US 20200140858A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2020/0140858 A1**

Saha et al. (43) **Pub. Date: May 7, 2020**

(54) **GENE CORRECTION OF POMPE DISEASE AND OTHER AUTOSOMAL RECESSIVE DISORDERS VIA RNA-GUIDED NUCLEASES**

(71) Applicant: **Wisconsin Alumni Research Foundation**, Madison, WI (US)

(72) Inventors: **Krishanu Saha**, Middleton, WI (US); **Jared Matthew Carlson-Stevermer**, Burlingame, CA (US); **Lucille Katherine Kohlenberg**, Elm Grove, WI (US)

(21) Appl. No.: **16/674,448**

(22) Filed: **Nov. 5, 2019**

Related U.S. Application Data

(60) Provisional application No. 62/755,980, filed on Nov. 5, 2018.

Publication Classification

(51) **Int. Cl.**

C12N 15/11	(2006.01)
C12N 9/22	(2006.01)
C12N 15/86	(2006.01)
A61K 35/545	(2006.01)
A61P 3/00	(2006.01)

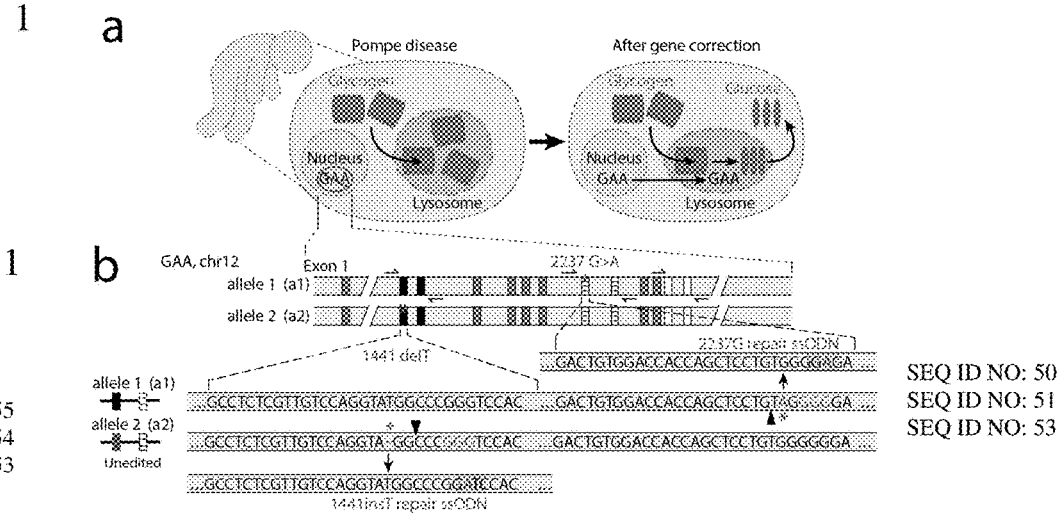
(52) **U.S. Cl.**

CPC ***C12N 15/11*** (2013.01); ***C12N 9/22*** (2013.01); ***C12N 15/86*** (2013.01); *C12N 2310/351* (2013.01); ***A61P 3/00*** (2018.01); *C12N 2310/20* (2017.05); *C12N 2800/80* (2013.01); ***A61K 35/545*** (2013.01)

(57) **ABSTRACT**

Described herein are guide RNAs and modified guide RNAs suitable for biallelic correction of Pompe disease. Also included are methods of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has an autosomal recessive disorder with compound heterozygous mutations, the methods including delivering a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide. The first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele, and the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele.

Specification includes a Sequence Listing.



SEQ ID NO: 55
SEQ ID NO: 54
SEQ ID NO: 53

SEQ ID NO: 50
SEQ ID NO: 51
SEQ ID NO: 53

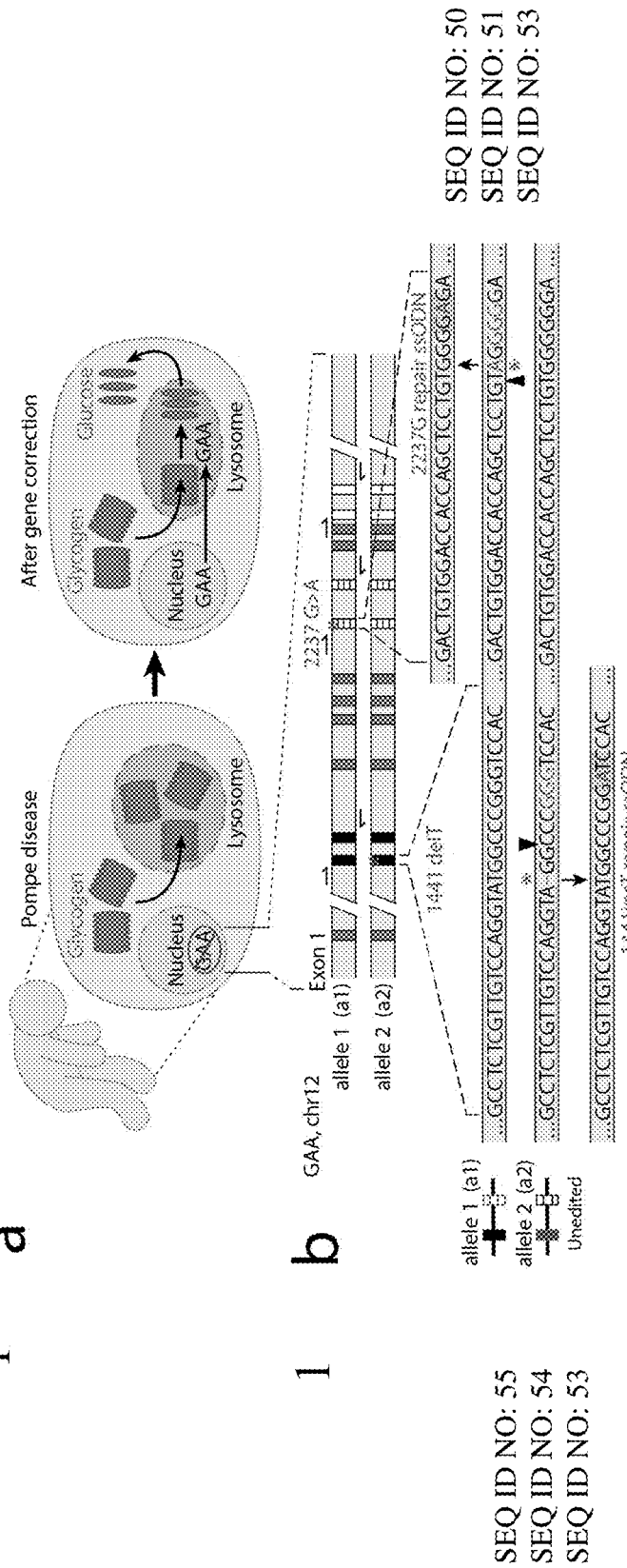

a
Pompe disease
Glycogen
Nucleus
GAA
Lysosome
After gene correction
Glycogen
Glucose
Nucleus
GAA
GAA
Lysosome
b
GAA, chr12
Exon 1
allele 1 (a1)
allele 2 (a2)
2237 G>A
1441 delT
2237G repair ssODN
...GACTGTGGACCACCAGCTCCTGTGGGGGGA...
...GCCTCTCGTTGTCCAGGTATGGCCCGGGTCCAC...
...GACTGTGGACCACCAGCTCCTGTGGGGGGA...
...GCCTCTCGTTGTCCAGGTA-GGCCCGGGTCCAC...
...GACTGTGGACCACCAGCTCCTGTGGGGGGA...
...GCCTCTCGTTGTCCAGGTATGGCCCGGATCCAC...
1441ins1 repair ssODN
allele 1 (a1)
allele 2 (a2)
Unedited
SEQ ID NO: 50
SEQ ID NO: 51
SEQ ID NO: 53
SEQ ID NO: 55
SEQ ID NO: 54
SEQ ID NO: 53

