce the increased ubiquitination for degrading aggregated proteins in the cytoplasm and nucleus, whereas in the lysosome PGRN deficiency causes the defect in the lysosomal delivery of GBA and in turn the accumulation of β-GlCer. OVA stimulation may accelerates this process. Recently, it was reported that RIPK3, a component of the TNFR1 signaling complex that mediates necroptosis, was also involved in the pathology of GD and inhibiting RIPK3 might be a novel therapeutic approach for GD. Thus, PGRN's anti-TNF and anti-cell death activities may also contribute to its therapeutic effects in GD and other LSDs.

**[000197]** Mutation of GRN gene are associated with front-temporal dementia. Insufficiency of PGRN has been associated with neuron degenerative diseases. Homozygous mutation of GRN was also linked to NCL. The finding that PGRN is an indispensable component of HSP70 pathways mediating the lysosomal delivery of GBA/LIMP2 may be also associated with neurodegenerative diseases. Mutation of the GRN gene may directly affect the HSP70 trafficking pathway and in turn lead to defects in the clearance of proteins such as TDP-43, and α-synuclein, or indirectly affect the function of lysosomes resulting from the impairment of GBA delivery and consequent accumulation of glucosylceramide. Thus, the identification of PGRN as a co-chaperone of HSP70 may also help us to better understand the putative molecular mechanisms underlying GRN mutations-associated disorders. In addition, PGRN physically binds to GBA whose mutations also associate with Parkinson’s Disease (PD), indicate that there may exist a functional and a genetically linkage between GRN and GBA genes, and their homozygous or heterozygous mutations may render some carriers vulnerable to rare (GD) and/or common (PD) diseases.

**[000198]** In summary, this study identifies PGRN as a previously-unrecognized molecule associated with and capable of causing GD, thus providing a solid foundation for future discoveries relating to this critical factor in GD and other lysosomal storage diseases. In addition, it also isolates PGRN as a novel co-chaperone of the prominent HSP70-mediated folding/trafficking pathway, thus uncovering a unique strategy to target this cardinal pathway of metabolic diseases. With the consideration that HSP70 folding and trafficking pathway is involved in a plethora of disease processes, the identification and manipulation of this new co-
chaperone of the HSP70 pathway may lead to innovative therapeutics for treating LSDs, especially GD, and other metabolic pathologies and conditions.

**METHODS SUMMARY**

[000199] *In vivo* assays for defining the essential role of PGRN in the lysosomal delivery of GBA using various animal models: Comparison of OVA-challenged wild type and PGRN-deficient mice; Comparison of 1-year old wild type and PGRN-deficient mice; Administration of imiglucerase or recombinant human PGRN (rPGRN) into OVA-challenged PGRN-deficient mice that develop Gaucher-like diseases.

[000200] In vitro cell-based assays for examining the β-GlcCer clearance by recombinant PGRN: Comparison of lipid-challenged wild type and PGRN null bone marrow derived macrophages (BMDMs) in the presence or absence of rPGRN; Comparison of PGRN correction of altered lysosomes in fibroblasts of various LSDs with or without lipid stimulation.

[000201] Assays for characterizing Gaucher-like diseases and for visualizing Gaucher-like cells: Histological analysis; Immunohistochemistry; Lipid composition analysis; GBA enzyme activity; Transmission Electron microscopy (TEM); Immunogold labeling TEM; Immunofluorescence staining and confocal microscope; Flow cytometry; Labeling of active lysosomal GBA in living cells using MDW933 inhibitory green probe.

[000202] Mass Spectrometry and Protein/protein interaction assays for identifying and characterizing the associations among PGRN, GBA, HSP70 and Sortilin: Co-immunoprecipitation; Solid-phase binding; Analytical Surface Plasmon Resonance with SensiQ Pioneer, Sensitive and conventional Mass Spectrometry.

**MATERIALS AND METHODS**

[000203] Materials: Fibroblasts from type I, II and III GD, Tay-Sachs disease, Farber disease, type IV and IV mucolipidosis (ML), type III and VI mucopolysaccharidosis (MPS), Niemann-Pick disease type B, and Fabry disease were purchased from Coriell Cell Repositories (Camden, NJ), and normal fibroblasts were purchased from Gibico. All fibroblasts were cultured in DMEM medium containing 10% FBS. Antibodies against GBA (sc-100544, sc-30844, and sc-32883), PGRN (SC-28928), Sortilin (sc-376576), a-GLA (sc-25823), HSP70 (sc-373867), Calregulin (sc-373863), TGN38 (sc-271624), EEA1 (sc-365652) LIMP2 (sc-55571), and LAMP2 (sc-18822), were purchased from Santa Cruz Biotechnology (Dallas, Texas). β-GlcCer antibody (Cat. No. RAS_0010) was purchased from Glycobio GmbH (Germany). Donkey anti-Mouse IgG labeled with Alexa Fluor® 488, Alexa Fluor 647, or Cyanine cy3, and donkey anti-Rabbit
labeled with Alexa Fluor® 488, or Cyanine cy3, and Donkey anti-sheep IgG labeled with Alexa Fluor 488, or Cyanine cy3 were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Recombinant His-tag PGRN protein was purified from 293T stable cell lines as described previously 13,14. Recombinant GBA (Cat. No. 7410-GH-010), sortilin (Cat. No. 3154-ST-050), and LIMP2 (Cat. No. 1966-LM-050) proteins and sheep anti-mouse PGRN antibody (AF2557) were purchased from R&D Systems (Minneapolis, MN). Human PGRN ELISA kit was purchased from AdipoGen (San Diego, CA). ERK inhibitor PD98059, PI3K inhibitor Wortmannin and mTOR inhibitor rapamycin were purchased from Life Technologies. Imiglucerase was provided by Dr. Pastores.

**Chronic lung inflammation model:** C57B/L6 WT and PGRN KO mice were hosted in the animal facility of New York University as previously described 13.55. 8 weeks-old mice were induced chronic lung inflammation by L.P. injection of OVA-Alum challenged at Day 1 and Day 15, followed by followed by intranasal challenge of 1% OVA beginning at Day 29 three times a week for four weeks 51. In PGRN rescue experiments frequency of intranasal challenge of OVA was increased to three times a week. The mice were sacrificed, and spleen, liver, leg, lung and bronchoalveolar lavage (BAL) were collected. In the PGRN rescue experiments, 4 mg/kg of recombinant PGRN or 60 u/kg imiglucerase were LP injected every week when intranasal challenge started.

In another experiment, WT and PGRN KO mice were hosted in animal facility of New York University until 1 year-old. Aged mice were sacrificed directly, and lung, spleen, liver, femur, and spine were collected for histology and micro-CT analysis.

**Histology and analysis:** After mice were sacrificed, one lobe of lung was cut without perfusion for future proteins and lipid analysis, the remaining part of lung was perfused with 4% paraformaldehyde (PFA). Spleen, liver, and femur were also collected and fixed with 4% PFA. All these tissues were embedded in paraffin, cut into slides, and stained with H&E and PAS by Mass Histology Service (Worcester, MA). Quantification of Gaucher-like cells number, and measurement of area of Gaucher-like cells were analyzed by Image J software.