FIG. 1 a and b

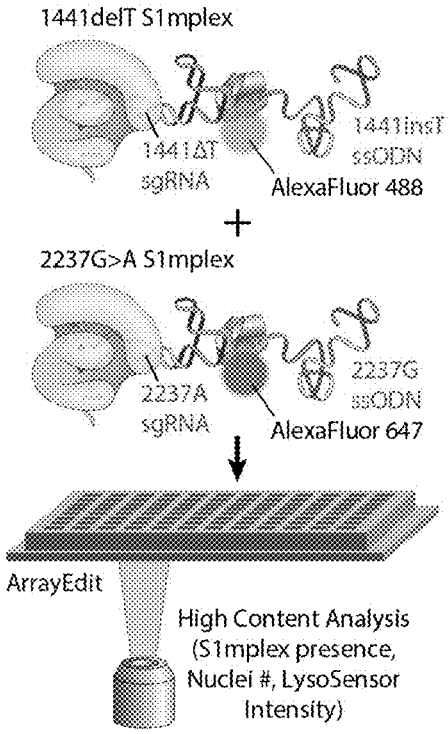

1441delT S1mplex
1441ΔT
sgRNA
1441insT
ssODN
AlexaFluor 488
+
2237G>A S1mplex
2237A
sgRNA
2237G
ssODN
AlexaFluor 647
ArrayEdit
High Content Analysis
(S1mplex presence,
Nuclei #, LysoSensor
Intensity)

FIG. 1c

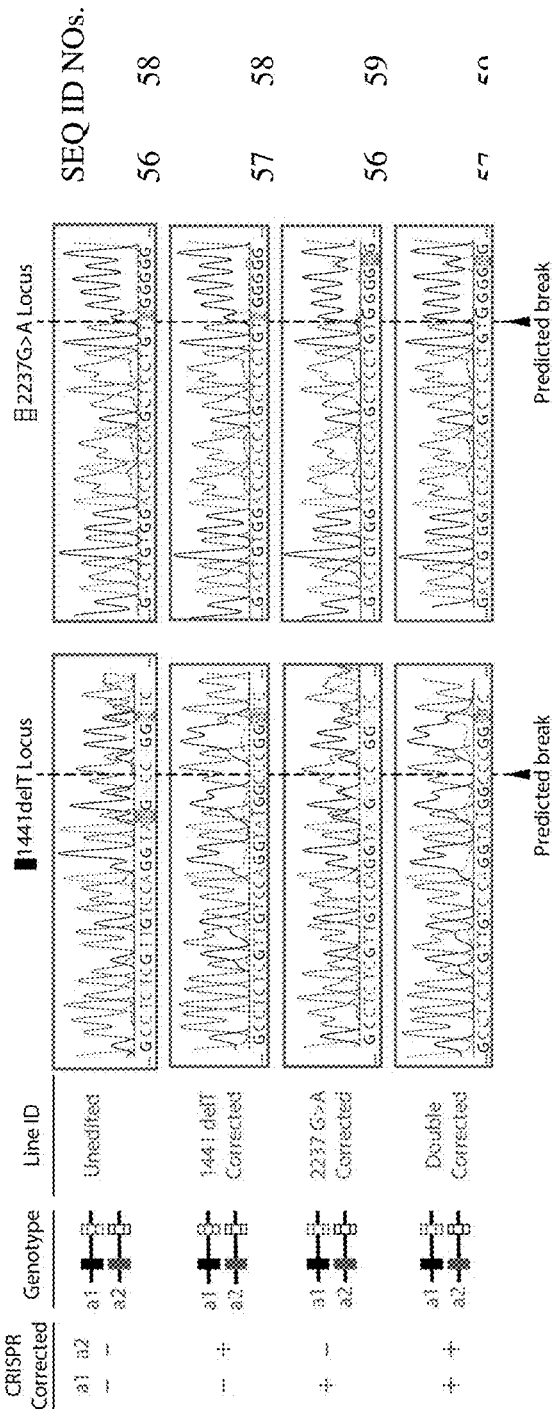

CRISPR Corrected
a1 a2
– –
+ –
– +
+ +
Genotype
a1
a2
Line ID
Uneditted
1441 delT Corrected
2237 G>A Corrected
Double Corrected
1441delT Locus
2237G>A Locus
Predicted break
Predicted break
SEQ ID NOs.
56 58
57 58
56 59

Fig. 1d

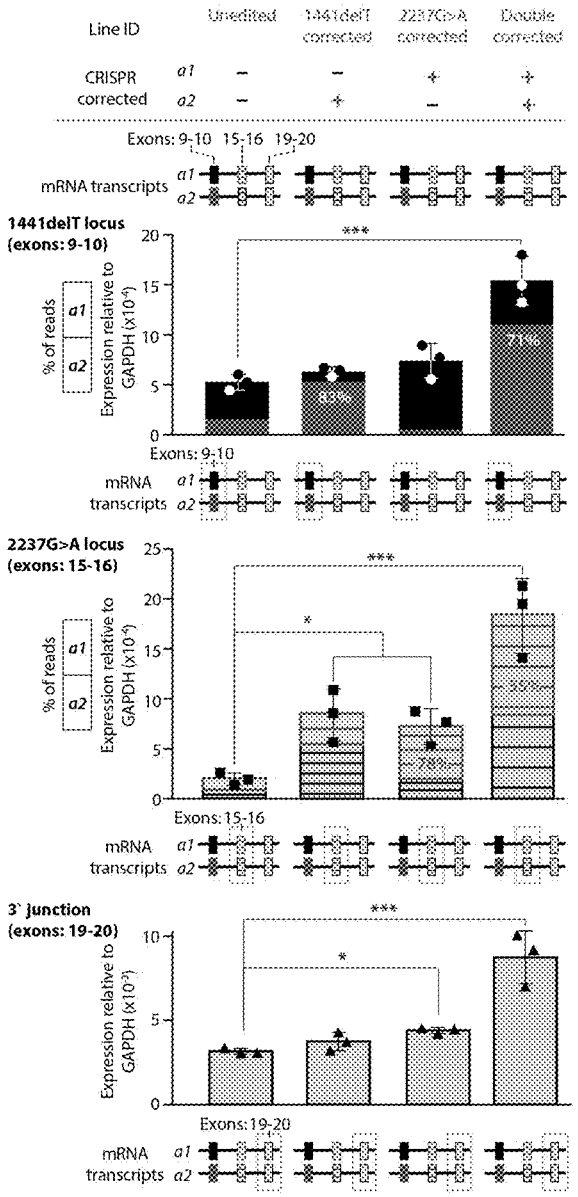

Line ID
Unedited
1441delT corrected
2237G>A corrected
Double corrected
CRISPR corrected
a1
a2
Exons: 9-10 15-16 19-20
mRNA transcripts
1441delT locus (exons: 9-10)
% of reads
Expression relative to GAPDH (x10⁻⁴)
***
83%
71%
Exons: 9-10
2237G>A locus (exons: 15-16)
*
Exons: 15-16
3` junction (exons: 19-20)
Expression relative to GAPDH (x10⁻³)
Exons: 19-20

FIG. 2a

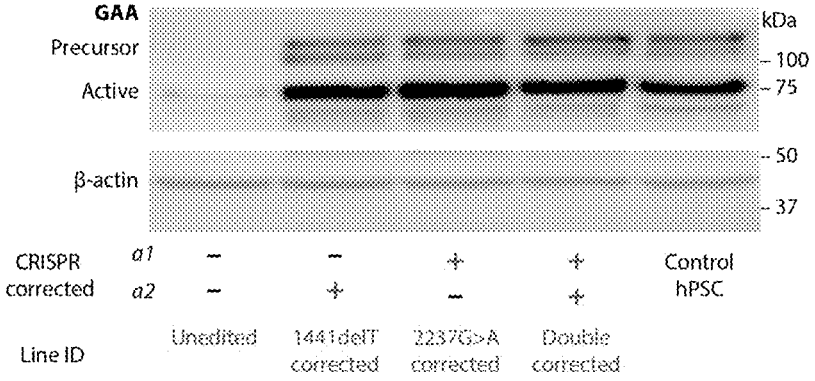

GAA
Precursor
Active
kDa
100
75
β-actin
50
37
CRISPR corrected
a1
a2
Control hPSC
Line ID
Unedited
1441delT corrected
2237G>A corrected
Double corrected

FIG. 2b

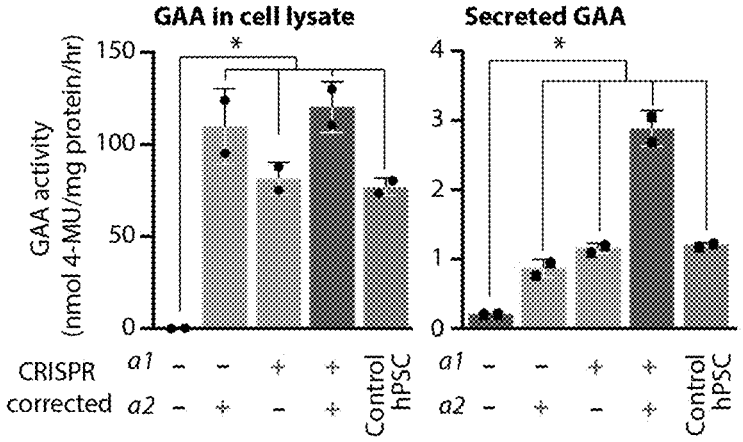

GAA in cell lysate
Secreted GAA
GAA activity (nmol 4-MU/mg protein/hr)
150
100
50
0
4
3
2
1
0
*
CRISPR corrected
a1
a2
Control hPSC

FIG. 2C

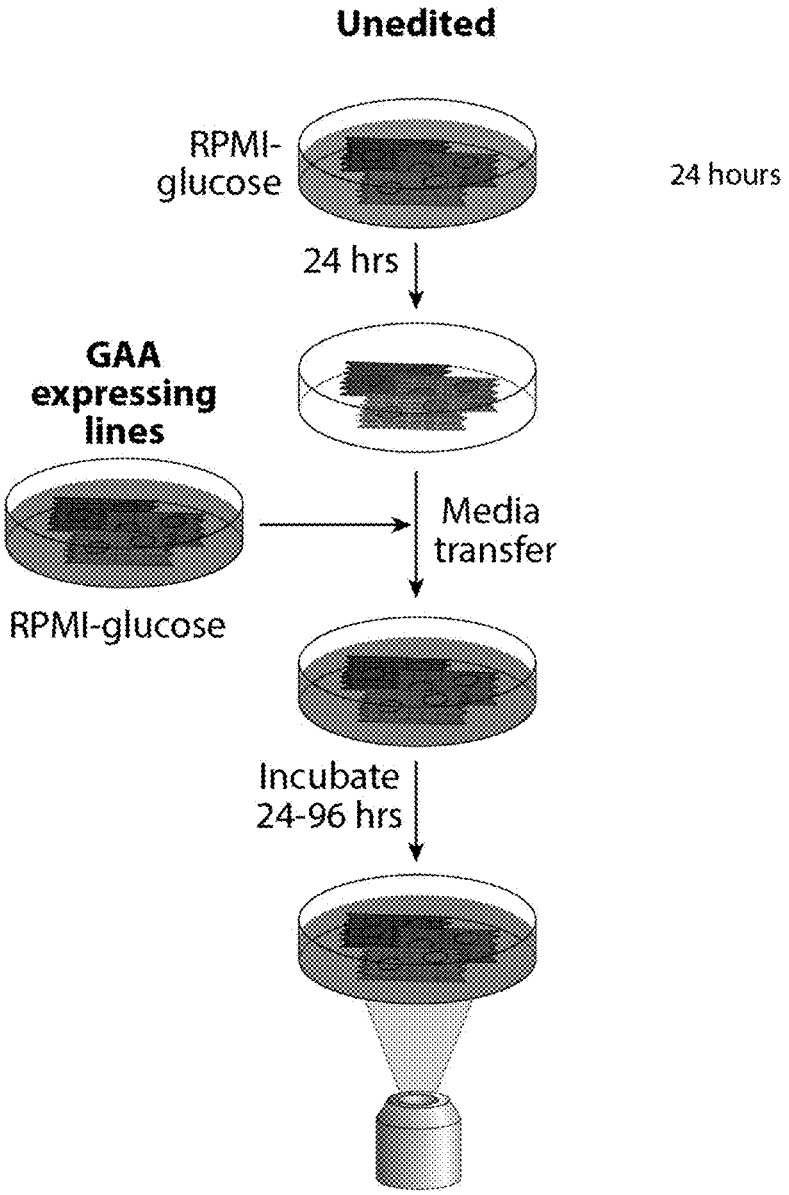

Unedited
RPMI-glucose
24 hours
24 hrs
GAA expressing lines
Media transfer
RPMI-glucose
Incubate 24-96 hrs