The femurs from indicated groups of mice were cleaned off soft tissue. Following routine fixation, decalcification, and paraffin embedding, tissue sections were prepared and stained with hematoxylin and eosin. We measured the bone volume in a standard zone, situated at least 0.5 mm from the growth plate, excluding the primary spongiosa and trabeculae connected to the cortical bone, and enumerated the osteoclasts and trabecular area in the same zone as that used for assessing bone volume (10xoriginal magnification), using BioQuant software.
Lipid composition analysis: Lung from WT and PGRN KO mice with or without OVA challenge was collected, and homogenized with RIPA lysis buffer containing proteinase inhibitors cocktail. In addition, lung and spleen from PGRN KO mice at different ages, and fibroblasts from health control and GD patients were also processed using the same method. Lmg total protein from each samples was used to measure the lipid composition by Lipidomics Core at Medical University of South Carolina. Levels of Ceramide, DAG, sphingomyelin, β-GlcCer, and glucosylsphingosine (GlcSph) were measured by the high-performance liquid chromatography/mass spectrometry (LC-MS/MS) methodology as previously described. Plasma from healthy control and GD patients, as well as from WT and PGRN KO mice were collected, and the levels of β-GlcCer and GlcSph were measured. Analytical results of lipids were expressed as: lipid level/total cellular protein: pmol/mg protein, or pmol/ml plasma.

GBA enzyme activity: Lung from WT and PGRN KO mice were lysed and 20 μg total protein were used to measure GBA activity as reported previously. Briefly GBA activity was quantified by cleavage artificial substrate 4-methyllumbiferyl -P-D-glucopyranoside (4 MUGP) into 4-methyllumbiferone at pH5.9 solutions (50 mM citrate phosphate buffer containing 0.15% Triton X-100 and 0.125% sodium taurocholate). The amount of 4-methyllumbiferone was measured at 360 nm excitation and 460 nm emission filters. GBA activity was expressed as nM/mg total protein/h.

Transmission electron microscope (TEM): WT and PGRN KO mice after OVA treatment, as well as aged PGRN KO mouse, were anesthetized and the lung was perfuse fixed with fixative containing 2.5% Glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 2 hours. After washing, the samples were fixed in 1% Os04 for 1 hour, block staining with 1% uranyl acetate for 1 hour, dehydration and embedded in Embed 812 (Electron Microscopy Sciences, Hatfield, PA). 60nm sections were cut, and stained with uranyl acetate and lead citrate by standard methods. Stained grids were examined under Philips CM- 12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan (4k x2.7k) digital camera (Gatan, Inc., Pleasanton, CA).

For immunoelectron microscopy, mice were perfused and fixed with 4% PFA in 0.1M phosphate buffer (pH7.4), and the lung was dissected and continuously fixed in the freshly made 3%, PFA in 0.1M phosphate buffer containing 0.1% glutaraldehyde and 4% sucrose (pH 7.4). After washing and dehydration, the tissue were embedded in Lowicryl K4M (Polysciences, Inc., Warrington, PA) and LR White (Electron Microscopy Sciences, Hatfield, PA). Polymerized will be under UV light (360nm) at -35°C for LK4M and -10°C for LR White. Ultrathin sections
were cut, mounted on Formvar-Carbon coated nickel grids. After incubation with primary antibodies at 4°C overnight, gold conjugated secondary antibodies (15nm Protein A Gold, Cell Microscopy Center, University Medical Center Utrecht, 35584 CX Utrecht, The Netherlands; 18nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were applied. The grids were stained with uranyl acetate and lead citrate by standard methods, and examined under Philips CM-12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan (4k x2.7k) digital camera (Gatan, Inc., Pleasanton, CA).

**Immunofluorescence staining and Confocal Microscope:** Frozen lung sections, or cover-slip cultured BMDM, were fixed with 4% formaldehyde for 5 min and washed twice with PBS. The cells were permeabilized by 0.1% Triton-100 PBS for 5 min and then wash with PBS. The tissues were blocked with 1:50 dilution of normal donkey serum for 30 min. Primary antibodies were probed on the slides at 4°C degree overnight. The next day slides were washed with PBS, fluorescence-labeled secondary antibodies (Alexa Fluor® 488-labeled donkey anti-mouse combined with Cyanine cy3-labeled donkey anti-rabbit antibody, or in some experiments different fluorescence were used) were added for 1 hour and wash with PBS. The tissues or BMDM cells were mounted on anti-fade medium containing DAPI. The images were taken by Leica TCS SP5 con-focal system.

**Flow cytometry:** BAL was collected when mice were sacrificed, and centrifuged at 1200 rpm for 5 min to collect cells. The cells were suspended and washed in ice-cold PBS containing 0.1% FBS for two times. The cells were stained with FITC-labeled CD1 1b antibody (eBioscience San Diego, CA) for 1 hour and analyzed by BD FACScan, and data were analyzed by FlowJo software.

**Measurement of bone mineral density (BMD):** We assessed the BMD (gm/cm2) of the whole skeletons of aged WT and PGRN-deficient mice, using a PIXImus bone densitometer (Lunar, Madison, WI). The instrument was calibrated before each scanning session, using a phantom with known BMD, according to the manufacturer’s guidelines. Mice were anesthetized by intraperitoneal injection of ketamine (90 µg/g of body weight) and xylazine (10 µg/g of body weight) and then were placed in the prone position on the specimen tray to allow scanning of the entire skeleton.

**Micro-CT:** The trabecular volume in the distal femoral metaphysis was measured using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland). A threshold of
300 was used for evaluation of all scans. 30 slices were analyzed, starting with the first slice in which condyles and primary spongiosa were no longer visible.

[000215] **Immunoprecipitation:** Lung tissue from OVA-challenged or unchallenged WT and PGRN KO mice with or without rPGRN treatment were lysed by RIPA lysis buffer containing protease inhibitors. 12000 rpm centrifuge 10 min to pellet the debris. The supernatant were transferred to a new tube and 10 seconds supersonic pulse were used to further release membrane proteins. Same amount of proteins from each group of mouse were mixed together to represent the protein profile of each group. 400 µg protein from mixed samples were used for immunoprecipitation. 2 µg/ml normal mouse and rabbit antibodies and 20 µl protein A/G agarose-beads were added, and incubated 1 hour at 4 °C. Centrifuge at 3000 rpm for 5 min to pellet the beads. The supernatant were transferred to a new tube and 2 µg/ml primary antibodies were added and incubated 1 hour at 4 °C, then 20 µl protein A/G agarose-beads were added and incubated overnight. The beads were washed with RIPA lysis buffer 6-8 times, the samples were run on SDS-PAGE, and targeted proteins were probed with antibody and visualized by western-blot. In some experiments, the samples after immunoprecipitation were sent to NYU core facility to do Mass Spectrometry.