FIG. 3a

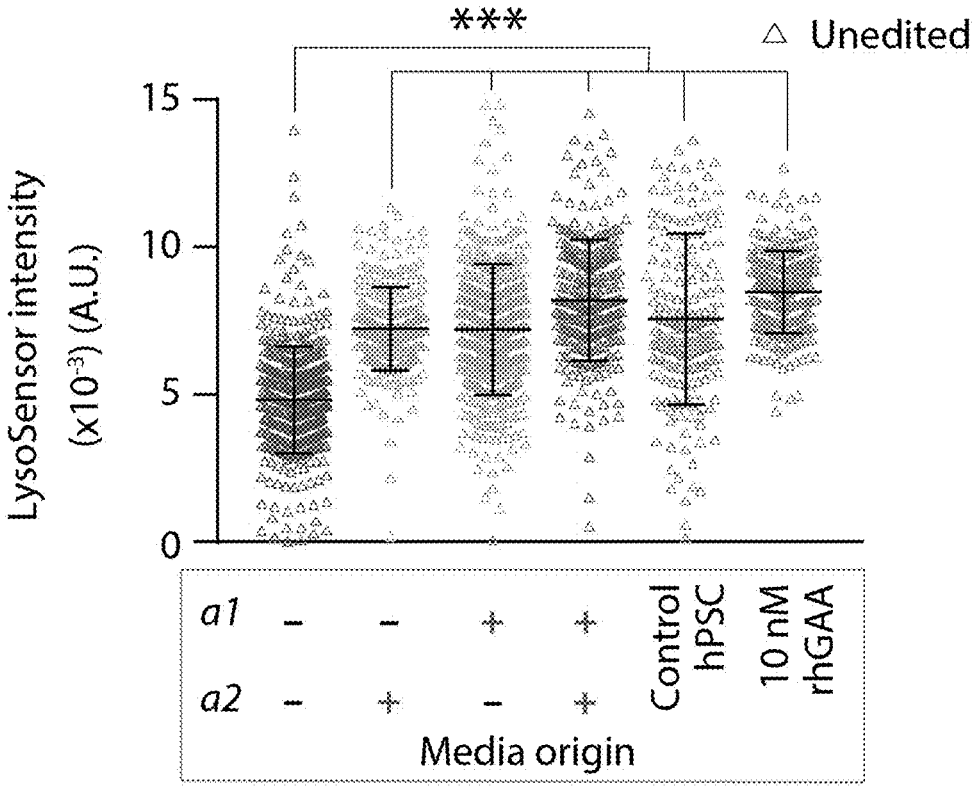

***
△ Unedited
LysoSensor intensity
(x10⁻³) (A.U.)
15
10
5
0
a1 – – + +
a2 – + – +
Control hPSC
10 nM rhGAA
Media origin

FIG. 3b

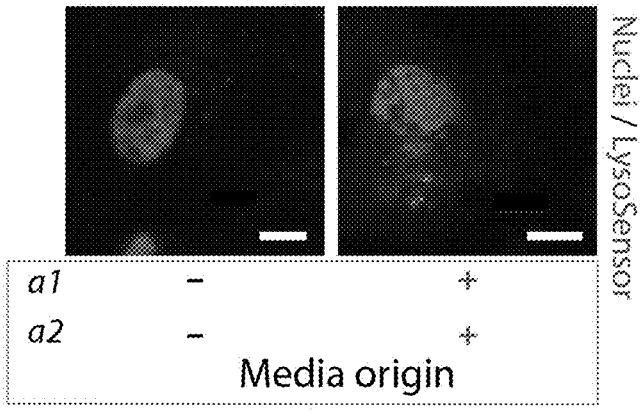

Nuclei / LysoSensor
a1 – +
a2 – +
Media origin

FIG. 3c

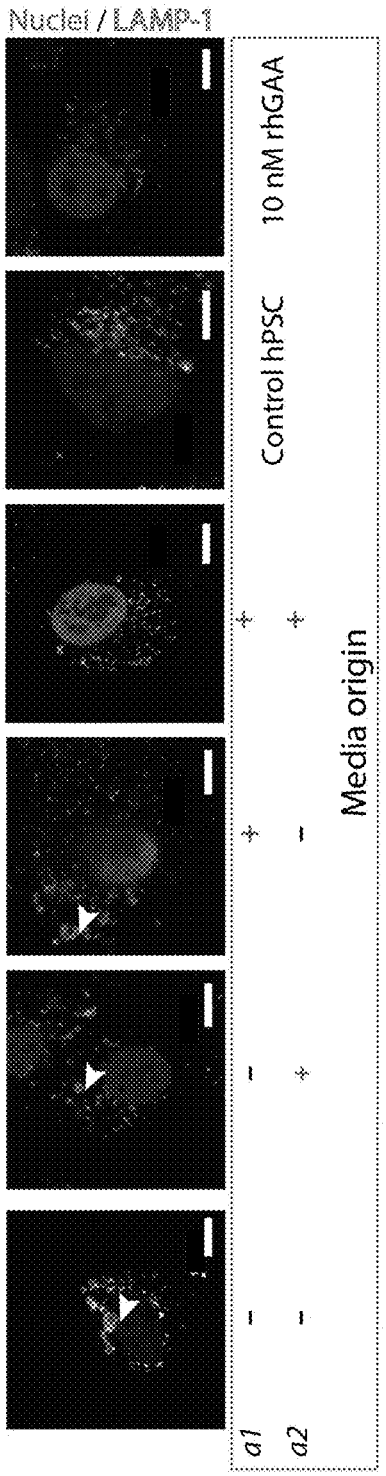


FIG. 3d

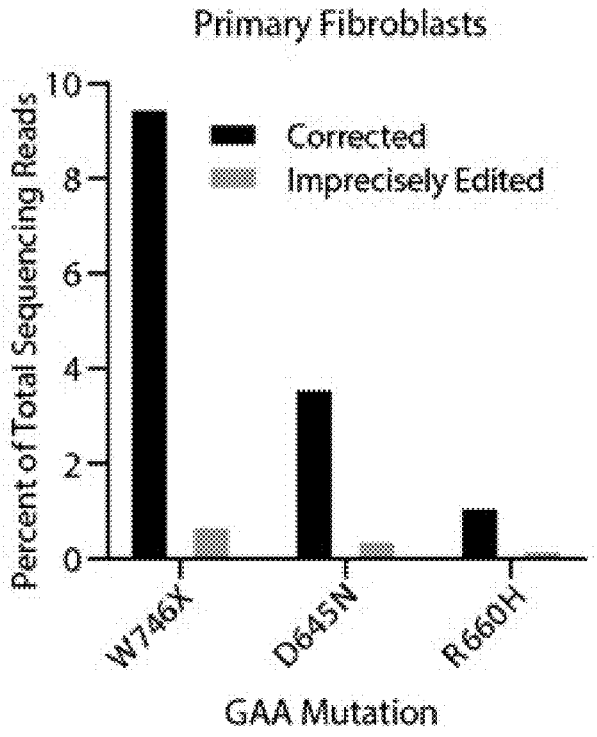

Primary Fibroblasts
Percent of Total Sequencing Reads
10
8
6
4
2
0
Corrected
Imprecisely Edited
W746X
D645N
R660H
GAA Mutation

FIG. 4a

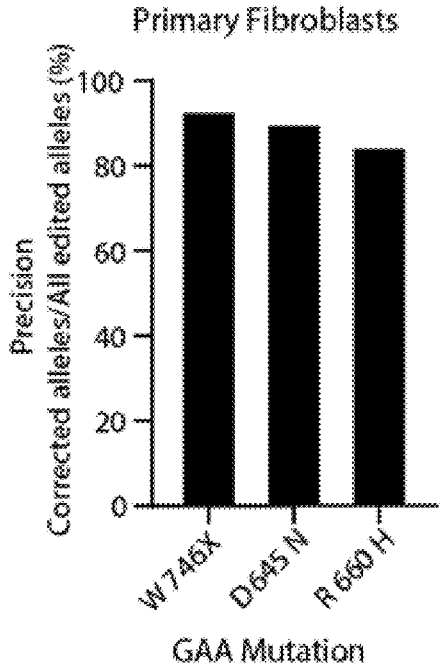

Primary Fibroblasts
Precision
Corrected alleles/All edited alleles (%)
100
80
60
40
20
0
W746X
D645N
R660H
GAA Mutation

FIG. 4b

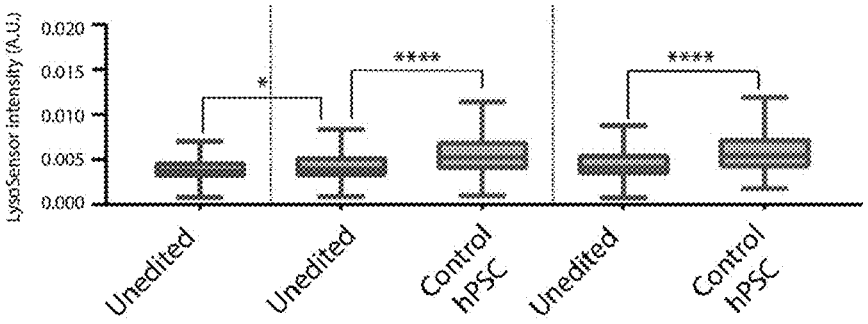

LysoSensor intensity (A.U.)
0.020
0.015
0.010
0.005
0.000
*
****
****
Unedited
Unedited
Control hPSC
Unedited
Control hPSC

FIG. 5

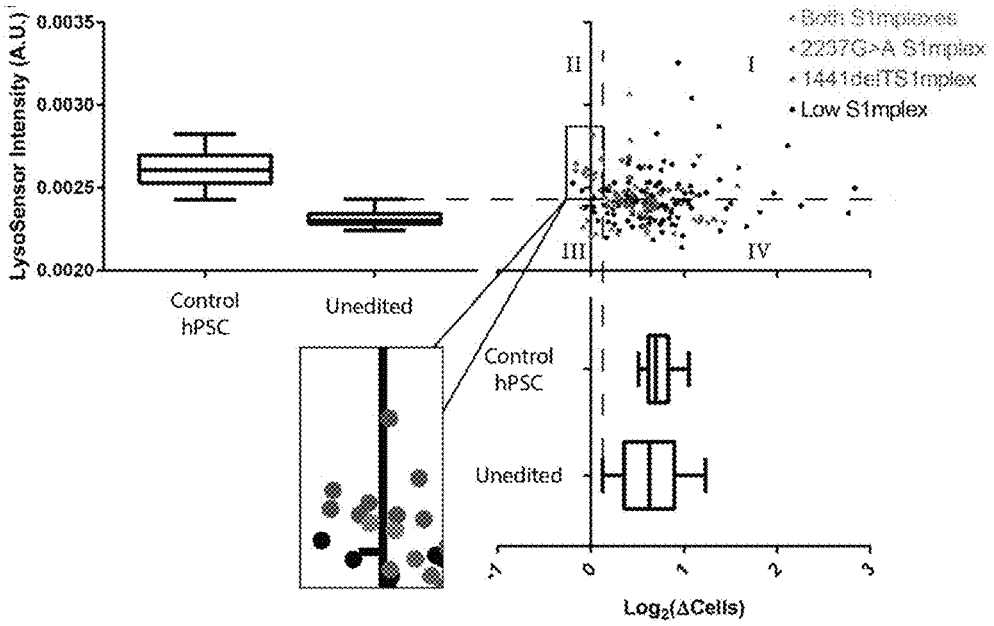

LysoSensor Intensity (A.U.)
0.0035
0.0030
0.0025
0.0020
Soft S1mplexes
2237G>A S1mplex
1441delTS1mplex
Low S1mplex
II
I
III
IV
Control hPSC
Unedited
Control hPSC
Unedited
-1
0
1
2
3
Log2(ΔCells)

FIG. 6

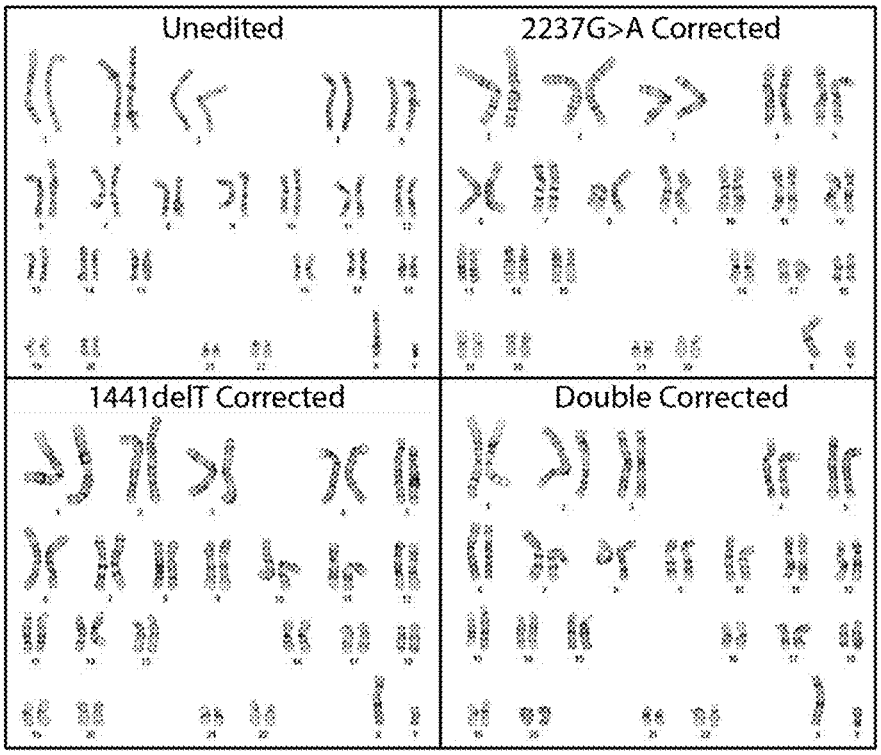


FIG. 7

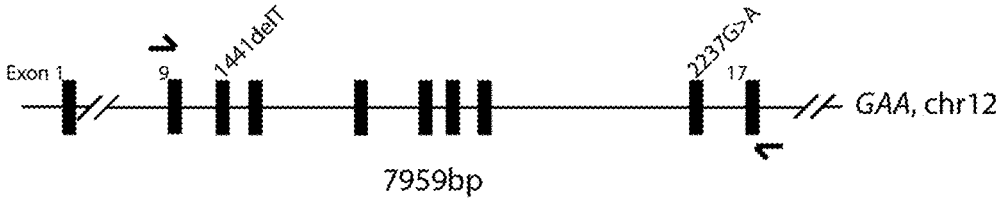


FIG. 8

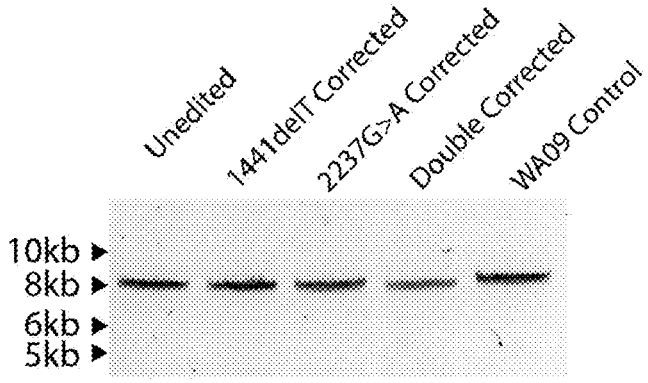
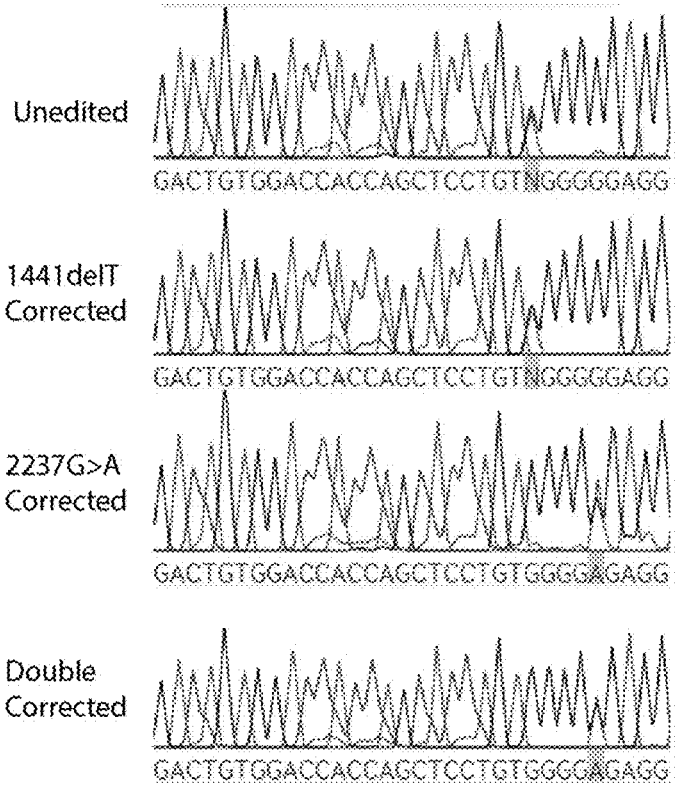


FIG. 9



SEQ ID NO: 70

SEQ ID NO: 70

SEQ ID NO: 71

SEQ ID NO: 71

FIG. 10

GENE CORRECTION OF POMPE DISEASE AND OTHER AUTOSOMAL RECESSIVE DISORDERS VIA RNA-GUIDED NUCLEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application 62/755,980 filed on Nov. 5, 2019, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH & DEVELOPMENT

[0002] This invention was made with government support under GM119644 awarded by the National Institutes of Health and CBET1350178 awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD OF THE DISCLOSURE

[0003] The present disclosure is related to compositions and methods for the treatment of autosomal recessive diseases.

BACKGROUND

[0004] Infantile-onset Pompe disease is an autosomal recessive glycogen storage disorder cause by mutations in the acid-α-glucosidase (GAA) gene that encodes an enzyme that breaks down glycogen within the lysosome (FIG. **1***a*). Over 400 different GAA mutations have been noted within ClinVar, and detailed case studies indicate a buildup of lysosomic glycogen, leading to clinical complications, most prominently in cardiac and muscle tissues. Left untreated, patients with infantile-onset Pompe disease typically die within the first year of life, and Pompe disease is now frequently included within newborn screening panels. Enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA) is currently the only approved clinical treatment for Pompe disease. However, patients require high levels of enzyme injected biweekly, rendering the treatment expensive and inconvenient. ERT may also be less effective in a subset of patients that are cross-reactive immunologic material (CRIM) negative.

[0005] Newer therapies in development for Pompe disease have primarily made use of integrated viral cassettes including transgenes to express GAA from exogenous promoters. Viral particles are injected either directly into muscle or administered systemically and transported to the liver. Direct injection to cardiac or skeletal muscle provided long term recovery of phenotype (10-fold reduction in glycogen content) to transduced cells but did not affect non-transduced cells and required high viral loads (>10^{10} viral genomes/kg). Silencing of the viral transgene, immune response to the viral vector, and insertional oncogenesis are outstanding concerns with these viral gene therapy approaches. Anti-sense oligonucleotides can be introduced to correct splicing in diseased patients that possessed mutations at splicing sites, but would only be beneficial to a subset of potential patients. Finally, autologous cell therapy has been proposed using cells engineered to constitutively overexpress GAA. None of these approaches retain endogenous GAA regulation nor have corrected the underlying GAA mutations.

[0006] What is needed are novel strategies for correction of the mutated alleles Pompe disease and other autosomal recessive disorders which could be a promising in vivo somatic gene editing strategy or an alternative strategy to generate gene corrected cells ex vivo for cell therapy.

BRIEF SUMMARY

[0007] In one aspect, a modified guide RNA comprises

[0008] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0009] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0010] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0011] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0012] wherein the single stranded protospacer region comprises

(SEQ ID NO: 1)
CTCGTTGTCCAGGTAGGCC,

(SEQ ID NO: 2)
TGGACCACCAGCTCCTGTAG,

(SEQ ID NO: 60)
GGACCACCAGCTCCTGTAGG,

(SEQ ID NO: 61)
GCCCAGGAAGCCGCAGACGT,
or

(SEQ ID NO: 62)
CAGAGGAGCTGTGTGTGCAC.

[0013] In another aspect, a guide RNA comprises

[0014] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0015] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0016] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0017] wherein the single stranded protospacer region comprises

(SEQ ID NO: 1)
CTCGTTGTCCAGGTAGGCCC,

(SEQ ID NO: 2)
TGGACCACCAGCTCCTGTAG,

(SEQ ID NO: 60)
GGACCACCAGCTCCTGTAGG,

(SEQ ID NO: 61)
GCCCAGGAAGCCGCAGACGT,
or

(SEQ ID NO: 62)
CAGAGGAGCTGTGTGTGCAC.

[0018] Also included herein are RNP complexes comprising the guide RNAs and modified guide RNAs, and a Cas9 polypeptide or active fragment thereof.

[0019] In another aspect, a method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has an autosomal recessive disorder with compound heterozygous mutations comprises

[0020] delivering to the patient or cell a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide, wherein each modified guide RNA comprises,

[0021] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0022] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0023] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0024] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0025] wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

[0026] wherein the first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele,

[0027] wherein the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele.

[0028] In an aspect, a method of treating a patient with an autosomal recessive disorder with compound heterozygous mutations comprises transplanting the cell made by the foregoing method.

[0029] In another aspect, a method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has Pompe disease, comprises

[0030] delivering to the patient or cell a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide, wherein each modified guide RNA comprises,

[0031] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0032] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0033] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0034] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0035] wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

[0036] wherein the first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele,

[0037] wherein the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele,

[0038] wherein the single stranded protospacer region of the first modified guide RNA comprises CTCGTTGTCCAGGTAGGCCC (SEQ ID NO: 1) and the single stranded protospacer region of the second guide RNA comprises TGGACCACCAGCTCCTGTA (SEQ ID NO: 2).