[000216] **Immunohistochemistry:** Paraffin-embedded lung slides from WT and PGRN KO mice de-paraffined by xylene and gradient ethanol. Antigen was retrieved by using 0.1% trypsin (diluted from 0.5% trypsin by 0.1% CaCl2) at 37 °C for 30 min. Endogenous hydrogen peroxidase was inactivated by 3% H2O2 in PBS for 10 minutes. The slides were blocked with 3%, BSA and 20% goat serum for 30 minutes. Primary antibodies were diluted at 1:20-50 with 2% goat serum, primed on the slides at 4 °C overnight. The next day slides were washed with PBS and secondary antibody were added (1:200 biotin-labeled goat-anti rabbit antibody or goat-anti mouse antibody) for 1 hour. The staining was visualized by Vector ABC peroxidase kit, followed by DAB substrates.

[000217] **Mass spectrum:** 1) Gel Separation and Digestion. Samples were reduced with DTT at 57 °C for 1 hour and were alkylated with Iodoacetamide at RT in the dark for 45 minutes. Each sample was loaded onto a NuPAGE® 4-12% Bis-Tris Gel 1.0 mm The gel was stained using GelCode Blue Stain Reagent (Thermo Scientific) and Coomassie stained gel bands were excised as indicated on the gel image. Excised gel pieces were destained with a 50:50 v/v solution of methanol and 100 mM ammonium bicarbonate. The gel pieces were partially dehydrated with an acetonitrile rinse and further dried in a SpeedVac concentrator for 20 minutes. 300 ng of sequencing grade modified trypsin (Promega) were added to each gel
sample. After the trypsin was absorbed 100 μl of 100 mM ammonium bicarbonate was added to cover the gel pieces. Digestion proceeded overnight on a shaker at RT.

[000218] (2) Protein Extraction. A slurry of R2 20 μm Poros beads (Life Technologies Corporation) in 5% formic acid and 0.2% trifluoroacetic acid (TFA) was added to each sample at an volume equal to that of the ammonium bicarbonate added for digestion. The samples shook at 4 °C for 2 hours. The beads were loaded onto equilibrated C18 ziptips (Millipore) using a microcentrifuge for 30 seconds at 6000 rpm. Gel pieces were rinsed three times with 0.1% TFA and each rinse was added to its corresponding ziptip followed by microcentrifugation. The extracted porors beads were further washed with 0.5% acetic acid. Peptides were eluted by the addition of 40% acetonitrile in 0.5% acetic acid followed by the addition of 80% acetonitrile in 0.5% acetic acid. The organic solvent was removed using a SpeedVac concentrator and the sample reconstituted in 0.5% acetic acid.

[000219] MS Analysis. 1/5th of each sample was analyzed individually with the mlgG analyzed first, then the KO GBA, and finally the WT GBA. Samples were injected for on-line LC-MS using the autosampler of a EASY-nLC 1000 (Thermo Scientific). Peptides were gradient eluted from the column directly to Q Exactive mass spectrometer (Thermo Scientific) using a 1 hour gradient Solvent A: 5% acetonitrile, 0.5% acetic acid Solvent B: 95% acetonitrile, 0.5% acetic acid.

[000220] MS Method. High resolution full MS spectra were acquired with a resolution of 70,000, an AGC target of le6, with a maximum ion time of 120 ms, and scan range of 300 to 1500 m/z. Following each full MS twenty data-dependent high resolution HCD MS/MS spectra were acquired. All MS/MS spectra were collected using the following instrument parameters: resolution of 17,000, AGC target of 2e5, maximum ion time of 250 ms, one microscan, 2 m/z isolation window, fixed first mass of 150 m/z, and NCE of 27. MS/MS spectra were searched against a uniprot mouse database using Sequest within Proteome Discoverer.

[000221] Surface Plasmon Resonance (SPR): All SPR experiments were done by SensiQ Technologies Inc. (Oklahoma City, OK) by using SensiQ Pioneer at a controlled analysis temperature of 25°C, and samples in the instrument sample racks were maintained at 18°C. The running buffer throughout the immobilization and the assay consisted of 10mM FEPES, 150mM NaCl, 0.005% Tween 20. Buffer pH was adjusted to pH 7.4, 6.5, 6.0 or 5.5 for individual runs, and for each pH the running buffer was used to prepare PGRN samples and sucrose diffusion standards.
A COOH1 chip was installed and conditioned via 10 second injections (2x each) of 10mM HCl, 50mM NaOH, and 0.1% SDS. Channel 3 was activated via a five minute injection of 4mM EDC and 1mM NHS in water at a 20uL/min flow rate. GBA (25ug/mL in 10mM sodium acetate pH 5.5) was then injected for -two minutes at a 10uL/min flow rate. Channels 1 and 2 were then activated with 4mM EDC and 1mM NHS in water for five minutes. Sortilin (10ug/mL in sodium acetate pH 4.0) was immobilized on channel 1 via a five minute injection at a 10uL/min flow rate. BSA (10 ug/mL in sodium acetate pH 4.3) was immobilized on the reference channel to reduce non-specific binding. All channels were capped with a four minute injection of IM Ethanolamine pH8.0.

The assay of PGRN was performed with a total of five buffer blank injections and two replicates of 200nM PGRN, all of which were given a 1 hour dissociation time. The OneStep™ injection was used for this assay to determine kinetic rate constants and the equilibrium dissociation constant from a single gradient inject. Two injections of 3% sucrose in running buffer were performed to serve as a diffusion standard.

Data was analyzed using the QDat Analysis Software (SensiQ Technologies and BioLogic Software). All data were double referenced to a reference channel (channel 2) and buffer blanks. The average signal of the buffer blanks was used to subtract injection artifacts. Referenced SPR data from the analysis channels were model fit to ascertain ka, kd, and K_D for the interactions.

Solid phase binding: 0.1, 1, 2, and 5 µg/ml PGRN proteins were coated in 96-wells with triplicate wells in PBS for overnight. The plate was washed with 0.1% tween/ PBS five times and then blocked with 2% BSA/PBS solution. Two µg BSA, LIMP2 and GBA protein were labeled with biotin followed the protocol of EZ-Link Sulfo-NHS-LC-Biotin and Biotinylation Kits (Thermo Scientific). Biotin-labeled LIMP2, GBA or BSA were added in the plate and incubate for 2 hours. Wash with 0.1% tween/PBS, and coated with streptavidin-HRP (1:2000 dilution) solution for 1 hour. After washing add the substrate and stop the reaction with 100 µl 2M H2SO4. Read result at UV 450nm in plate reader.