[0039] In another aspect, a method of making an RNP complex, comprises

[0040] selecting a single stranded protospacer sequence by identifying a mutant allele to be corrected, wherein the mutant allele is within 0 to 100 bases of a protospacer adjacent motif,

[0041] producing a modified guide RNA comprising the selected single stranded protospacer region, wherein the modified guide RNA comprises

[0042] a crRNA comprising the single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0043] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0044] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0045] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0046] selecting a donor polynucleotide to correct the mutant allele and complementary to an anti-sense strand of genomic DNA, wherein the donor polynucleotide comprises a silent mutation in a constant region of the protospacer adjacent motif, e.g., within the final two nucleotides of the protospacer adjacent motif for *Streptococcus pyogenes,*

[0047] producing a biotinylated donor polynucleotide, wherein the donor polynucleotide is biotinylated at the 5' end or the 3' end, and

[0048] assembling the modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, and the biotinylated donor polynucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0050] FIG. **1***a* is a schematic of the cause of Pompe disease. Pompe disease is caused by two defective copies of acid-α-glucosidase (GAA). This enzyme is responsible for breakdown of glycogen within lysosomes. Without GAA, glycogen build-up can cause downstream health issues. After correction, GAA expresses a functional protein leading to a reduction in lysosomal size.

[0051] FIG. **1***b* is a schematic of editing locations within the GAA locus. Pompe mutants are compound heterozygous at GAA. Allele one contains a point mutation that causes a premature stop codon (GAA:c.[1441=;2237G>A]) while allele two carries a one basepair deletion (GAA:c.[1441delT;2237=]). sgRNAs (underline) were designed to

be specific to only the diseased allele by containing mutants (red) within the seed region. ssODNs used for genomic repair contained the wildtype sequence at the mutation site as well as a silent mutation (blue) to remove the PAM site (green) to prevent re-cutting of the corrected allele.

[0052] FIG. **1***c* is a schematic of S1mplex design for repair of compound heterozygous mutations. S1mplexes targeting the 1441delT mutant were labelled with an AlexaFluor®488 compound while S1mplexes targeting the 2237G>A mutation were labelled with an AlexaFluor®647. These RNP species were mixed prior to transfecting into cells and subsequently plated on ArrayEdit platform to conduct high-content analysis.

[0053] FIG. **1***d* shows Sanger sequencing traces of all corrected lines. The unedited line contains mutations at both alleles: 1441delT mutation causes a breakdown of sequence trace, whereas a single point mutation demonstrates a heterozygosity 2237G>A locus. Single corrected clones remain identical to unedited line at unedited locus and contained PAM wobble on the corrected allele. Double corrected line contained PAM wobble at both loci. Wobble A bases in the corrected lines are highlighted to indicate repair from the ssODN. SpCas9 cut site denoted by dotted line, and sequencing chromatograms do not show evidence of undesired NHEJ products.

[0054] FIG. **2***a*, top panel, is a schematic of GAA mRNA used for qRT-PCR. mRNA was analyzed at 3 locations, around the 1441delT locus (solid), the 2237G>A locus (hashed), and at the final 3' junction (outlined). The middle two panels show an overlay of qRT-PCR and deep sequencing data around each edited locus: analysis around 1441delT loci (solid bars) and analysis around 2237G>A locus (hashed bars). Bars are color coded by sequence identity, either wildtype, mutant, or corrected, from deep sequencing analysis. Bar heights are equivalent to qRT-PCR quantification relative to GAPDH. In all corrected lines, the corrected allele was expressed at a higher frequency than the unedited allele. Double corrected line expressed the highest level of overall mRNA and expressed each allele at approximately equal amounts. The bottom panel shows quantification of total GAA mRNA in unedited, single corrected, and double corrected lines via qRT-PCR. The double corrected line had a significantly higher amount of mRNA than any of the other isolated line (n=3 technical replicates). This is consistent with expression from two active alleles (*$p<0.05$ ***$p<0.005$, two-tailed t-test, $\alpha=0.05$, heteroscedastic). Bar graphs are plotted with standard deviation.

[0055] FIG. **2***b* shows a Western blot for GAA protein. Each of the corrected lines expressed high levels of active protein as well as detectable levels of precursor protein. Unedited cells expressed significantly lower levels of GAA protein but was still above the limit of detection.

[0056] FIG. **2***c* shows GAA activity in cell lysate and cell culture media supernatant as measured by 4-MUG cleavage in acidic conditions. Unedited cells were unable to cleave this substrate, showing there was little to no active protein. All corrected lines had significantly higher activity than unedited cells but were indistinguishable from each other (n=2 technical replicates, *$p<0.05$, two-tailed t-test, $\alpha=0.05$, heteroscedastic; Bar graphs plotted with standard deviation).

[0057] FIG. **3***a* is a schematic of enzymatic cross-correction experiments using gene-corrected cardiomyocytes. Unedited iPSC-CMs (red) were supplied media without glucose for 24 hours (orange). After 24 hours media was replaced with media (pink) that had previously been exposed to corrected cell lines (blue) or supplemented with rhGAA. 96 hours after replacement, unedited cells were stained with LysoSensor™ and imaged using confocal microscopy for dye intensity.

[0058] FIG. **3***b* shows quantification of LysoSensor™ intensity in cross-corrected lines 96 hours post media exchange. Each triangle represents a corrected cell identified using CellProfiler™. After 96 hours of daily media changes or supplementing with rhGAA all conditions had significant increase in dye intensity over control conditions. Unedited cells were modified to express histone 2B (H2B)-mCherry to facilitate imaging of the nuclei in these assays. (***$p<0.005$, $n>134$, two-tailed t-test, $\alpha=0.05$, heteroscedastic; bar graphs plotted with standard deviation).

[0059] FIG. **3***c* shows representative images of unedited iPSC-CMs stained with LysoSensor™ in media from unedited and double corrected iPSC-CMs.

[0060] FIG. **3***d* shows representative images of LAMP1 staining in unedited, single corrected, double corrected cells and control PSC-CM and unedited iPSC-CM treated with rhGAA. (scale bars: 10 μm).

[0061] FIGS. **4***a* and *b* show highly precise gene correction using S1mplex strategy in primary fibroblasts from three Pompe disease patients. FIG. **4***a* shows the percentage of total sequencing reads from S1mplex-edited, primary fibroblasts from Pompe diseased patients. Results indicate gene correction and imprecise editing for 3 different mutations. FIG. **4***b* shows that percentage of edited alleles that are precisely edited, indicating that S1mplex genome editors can perform as precise-90 editors. Primary fibroblasts were obtained from Coriell Institute. W746X mutation was from Coriell ID: GM04912. D645N mutation was from Coriell ID: GM20090. R660H was from Coriell ID: GM13522.

[0062] FIG. **5** shows quantification of LysoSensor™ intensity in each co-culture condition. LysoSensor™ intensity was measured on a per-cell basis using confocal microscopy. Control hPSCs had significantly higher intensity than unedited cells in all conditions. Unedited cells co-cultured with hPSCs also had an increased LysoSensor™ intensity when compared to those cultured alone (*$p<0.05$, ****$p<0.001$).

[0063] FIG. **6** shows Left: LysoSensor™ quantification per μFeature of two mock transfections after 7 days of growth. hPSCs were significantly more intense than unedited iPSCs on ArrayEdit. Bottom Right: Growth rate of unedited and control hPSCs following a mock transfection to establish a baseline for growth. Growth rates were calculated by measuring the per-day change in the number of cells of the μFeature. Features were graphed as an average of these per day changes. Top Right: LysoSensor™ intensity was plotted against growth rate per μFeature to identify edited colonies. Individual plotted colonies were also assayed for presence of either genome editor (represented in either purple or green), both genome editors (red) and low amounts of genome editors (black). Colonies of interest are identified as those with high genome editor expression and lower growth rates, presumably arising from the stress of genome editing. Dashed lines indicate regions of interest. Also included is a magnification of quadrant II from panel. μFeatures in this region were selected for genomic analysis to isolate edited clones.

[0064] FIG. **7** shows karyotypes of all isolated gene-corrected lines as well as unedited cells. No abnormalities were detected at a band resolution of 500.

[0065] FIG. **8** shows a schematic for long PCR covering both Cas9 cut sites. Primers are denoted by arrows. The expected PCR amplicon is 7959 bp in length.

[0066] FIG. **9** shows a gel analysis of long range PCR described in panel c and FIG. **2***a* in each isolated cell line. No significant deviances from the expected length were detected, and no other significant bands were observed. WA09 control cells are hPSCs.