BMDM differentiation and in vitro GD model: Differentiation of BMDMs was performed by following protocol reported previously 25-53. Briefly monocytes were isolated from WT and KO bone marrow and cultured in RPMI1640, supplemented with L929 condition medium for 5 days to differentiation into macrophages. To mimic development of Gaucher cells in vitro, 50 µg/ml brain lysates (lg of mouse brain tissues were homogenized in 10ml of DMEM medium by Bio-Gen PRO200 Homogenizer from 1 min at highest speed) containing
various kinds of lipids, including sphingolipid, were added in the cell culture supernatant for 10 days. In the case of the in vitro rescue experiments, 0.1 and 0.4 g/ml PGRN were added at the same time with lipid. Cell culture mediums were replenished every three days. The levels of β-GlcCer were stained by immunofluorescence staining.

**[000227] Fluorescence labeling of active form of lysosomal GBA:** MDW933, a specific sensitive fluorescence dye for labeling active lysosomal GBA, was generously provided by Dr. Hermen E. Overkleeft at University of Leiden. BMDMs were cultured on cover glass, and MDW933 (50 nM) were added in culture medium for 2 hours to label lysosomal GBA. Cells were then fixed with 3% (v/v) paraformaldehyde in PBS for 15 min, and permeabilized by 0.1 mM NH₄Cl in PBS for 10 min. BMDMs were mounted with DAPI-medium, and fluorescence were visualized under confocal microscope.

**[000228] Knockdown of PGRN and HSP70 by siRNA approach:** siRNAs against mouse PGRN and HSP70 were purchased from Life Technology. RAW264.7 cells were transfected with 20 pmol of corresponding siRNA using Lipofectamine 2000. The cells were then treated with lipid mixture (50 µg/ml) for 24 hours and the level of active form of GBA were measured by MDW933 dye, and the knockdown efficiency of PGRN or HSP70 was examined by immunofluorescence staining using their specific antibodies.

**[000229] Lysosome staining in LSD fibroblasts:** Fibroblasts from different LSDs and healthy control were cultured on coverslip in 24-well plates in the absence or presence of recombinant PGRN protein (0.4 µg/ml), lipid lysis (50µg/ml), and PGRN plus lipid lysis for 24 hours. The next day fresh medium containing 100nM LysoTracker® Red was added for 1 hour. The cells were washed with PBS and fixed in 2% PFA. The coverslips were mounted on slides and the staining of lysosomes was imaged by confocal microscopy. Ten images were randomly taken from each sample, and fluorescence intensities were measured by Image J software.

**[000230] Human Study Participants:** 115 GD samples were collected from New York University School of Medicine by Dr. Pastores. 44 healthy controls from the general population and 55 healthy controls of Ashkenazi Jewish were provided by Dr. Saunders-Pullman from Beth Israel Medical Center. Each of the patients signed a consent form. This study was approved by Institutional Review Boards of New York University School of Medicine and Beth Israel Medical Center.

**[000231] Serum levels of PGRN in GD patients:** Serum levels of PGRN were measured by ELISA kit from Adipogen (San Diego, CA). Briefly, the ELISA plated were blocked with 300 µl blocking buffer for 30 min. Serum was diluted 200 fold by PBS. After blocking, load 100 µl
samples and standards for 2 hours. Wash the plates with PBS/tween for 5 times and add 100 µl Detection Antibody for 1 hour. Wash the plates again and add 100 µl Detector for another hour. Rinse the plates and add 100 µl TMB Substrate Solution, and the reaction was terminated by Stop solution. The results were recorded at 450 nm by plate reader. The concentrations of PGRN were calculated based on the standard curve.

[000232] Sequencing GRN gene: Genomic DNAs from 40 GD patients were used as templates to amplify full-length GRN gene, including 1kb promoter region and 8kb full-length GRN gene, by Phusion® High-Fidelity DNA Polymerases (NEB Inc, Ipswich, MA). All PCR products were mixed at equal molar ratio into one tube. The final sample was sent to Genomic facility of Yale University for gene sequencing by a novel technology, PacBio RS II Sequencing System 54. The sequence of each patient was sorted out by their barcode sequence, and full-length GRN sequence was aligned using basic local alignment with successive refinement (blatt) from Pacific Biosciences (github, Pacific Biosciences; Chaisson, MJ and Tesler, G (2012) BMC Bioinformatics 13:238), and then SAMtools (samtools sourceforge) were used to detect the variants in the patients samples.

[000233] Statistical analysis: For comparison of treatment groups, we performed unpaired t-tests, and one-way or two-way ANOVA (where appropriate). All statistical analysis was performed using SPSS Software. Statistical significance was two-sided and was achieved when at p<0.05.

[000234] REFERENCES

44 Wei, H. et al. ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders and are alleviated by chemical chaperones. Human molecular genetics 17, 469-477, doi:10.1093/hmg/ddm324 (2008).


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**EXAMPLE 2**

**PGRN DERIVATIVE PEPTIDE ATSTTRIN**

PGRN peptides, particularly including the PGRN derivative peptide denoted Atsttrin, have been evaluated and identified as having overlapping PGRN activity and, in some instances enhanced activity versus wild-type or full length PGRN. The PGRN derivative peptide Atsttrin is active as a modulator of TNF/TNFR activity and signaling, inhibiting or blocking TNF-mediated signaling or response, including TNF-a-induced inflammatory arthritis (Tang W et al (201 i) Science 332:478-484; WO 2010120374). Atsttrin is a PGRN-derived engineered protein (Antagonist of TNF/TNFR Signaling via Targeting TNF Receptors), comprising combinations of half units of PGRN units A, C and F in combination with linker units P3, P4 and P5 (US Patent 8,362,218; WO 2010120374). Atsttrin provides a PGRN-derived active peptide having overlapping activity and capability with the full length PGRN molecule.
PGRN derivative peptide Atstrrin was evaluated for activity and effects like PGRN in LSDs, including in the PGRN KO induced Gaucher's disease. Both PGRN and Atstrrin, the PGRN-derived engineered protein, were found to rescue β-GlcCer accumulation in PGRN KO macrophages. BMDM from PGRN KO mice were treated with 5C^g/ml lipid for 10 days, with or without various amounts of PGRN or Atstrrin (100 ng/ml or 400 ng/ml) and assessed (FIGURE 39). Under a light microscope, BMDM looks messy and the cells become disorganized after lipid treatment, and these morphological changes was partially rescued by either of PGRN and Atstrrin in a dose-dependent manner (upper panel) (FIGURE 39 first set of panels (LM)). β-GlcCer is accumulated with lipid treatment, and the accumulation is prevented by addition of either recombinant PGRN and Atstrrin in a dose-dependent manner (FIGURE 39).

PGRN and Atstrrin were found to rescue β-GlcCer accumulation in fibroblasts from GD patients. Fibroblasts were isolated from Type II GD patients and evaluated with no treatment, lipid mixture alone, or the lipid mixture combined with treatment with either PGRN (400ng/ml) or Atstrrin (400ng/ml). The fibroblasts were treated with 50 μg/ml lipid mixture, with or without added PGRN or Atstrrin for 2days. The expression of GBA and β-GlcCer were then measured by immunofluorescence staining, and images were acquired by confocal microscopy. The results (FIGURE 40) show that lipid treatment significantly induced GBA aggregation and β-GlcCer accumulation in GD fibroblasts, and that 400ng/ml PGRN treatment almost completely prevented the GBA aggregation and β-GlcCer storage. Unlike in BMDM from PGRN KO mice, Atstrrin exhibited less efficiency versus PGRN in rescuing the accumulation phenotype.

We found that both PGRN and Atstrrin works well in BMDM from PGRN KO mice, however, Atstrrin shows a somewhat lower efficacy at the same dose in GD fibroblast cells. It suggests that PGRN can get into fibroblasts successfully, while Atstrrin enters fibroblasts with less efficiency. The endocytosis of PGRN is mediated by Sortilin, and PGRN binds Sortilin with its last three C-terminal amino acids, while Atstrrin does not have this motif. We hypothesize that Atstrrin may not enter fibroblasts via a Sortilin-dependent endocytosis pathway. It is notable that increased concentrations of Atstrrin (see below) are effective to rescue β-GlcCer accumulation in GD fibroblasts. In contrast, both PGRN and Atstrrin can be taken up by macrophages, so both proteins are equally effective in macrophages.

Further studies showed that Atstrrin rescues β-GlcCer accumulation in a dosage-dependent manner, particularly at higher doses. Fibroblasts from GD patients were treated with 50 μg/ml lipid mixture (as described above), together with various amounts of Atstrrin (0^g/ml, 5μg/ml, and 50μg/ml). β-GlcCer level was evaluated and viewed by immunofluorescence...
staining (FIGURE 41). Atstrrin rescues β-GlcCer accumulation in a dose-dependent manner, and especially at 50 µg/ml.

**EXAMPLE 3**

**PGRN DERIVATIVE PEPTIDE ATSTRTRIN EFFECTIVENESS IN VARIOUS LYSOSOMAL STORAGE DISEASES**

Next, fibroblasts from LSD patients were obtained and treated with either PGRN or Atstrrin. Fibroblasts were evaluated from patients with various lysosomal storage diseases, including Gaucher's disease (GD); Tay-sachs disease (TSD); mucolipidosis (ML); mucopolysaccharidosis (MPS); metachromatic leukodystrophy (MLD); and Farber disease (FD). For each set of LSD disease fibroblasts, LSD fibroblasts were challenged with lipid alone or in combination with recombinant PGRN or Atstrrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red. Ten images for each sample were randomly taken under confocal microscope, and lysosome storage were quantified based on fluorescence intensity by image J software. The results are depicted in FIGURE 43 and FIGURE 44. As shown in the results, both Atstrrin and PGRN were effective and lysosome storage was significantly reduced in all LSD disease fibroblasts for Gaucher's disease Type I and II (GDI and GDII), Tay-sachs disease (TSD); mucolipidosis III (MLIII), mucopolysaccharidosis II, III and VI (MPSII, MPSIII, MPSVI), metachromatic leukodystrophy (MLD), and Farber disease (FD).

**[000242]** Fibroblasts from the chronic neuropathic form Gaucher's disease type III (GDIII) were evaluated. Fibroblasts from GD III patients were challenged with lipid alone or with recombinant PGRN or Atstrrin (0.4 µg/ml), respectively for 24 hours. The lysosome was stained with lysotracker-red and ten images for each sample were randomly taken and lysosome storage quantified based on fluorescence intensity. In this study, PGRN was significantly effective,
however lysosome storage, as evaluated by fluorescence intensity, was not significantly reduced with Atstrin treatment (FIGURE 45).

[000243] In LSD fibroblast studies, some diseased fibroblasts showed more minimal effects of PGRN or Atstrin to reduce lysosome storage. These are only a single set of experiments, however. Using the same approach as noted above, LSD fibroblasts from each of Fabry disease (FABRY) mucolipidosis IV (MLIV); mucopolysaccharidosis IVA and VII (MPSIVA and MPSVII) and Niemann-Pick disease A and B (NPDA and NPDB) were evaluated by challenging the LSD fibroblasts with lipid alone or recombinant PGRN or Atstrin (0.4 μg/πl) (FIGURE 46). In each of these specific LSD fibroblasts, effects of neither PGRN or Atstrin were found to be significant.

[000244] LSD fibroblasts from Krabbe Disease patients were similarly evaluated (FIGURE 47). Both PGRN and Atstrin were effective to significantly reduce lysosome lipid storage in Krabbe Disease patient fibroblasts.

[000245] Taken together, these studies demonstrate remarkable effectiveness of both progranulin PGRN and progranulin derivative peptides, as exemplified by PGRN peptide Atstrin, in reducing lysosomal storage in various recognized lysosomal storage diseases. Both PGRN and Atstrin significantly reduce lipid buildup in lysosomes in cells derived from patients with LSDs including Gaucher's disease, Tay-Sachs disease, metachromatic leukodystrophy, Farber disease, Krabbe disease and in forms of mucolipidosis and mucopolysaccharidosis.

EXAMPLE 4
EVALUATIONS OF PROGRANULIN IN THE LYSOSOMAL STORAGE DISEASE
TAY-SACHS DISEASE

[000246] Tay-Sachs disease (also known as GM2 gangliosidosis or hexosaminidase A deficiency) is a rare autosomal recessive inherited disorder that progressively destroys nerve cells in the brain and spinal cord. Tay-Sachs disease results when an enzyme (β-hexosaminidase A) (FIGURE 1) that helps break down fatty substances is absent and the disease occurs when harmful quantities of gangliosides accumulate in the brain's nerve cells, eventually leading to the premature death of the cells. A ganglioside is a form of sphingolipid, thus Tay-Sachs disease a member of the sphingolipidoses.

[000247] Tay-Sachs can become apparent in infancy (infantile Tay-Sachs disease), where infants lose motor skills such as turning over, sitting and crawling after age 3-6 months. As the
disease progresses, the child's body loses function, leading to blindness, deafness, paralysis and death. Later-onset forms of Tay-Sachs disease also occur but are very rare, initially seen in ages 2-10 (juvenile Tay-Sachs disease) or a later rare form seen in adults aged 30s or 40s (adult/late-onset Tay-Sachs disease). Characteristic features include muscle weakness, loss of muscle coordination (ataxia), spasticity, and other movement problems, speech problems, cognitive decline, and mental illness. Mutations in the \( \text{HEXA} \) gene encoding the alpha subunit of the enzyme \( \beta \)-hexosaminidase A cause Tay-Sachs disease. \( \beta \)-hexosaminidase A enzyme is located in lysosomes and helps break down GM2 ganglioside. GM2 ganglioside accumulates in lysosomes to toxic levels. Patients with and carriers of Tay-Sachs can be identified by a blood test that measures hexosaminidase A activity or by evaluating for mutations in the \( \text{HEXA} \) gene. Tay-Sachs disease is noted as increased in prevalence in Ashkenazi Jewish people. A four base pair insertion in exon 11 (1278insTATC) resulting in an altered reading frame for the \( \text{HEXA} \) gene has been identified as the most prevalent \( \text{HEXA} \) mutation in the Ashkenazi population. This mutation is also found in Cajun people in Louisiana. Two other unrelated mutations have been identified in French Canadians and associated with Tay-Sachs.