[0067] FIG. **10** shows Sanger sequencing traces of long range PCR shown in panel FIG. **9**. SNPs were observed showing that PCR products were a result of amplification from both alleles within the cells.

[0068] The above-described and other features will be appreciated and understood by those skilled in the art from the following detailed description, drawings, and appended claims.

DETAILED DESCRIPTION

[0069] For individuals with an autosomal recessive disease (e.g., cystic fibrosis, sickle cell anemia, and Tay-Sachs disease), the mutated gene is located on one of the nonsex chromosomes (autosomes), and both alleles of the gene carry mutations. The parents of an individual with symptoms of an autosomal recessive disease generally each carry one copy of the mutated gene, but they do not show obvious symptoms of the disease. Therefore, it has been assumed that correcting only one allele of the mutated gene would be sufficient to rescue the disease, and that additional genomic surgery to repair the second allele may subject a patient to undue risk. The inventors have tested this assumption with CRISPR-Cas9 gene editing to systematically correct both mutated alleles within the same cell from an autosomal recessive, infantile-onset case of Pompe disease. Unexpectedly, the inventors have shown that a CRISPR-Cas9 gene editing system can correct both mutated alleles.

[0070] The inventors previously developed a S1mplex strategy for modified guide RNAs such as sgRNAs and their RNP complexes with Cas9. The S1mplex tool exploits high affinity interactions between a short RNA aptamer and streptavidin to promote more faithful writing of the human genome. S1mplex modified guide RNAs or traditional guide RNAs can be used in the methods described herein.

[0071] In an aspect, these RNP-containing complexes can be assembled outside the cell to a desired stoichiometry and delivered as an all-in-one gene-editing nanoparticle together with a donor nucleic acid template. In addition, the complexes can be easily decorated with additional moieties such as fluorophores or Qdots to enrich for edited cells. Use of these particles with a biotinylated ssODN reduced heterogeneity in delivery among RNPs and nucleic acids within human cells and enriches the ratio of precisely-edited to imprecisely-alleles edited alleles up to 18-fold higher than standard RNP methods, approaching a ratio of four precise edits to every one imprecise edit. Further functionalization with a unique fluorophore enables multiplexed editing and enrichment of precisely edited populations through cell sorting. Taken together, advances with the S1mplex tool generates chemically-defined reagents to promote precise editing of the human genome.

[0072] The S1mplex strategy is inspired by CRISPR display that leverages structural studies of the RNP to identify locations in the sgRNA sequence where RNA aptamers could be tolerated.

[0073] CRISPR refers to the Clustered Regularly Interspaced Short Palindromic Repeats type II system used by bacteria and archaea for adaptive defense. This system enables bacteria and archaea to detect and silence foreign nucleic acids, e.g., from viruses or plasmids, in a sequence-specific manner. In type II systems, guide RNA interacts with Cas9 and directs the nuclease activity of Cas9 to target DNA sequences complementary to those present in the guide RNA. Guide RNA base pairs with complementary sequences in target DNA. Cas9 nuclease activity then generates a double-stranded break in the target DNA.

[0074] CRISPR/Cas9 is an RNP complex. CRISPR RNA (crRNA) includes a 20 base protospacer element that is complementary to a genomic DNA sequence as well as additional elements that are complementary to the transactivating RNA (tracrRNA). The tracrRNA hybridizes to the crRNA and binds to the Cas9 protein, to provide an active RNP complex. Thus, in nature, the CRISPR/Cas9 complex contains two RNA species.

[0075] sgRNA refers to a single RNA species which combines the tracrRNA and the crRNA and is capable of directing Cas9-mediated cleavage of target DNA. An sgRNA thus contains the sequences necessary for Cas9 binding and nuclease activity and a target sequence complementary to a target DNA of interest (protospacer sequence). In general, in an sgRNA, the tracrRNA and the crRNA are connected by a linker loop sequence. sgRNAs are well-known in the art. While sgRNA is generally used throughout this disclosure, two-part guide RNAs containing a crRNA and a tracrRNA can also be employed.

[0076] As used herein, a guide RNA protospacer sequence refers to the nucleotide sequence of a guide RNA that binds to a target DNA sequence and directs Cas9 nuclease activity to the target DNA locus. In some embodiments, the guide RNA protospacer sequence is complementary to the target DNA sequence. As described herein, the protospacer sequence of a single guide RNA may be customized, allowing the targeting of Cas9 activity to a target DNA of interest.

[0077] Any desired target DNA sequence of interest may be targeted by a guide RNA target sequence. Any length of target sequence that permits CRISPR-Cas9 specific nuclease activity may be used in a guide RNA. In some embodiments, a guide RNA contains a 20 nucleotide protospacer sequence.

[0078] In addition to the protospacer sequence, the targeted sequence includes a protospacer adjacent motif (PAM) adjacent to the protospacer region which is a sequence recognized by the CRISPR RNP as a cutting site. Without wishing to be bound to theory, it is thought that the only requirement for a target DNA sequence is the presence of a protospacer-adjacent motif (PAM) adjacent to the sequence complementary to the guide RNA target sequence. Different Cas9 complexes are known to have different PAM motifs. For example, Cas9 from *Streptococcus pyogenes* has a NGG trinucleotide PAM motif; the PAM motif of *N. meningitidis* Cas9 is NNNNGATT; the PAM motif of *S. thermophilus* Cas9 is NNAGAAW; and the PAM motif of *T. denticola* Cas9 is NAAAAC.

[0079] A modified guide RNA is a one-part or two-part RNA capable of directing Cas-9-mediated cleavage of target DNA. A modified sg RNA is a single RNA species capable

of directing Cas9-mediated cleavage of target DNA. A modified sgRNA, for example, comprises sequences that provide Cas9 nuclease activity, a protospacer sequence complementary to a target DNA of interest, and an aptamer that binds a biotin-binding molecule. The linker loop that connects the tracrRNA and the crRNA in an sgRNA can be replaced with an aptamer that binds a biotin-binding molecule such as a streptavidin-binding aptamer. Unexpectedly, the modified sgRNAs can bind both Cas9 protein and streptavidin, and form active RNP complexes which induce error-prone DNA repair less frequently than standard CRISPR-Cas9 RNP complexes.

[0080] In an aspect, a modified guide RNA, comprises

[0081] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0082] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0083] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0084] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0085] wherein the single stranded protospacer region comprises

(SEQ ID NO: 1)
CTCGTTGTCCAGGTAGGCCC,

(SEQ ID NO: 60)
GGACCACCAGCTCCTGTAGG,

(SEQ ID NO: 61)
GCCCAGGAAGCCGCAGACGT,
or

(SEQ ID NO: 62)
CAGAGGAGCTGTGTGTGCAC

(SEQ ID NO: 2)
TGGACCACCAGCTCCTGTAG.

[0086] In another aspect, the crRNA and the tracrRNA form an sgRNA, the sgRNA comprising from 5' to 3',

[0087] the single-stranded protospacer sequence,

[0088] the first complementary strand of a binding region for the Cas9 polypeptide,

[0089] the aptamer that binds a biotin-binding molecule, and

[0090] the second complementary strand of the binding region for the Cas9 polypeptide.

[0091] More specifically, a modified sgRNA comprises, from 5' to 3', a single-stranded protospacer sequence, a first complementary strand of a binding region for the Cas9 polypeptide, an aptamer that binds a biotin-binding molecule, and a second complementary strand of the binding region of the Cas9 protein. In an embodiment, in the secondary structure of the modified sgRNA, the stem forms a stem-loop structure with the aptamer that binds the biotin-binding molecule.

[0092] In another aspect,

[0093] a crRNA comprises a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0094] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0095] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0096] wherein the single stranded protospacer region comprises

(SEQ ID NO: 1)
CTCGTTGTCCAGGTAGGCCC,

(SEQ ID NO: 2)
TGGACCACCAGCTCCTGTAG,

(SEQ ID NO: 60)
GGACCACCAGCTCCTGTAGG,

(SEQ ID NO: 61)
GCCCAGGAAGCCGCAGACGT,
or

(SEQ ID NO: 62)
CAGAGGAGCTGTGTGTGCAC.

[0097] The single-stranded protospacer region can comprise 17 to 20 nucleotides. Exemplary binding regions for Cas9 polypeptides comprise 10 to 35 base pairs.

[0098] In an aspect, the aptamer that binds a biotin-binding molecule forms a stem-loop structure. The stem portion of the stem-loop structure optionally forms a contiguous double strand with the double-stranded binding region for the Cas9 polypeptide. The stem portion of the aptamer can comprise 9 to 15 base pairs, while the loop comprises 30 nucleotides. The aptamer may contain more than one stem-loop structure. The length of the stem portion of the aptamer is not critical and can be adjusted depending on the application of the modified guide RNA.