[000248] There is currently no cure or treatment for Tay-Sachs disease. Even with the best care, children with infantile Tay-Sachs disease die by the age of 4. Patients receive supportive care to ease the symptoms or extend life. In late-onset Tay-Sachs, medication (e.g., lithium for depression) can sometimes control psychiatric symptoms and seizures. Recently, researchers discovered that Pyrimethamine can increase \( \beta \)-hexosaminidase activity, thus potentially slowing down the progression of Late-Onset Tay-Sachs disease (Clarke JT, et al (2004) \emph{Molecular Genetics and Metabolism} 102 (1): 6-12; Osher E, et al (2011) \emph{Mol. Genet. Metab.} (Molecular Genetics and Metabolism) 102 (3): 356-63).

[000249] The above example studies and FIGURE 33 evaluated lysosomes in fibroblasts from LSD patients, including Tay-Sachs disease patients. Fibroblasts from healthy control and different LSDs including Tay-Sachs disease were treated with progranulin (PGRN) with and without lipid stimulation. PGRN significantly corrected lysosomes in Tay-Sachs disease patient fibroblasts. The following studies were undertaken to additionally evaluate the role of PGRN in Tay-Sachs disease and as a modulator.

[000250] First, levels of the HexA protein and also HexB (which encodes the beta subunit of the enzyme (\( \beta \)-hexosaminidase A) were evaluated in PGRN KO mice. Lung tissues from WT and PGRN KO mice after OVA challenge (described above in earlier examples) were stained for HexA and HexB by immunohistochemistry (HexA antibody sc-376777, and HexB antibody sc-
134581are from Santa Cruz). The results are depicted in FIGURE 37 and demonstrate that HexA but not HexB is aggregated in PGRN mice.

[000251] Also, aged PGRN KO mice were evaluated for GM2 ganglioside in their brains. Brain tissue from aged PGRN KO mice was stained with a GM2 antibody by immunohistochemistry and compared to WT mice (GM2 antibody, Cat. No. 345759, EMD Millipore). FIGURE 38 shows elevated GM2 in the brain of aged PGRN KO mice. Thus we conclude that with PGRN knockout, HexA is aggregated and GM2 accumulated in the brain. Absence of PGRN results in accumulation of GM2 ganglioside and altered HexA, which are both indicators of Tay-Sachs disease, further indicating a role of PGRN in Tay-Sachs disease and providing further reasoning for PGRN effects in Tay-Sachs disease patients and patient cells.

[000252] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[000253] Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.
WHAT IS CLAIMED IS:

1. A composition for treatment or alleviation of a lysosomal storage disease comprising isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50.

2. The composition of claim 1 further comprising an enzyme replacement therapy agent or substrate reduction therapy agent for a lysosomal storage disease.

3. The composition of claim 2 further comprising one or more of glucocerebrosidase, α-galactosidase, β-galactosidase, β-hexosaminidase and sphingomyelinase.

4. The composition of claim 2 comprising PGRN or atsttrin in combination with glucocerebrosidase for treatment or alleviation of Gaucher’s Disease.

5. The composition of claim 2 comprising PGRN or atsttrin in combination with β-hexosaminidase A for treatment or alleviation of Tay-Sachs Disease.

6. The composition of claim 1, further comprising one or more molecular chaperone or lysosomal delivery protein.

7. The composition of claim 6 wherein the molecular chaperone or lysosomal delivery protein is HSP70.

8. The composition of any of claims 1-7 which is a pharmaceutical composition and further comprises a pharmaceutically acceptable carrier, vehicle, diluent or excipient.

9. A method for facilitating lysosomal delivery of a protein or enzyme in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50.
10. The method of claim 9 for facilitating delivery of glycocerebrisidase (GBA) in a patient with Gaucher's Disease comprising administering to said patient isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50.

11. The method of claim 9 for facilitating delivery of β-hexosaminidase A (HexA) in a patient with Tay-Sachs disease comprising administering to said patient isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50.

12. A method for treating or alleviating a lysosomal storage disease in an animal comprising administering to said animal isolated PGRN, or active fragments thereof including atsttrin, wherein said PGRN or active fragment comprises an amino acid sequence as set out in any of FIGURES 49 or 50.

13. The method of claim 12 comprising additionally administering one or more lysosomal enzyme which is reduced, absent, mutated or altered in the lysosomal storage disease.

14. The method of claim 12 wherein the lysosomal storage disease is selected from Gaucher's Disease (GD), Tay-Sachs disease, Fabry disease, Farber disease, Sandhoff disease, G_{M1} gangliosidosis, Krabbe disease, Niemann-Pick Disease (Type A, Type B, Type C), Pompe disease, mucolipidosis Type II (Hunter syndrome), mucolipidosis Type IIIA, infantile free sialic acid storage disease (ISSD), lysosomal acid lipase deficiency, Juvenile Hexosaminidase A deficiency, Wollman disease and Salla disease.

15. The method of claim 12 wherein the lysosomal storage disease is selected from Gaucher's disease (GD), Tay-sachs disease (TSD), mucolipidosis (ML), mucopolysaccharidosis (MPS), metachromatic leukodystrophy (MLD), Farber disease (FD) and Krabbe disease (KD).
16. The method of claim 12 comprising additionally administering the lysosomal enzyme glycocerebrosidase (GBA) or an active fragment or recombinant form thereof for treating or alleviating Gaucher's Disease.

17. The method of claim 12 comprising additionally administering the lysosomal enzyme β-hexosaminidase A (HexA) or an active fragment or recombinant form thereof for treating or alleviating Tay-Sachs disease.

18. The method of claim 12 wherein the lysosomal enzyme is selected from one or more of a glucocerebrosidase, α-galactosidase, β-galactosidase, β-hexosaminidase and sphingomyelinase.

19. A method for diagnosing or evaluating lysosomal storage disease in an animal comprising determining the expression or activity of PGRN or detecting one or more mutation in the genomic DNA or gene encoding PGRN in said animal.

20. The method of claim 19 comprising additionally determining the expression or activity of one or more lysosomal enzyme or detecting one or more mutation in the genomic DNA or gene encoding one or more lysosomal enzyme in said animal.

21. The method of claim 19 for diagnosing or evaluating Gaucher's disease in an animal.

22. The method of claim 19 for diagnosing or evaluating Gaucher's disease comprising additionally determining the expression or activity of GBA or detecting one or more mutation in the genomic DNA or gene encoding GBA in said animal.

23. The method of any of claims 19-22 wherein one or more PGRN mutation selected from rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A is determined.
24. The method of claim 22 wherein one or more PGRN mutation selected from rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A is determined.

25. The method of claim 24 wherein the PGRN SNP sites are determined by Tagman genotyping methods wherein the primers are selected from: for mutation rs4792937 forward primer, 5'-TGTCCTGGAAA CCATCCTTC-3' (SEQ ID NO: 11), reverse primer 5'-CTCCCAAAGC GATTTCTCTA-3' (SEQ ID NO: 12), and Taqman tag sequence 5'-TCAGTAGCTCACA[T/C]TTGTAA-3' (SEQ ID NO: 13); for mutation rs850713 forward primer 5'-CCTTCCC T GAGTGGGCTGGTA-3' (SEQ ID NO: 14), reverse primer 5'-AGT GCACCCGTCTCTCACAG C-3' (SEQ ID NO: 15), and Taqman tag sequence 5'-AGGTGACCTGGG GAGATGGGG[A/G]TATGTGGAGGAAGTGGG GGCAGAG-3' (SEQ ID NO: 16); for mutation rs78403836 forward primer 5'-CTGTCCTCTCCATGGCTAC-3'(SEQ ID NO: 17), reverse primer 5'-GCGGACCTGTAAGCATGAAT-3' (SEQ ID NO: 18), and Tagman tag sequence 5'-AGGAAGAC[G/C]TGATTTT-3' (SEQ ID NO: 19); for mutation rs5848 forward primer 5'-CCAGGGGTACCAAGTGTTTG-3' (SEQ ID NO: 20), reverse primer 5'-CACAGGGGTCCACTGACCAG-3' (SEQ ID NO: 21), and Taqman tag sequence TCTGCTCAGGCCTCCCTAGCAACCT[GC/ TJCCCTAACCAATTCTCCCTGGACCC (SEQ ID NO: 22); and for point mutations of p.C315S, p.E316Q, and p.P365A wherein the primers for PCR amplification comprise forward primer 5'-GGTGGTGTAAGCGGTAC-3' (SEQ ID NO: 23) and reverse primer 5'-ACCTGCCCAGCAGGATGC-3' (SEQ ID NO: 24), followed by sequencing.

26. A kit for diagnosing or evaluating lysosomal storage disease in an animal by detecting the presence or activity and amount of PGRN comprising

(a) a predetermined amount of a detectably labelled specific binding partner of or antibody directed against PGRN;

(b) other reagents; and

(c) directions for use of said kit.
27. A kit for diagnosing or evaluating lysosomal storage disease in an animal by detecting the presence of a PGRN mutation in said animal comprising
   (a) one or more nucleic acid probe or primer specific for or directed against the PGRN gene or encoding DNA;
   (b) other reagents; and
   (c) directions for use of said kit.

28. The kit of claim 27 wherein one or more nucleic acid primer or probe is specific for or suitable for detection or determination of a PRN mutation selected from rs4792937, rs850713, rs78403836, rs5848, and three point mutations, p.C315S, p.E316Q, and p.P365A

29. The kit of any of claims 26-28 for diagnosing or evaluating Gaucher’ s disease, wherein said kit further comprises a detectably labelled specific binding partner of or antibody directed against GBA or one or more nucleic acid probe or primer specific for or directed against the GBA gene or encoding DNA

30. An animal model for Gaucher’ s Disease wherein the animal comprises altered PGRN, wherein PGRN is null, absent or mutated and lysosomal substrate β-GlcCer is increased in macrophages.

31. The animal model of claim 30 additionally comprising a GBA mutation associated with Gaucher’ s Disease or wherein GBA is null or absent.

32. An animal model for Tay-Sachs disease wherein the animal comprises altered PGRN, wherein PGRN is null, absent or mutated and lysosomal substrate β-hexosaminidase A is increased in macrophages.
FIG. 3

KO

WT

Macrophage

lysosome
FIG. 5

a

Imiglucerase  OVA  Ctrl

H&E  PAS

b

pmol/mg protein

0  500  1000  1500

C16  C22  C24  C24

Ctrl  OVA  OVA+Imig.

c

Spleen/BW (mg/g)  Liver/BW (mg/g)  Cyto/Nu ratio  Gaucher cells (mm²)

WT  PBS Imiglucerase  NO OVA  PBS Imiglucerase

WT  PBS Imiglucerase  NO OVA  PBS Imiglucerase

*  **  ***  ****
FIG. 6
FIG. 9
FIG. 11

[Image of a bar graph showing GBA activity (nM/mg/h) for WT and KO groups, along with a Western blot image comparing Actin and GBA expression in WT and KO conditions.]
FIG. 13
FIG. 14
FIG. 16
FIG. 17
FIG. 19
FIG. 20

(a) DAG (pmol/mg protein)

(b) SM (pmol/mg protein)

(c) Ceramide (pmol/mg protein)
FIG. 23

a) GlcCer (pmol/ml plasma)

- 3M
- 6M
- 15M

No significant difference (NS)

b) GlcCer (pmol/mg protein)

- 3M
- 9M
- 15M

Significant difference (***, *)

c) GlcCer (pmol/mg protein)

- 3M
- 9M
- 15M

Significant difference (***, *)
FIG. 29

<table>
<thead>
<tr>
<th>LAMP2 PGRN</th>
<th>LIMP2 PGRN</th>
<th>GBA PGRN</th>
<th>EEA1 PGRN</th>
<th>PGRN</th>
</tr>
</thead>
</table>

0  
10
30
60
120 (min)
FIG. 33

a

ML III FD TSD GD II GD I WT

b

Lipid PGRN Ctrl

Fluorescence intensity

ML III FD TSD GD II GD I WT

Fluorescence intensity

Fluorescence intensity

Fluorescence intensity

Fluorescence intensity
FIG. 35
FIG. 40

Lipid 50 (μg/ml)
PGf200 (μg/ml)
Astaxin 400 (μg/ml)

Merge

DAPI

GlCeCer

GBA

LM
FIG. 41

The figure shows a series of images under various conditions. The images are labeled as 'Merge', 'DAPI', and 'GlcCer' with corresponding lipid 50 and Aestrin concentrations.

- **Merge** images show the combined view of the samples.
- **DAPI** images highlight the cell nuclei.
- **GlcCer** images display the distribution of GlcCer.

Each row represents a different concentration of Aestrin (µg/ml) and lipid 50 (µg/ml) as indicated by '+' symbols.

- **0µg/ml** and **0µg/ml** (top row).
- **0µg/ml** and **0.5µg/ml** (second row).
- **0µg/ml** and **5µg/ml** (third row).
- **0µg/ml** and **50µg/ml** (fourth row).

The images provide a visual comparison of the effects of these concentrations on the samples.
FIG. 45

GD III

Ctrl
lipid
lipid+P
lipid+Ats

GD III

Fluorescence intensity

Ctrl
lipid
lipid+P
lipid+Ats

45/53
FIG. 46

NPD B  NPD A  ML IV  MPS VII  MPS IVA  FABRY

Ctrl
lipid
lipid+P
lipid+Ats

NPD A  MPS VII  FABRY

Fluorescence intensity

MPS IVA

Fluorescence intensity

ML IV

Fluorescence intensity

NPD B
FIG. 49

A  Human PGRN

1  MWTILSVWALTAAGVLGTRCPDGCFCVFACCLDPGGASYSSCCRPLLDKWPTTLSRHLGGF
61  CQVDAHCSAGHSCITFVSGTSSCPCFEEAVACDGHHCCPRGFCRGCSADGRSCFQRSQGNNS
121  VGAICQCPDSQFECRDSTCCVMVDSWGCPCMPQASCCEEDRHCPCPHAGACDVLHVCRTIT
181  PTGTHPLAKLPAQRNTAVALSVMCPDARSKCPDGSTCELFSGKYGCMPMNATCC
241  SDHELCCPQGPTVCDLIQSKLCSSKNATDILTKPLAHTVGDVKGCMEVSCPDGYTCCRLQ
301  SGAWGCPCPTQAVCCEDHLHCPAGFTCDTQKGTCEQPQFQPVFMKAPALSLPQAL
361  KRDVPCDNVSSCPSDDTCQLTSGEGWGCPIPFEAVCCSDHQCHCPQGYTCVAEGQQQRS
421  EIVAGLEKMPARRALSHPRDIGCDQHTSACPQGTCCPSLGGSWACCQPHAVCCEDRQH
481  CCPAGYTNCVKARSCEKEVVSAQPFATFLARSHPHVGVKDVCEGCHFCHDNQTCRDNQRG
541  WACCYPQGVCADRRHCCPAGFRCAARGTKCLRREAPRDAFPLDPATLQRL

B  Mouse PGRN

1  M#ILSVWLALVARLVAGTQC PDQQCFCVAC CLDQGGANYS CNCPLLDTWP IITSRRLDGS
61  CQIRDHCPCYSSCLLYGSTSSCSSFPFESGV SCDGQCHCPP RGFIHCSDAGXK SCQSISDSLL
121  GAVQCPGSQF ECPDSATCCI MIDGSCCPC MPQASCCEBDR VHCCPHGASC DLVHTRCISP
181  TGHYPLKLFPAQRNTRAVA SFSVCPDAD TQCPDDSTCC ELPTKGYGCC MPNACCSSD
241  HLHCCPQDTV CIDLQSKCIS KDTYDLMTRT LPYGPVNEVK CDELVSCPDD YTCCRLNTGA

GrnA

301  WGCPPTFKA VCCEDHHCFF AGFQCHTETG TCELVYQLQP WMKTVANALS LFDPQLKND
361  VPCEFDSSCP SNNTCCRLSS GDWGCCHHEP AYCCIDQHCFC QFGFKCHMD GCQCKGDV

GrnC

421  AGLEKMPVRQ TTLLQHGFDC CDQHTSCPVQ QTCPSTLKS WACQQLPHAV CEDQRCHCCP

GrnD

481  AGYTCNKAR TCEKDAGVSQ PSMDLTFGSK VGNECGAGH FCHEADQSCCK DSQGGMACCF

GrnE

541  YVKGVCCRDG RHCCPIGFHC SAGTCLKLK KTPRWDILLR DPAPRELL
**FIG. 50**

**Atettrin peptide sequence** (1/2F+P3+P4+1/2A+P5+1/2C):

PQASCCEDRVHCCPHGAFCDLVHTRCITPTGTHPLAKKLPQRTNRVALSSASSKเKENATDDLTLTKPAHTVGDVKCDMEVSCPDGYTCRLoQS GoodmanQIPwCEQGPHQVPwMEKAPAHLSLPDPQAŁKRDVPQCDNVSSCPSSDTCCQLTSGENGCCPIP

**Other peptide Sequences:**

F + P3:

IQCPDSQFECPDFSTCCVMVGSGAGCCPMQPQASCCEDRVHCCPHGAFCDLVHTRCITPTGTHP

P4 + A:

SKENATDDLTLTKPAHTVGDVKCDMEVSCPDGYTCRLoQS GoodmanQIPwCEQGPHQVPwMEKAPAHLSLPDPQAŁKRDVPQCDNVSSCPSSDTCCQLTSGENGCCPIP

P5 + C:

Q6PHQVPwMEKAPAHLSLPDPQAŁKRDVPQCDNVSSCPSSDTCCQLTSGENGCCPIPwCEQGPHQVPwMEKAPAHLSLPDPQAŁKRDVPQCDNVSSCPSSDTCCQLTSGENGCCPIP

1/2F+P3+P4+1/2A:

PQASCCEDRVHCCPHGAFCDLVHTRCITPTGTHPLAKKLPQRTNRVALSSASSKเKENATDDLTLTKPAHTVGDVKCDMEVSCPDGYTCRLoQS GoodmanQIPwCEQGPHQVPwMEKAPAHLSLPDPQAŁKRDVPQCDNVSSCPSSDTCCQLTSGENGCCPIP

P4+1/2A+P5+1/2C:

SKENATDDLTLTKPAHTVGDVKCDMEVSCPDGYTCRLoQS GoodmanQIPwCEQGPHQVPwMEKAPAHLSLPDPQAŁKRDVPQCDNVSSCPSSDTCCQLTSGENGCCPIP
## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

**IPC(8) - A61 P 3/30 (2015.01)**

**CPC - A61K 38/00 (2015.04)**

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

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

- IPC(8) - A61K 38/00, 38/17; A61P 3/30 (2015.01)
- CPC - A61K 38/00, 38/17, 38/1709; C12Q 1/6883, 2600/16, 2600/156; C07K 14/47, 14/435, 14/ (2015.04)

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

- CPC - A61K 38/00, 38/17, 38/1709; C12Q 1/6883, 2600/16, 2600/156; C07K 14/47, 14/435, 14/ (2015.04) (keyword delimited)
- USPC - 435/69.1, 375; 514/1.1

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

- PatBase, Google Patents, PubMed

Search terms used: Progranulin, PGRN, PC-cell derived growth factor, PCDGF, acrogranin, Granulin/epithelin, precursor, proepithelin, GP80

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>US 2013/0157945 A1 (LIU) 20 June 2013 (20.06.2013) entire document</td>
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<td>Y</td>
<td>US 2013/0230506 A1 (JENSEN et al) 05 September 2013 (05.09.2013) entire document</td>
<td>6, 7</td>
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</table>

**X** Further documents are listed in the continuation of Box C. [1]

- "A" Special categories of cited documents:
  - "E" earlier application or patent but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "Z" document member of the same patent family

Date of the actual completion of the international search: 11 May 2015

Date of mailing of the international search report: 1 JUN 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer: Blaine R. Copenhaver
PCT Helpdesk: 571-272-4300
PCT.GSD: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)
**DOCUMENTS CONSIDERED TO BE RELEVANT**

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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>Box No. I</td>
<td>Nucleotide and/or amino acid sequence(s) (Continuation of item 1c of the first sheet)</td>
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1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)
   - [ ] on paper
   - [x] in electronic form

   b. (time)
   - [x] in the international application as filed
   - [ ] together with the international application in electronic form
   - [x] subsequently to this Authority for the purposes of search

2. [x] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 2-9 and 11-24 were searched.