[0099] A "Cas9" polypeptide is a polypeptide that functions as a nuclease when complexed to a guide RNA, e.g., an sgRNA or modified sgRNA. The Cas9 (CRISPR-associated 9, also known as Csn1) family of polypeptides, for example, when bound to a crRNA:tracrRNA guide or single guide RNA, are able to cleave target DNA at a sequence complementary to the sgRNA target sequence and adjacent to a PAM motif as described above. Cas9 polypeptides are characteristic of type II CRISPR-Cas systems. The broad term "Cas9" Cas9 polypeptides include natural sequences as well as engineered Cas9 functioning polypeptides. The term "Cas9 polypeptide" also includes the analogous Clustered Regularly Interspaced Short Palindromic Repeats from *Prevotella* and *Francisella* 1 or CRISPR/Cpf1 which is a DNA-editing technology analogous to the CRISPR/Cas9 system. Cpf1 is an RNA-guided endonuclease of a class II CRISPR/Cas system. This acquired immune mechanism is found in *Prevotella* and *Francisella* bacteria. Additional Class I Cas proteins include Cas3, Cas8a, Cas5, Cas8b, Cas8c, Cas 10d, Case1, Cse 2, Csy 1, Csy 2, Csy 3, GSU0054, Cas 10, Csm 2, Cmr 5, Cas10, Csx11, Csx10, and Csf 1. Additional Class 2 Cas9 polypeptides include Csn 2, Cas4, C2c1, C2c3 and Cas13a.

[0100] Exemplary Cas9 polypeptides include Cas9 polypeptide derived from *Streptococcus pyogenes*, e.g., a polypeptide having the sequence of the Swiss-Prot accession Q99ZW2 (SEQ ID NO: 3); Cas9 polypeptide derived from *Streptococcus thermophilus*, e.g., a polypeptide having the sequence of the Swiss-Prot accession G3ECR1 (SEQ ID NO: 4); a Cas9 polypeptide derived from a bacterial species within the genus *Streptococcus*; a Cas9 polypeptide derived from a bacterial species in the genus *Neisseria* (e.g., Gen-

Bank accession number YP_003082577; WP 015815286.1 (SEQ ID NO: 5)); a Cas9 polypeptide derived from a bacterial species within the genus *Treponema* (e.g., GenBank accession number EMB41078 (SEQ ID NO: 6)); and a polypeptide with Cas9 activity derived from a bacterial or archaeal species. Methods of identifying a Cas9 protein are known in the art. For example, a putative Cas9 protein may be complexed with crRNA and tracrRNA or sgRNA and incubated with DNA bearing a target DNA sequence and a PAM motif.

[0101] The term "Cas9" or "Cas9 nuclease" refers to an RNA-guided nuclease comprising a Cas9 protein, or a fragment thereof (e.g., a protein comprising an active, inactive, or partially active DNA cleavage domain of Cas9, and/or the gRNA binding domain of Cas9). In some embodiments, a Cas9 nuclease has an inactive (e.g., an inactivated) DNA cleavage domain, that is, the Cas9 is a nickase. Other embodiments of Cas9, both DNA cleavage domains are inactivated. This is referred to as catalytically-inactive Cas9, dead Cas9, or dCas9.

[0102] Functional Cas9 mutants are described, for example, in US20170081650 and US20170152508, incorporated herein by reference for its disclosure of Cas9 mutants.

[0103] In addition, to the modified sgRNA and the Cas9 polypeptide or active fragment thereof, an RNP complex may further comprise a biotin-binding molecule such as an avidin such as avidin, streptavidin, or neutravidin which bind with high affinity to the aptamer that binds the biotin-binding molecule in the modified sgRNA. Avidin, streptavidin and neutravidin are a tetramers and each subunit can bind biotin with equal affinity. Avidin, streptavidin and neutravidin variants that contain one, two or three biotin binding sites are also available and may be employed in the complex.

[0104] When the RNP complex comprises a biotin-binding molecule, the complex can further comprise a biotinylated molecule which associates with the complex via the biotin-binding molecule. The biotinylated molecule can target the RNP complex to a specific cell type, organ or tissue. For example, PEG-coated gold nanoparticles exhibit size-dependent in vivo toxicity; the renal clearance of quantum dots can be controlled; and the accumulation of PEGylated silane-coated magnetic iron oxide nanoparticles has been shown to be size dependent.

[0105] In one embodiment, the biotinylated molecule is a biotinylated oligodeoxynucleotide, such as a biotinylated donor DNA template. Homologous recombination can insert an exogenous polynucleotide sequence into the target nucleic acid cleavage site. An exogenous polynucleotide sequence can be called a donor polynucleotide or a donor sequence. In some embodiments, a donor polynucleotide, a portion of a donor polynucleotide, a copy of a donor polynucleotide, or a portion of a copy of a donor polynucleotide can be inserted into a target nucleic acid cleavage site. A donor polynucleotide can be single-stranded DNA, double-stranded DNA, RNA, or a duplex of RNA and DNA. A donor polynucleotide can be a sequence that does not naturally occur at a target nucleic acid cleavage site. In some embodiments, modifications of a target nucleic acid due to NHEJ and/or HDR can lead to, for example, mutations, deletions, alterations, integrations, gene correction, gene replacement, transgene insertion, nucleotide deletion, gene disruption, and/or gene mutation. The process of integrating non-native nucleic acid(s) into genomic DNA can be referred to as "genome engineering".

[0106] In an embodiment, the biotinylated molecule is a nanoparticle, such as a quantum dot, a gold particle, a magnetic particle, a polymeric nanoparticle. In another embodiment, the biotinylated molecule is a biotinylated fluorescent dye such as Atto 425-Biotin, Atto 488-Biotin, Atto 520-Biotin, Atto-550 Biotin, Atto 565-Biotin, Atto 590-Biotin, Atto 610-Biotin, Atto 620-Biotin, Atto 655-Biotin, Atto 680-Biotin, Atto 700-Biotin, Atto 725-Biotin, Atto 740-Biotin, fluorescein biotin, biotin-4-fluorescein, biotin-(5-fluorescein) conjugate, and biotin-B-phycoerythrin, Alexafluor® 488 biocytin, Alexafluor® 546, Alexa Fluor® 549, lucifer yellow cadaverine biotin-X, *Lucifer* yellow biocytin, Oregon green 488 biocytin, biotin-rhodamine and tetramethylrhodamine biocytin. Biotinylated molecule may also be a peptide, proteins or protein domains, specifically antibodies and Fab domains.

[0107] In another embodiment, the biotinylated donor polynucleotide comprises a contrast agent, a cell targeting ligand, a tissue targeting ligand, or a peptide.

[0108] In another aspect, the biotin-binding molecule can be covalently linked to a donor polynucleotide, a nanoparticle, or a dye molecule either directly or via a linker molecule, using, for example a disulfide linker. The bound biotin-binding molecule can then bind the aptamer of the modified sgRNA. Additional biotinylated donor polynucleotides, nanoparticle, contrast agent, or dye molecules can then be associated with the bound biotin-binding molecule. Alternatively, the biotin-binding molecule can be associated with the biotinylated molecule prior to adding to modified sgRNA.

[0109] A method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has an autosomal recessive disorder with compound heterozygous mutations comprises

[0110] delivering to the patient or cell a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide, wherein each modified guide RNA comprises,

[0111] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0112] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0113] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0114] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0115] wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

[0116] wherein the first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele,

[0117] wherein the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele.

[0118] Exemplary patient-derived cells comprises an induced pluripotent stem cell, a progenitor cell, a mesenchymal stem cell, or a tissue-specific stem cell. Exemplary tissue-specific stem cells comprises a skeletal stem cell, a hematopoietic stem cell, an epithelial stem cell, or a neural stem cell.

[0119] In an embodiment, a first RNP complex comprises the first modified guide RNA, the Cas9 polypeptide, the biotin-binding molecule and the first biotinylated donor polynucleotide; and a second RNP complex comprises the second modified guide RNA, the Cas9 polypeptide, the biotin-binding molecule and the second biotinylated donor polynucleotide.

[0120] In another embodiment, the first modified guide RNA, the second modified guide RNA and the Cas9 polypeptide are expressed from one or more viral vectors. For example, a first viral vector expresses the first modified guide RNA, and a second viral vector expresses the second modified guide RNA, and a third viral vector expresses the Cas9 polypeptide.

[0121] Exemplary patients are human patients. Exemplary human autosomal recessive disorders are aspartylglucosaminuria, Batten disease, cystinosis, Fabry disease, Gaucher Disease Type I, II or III, Pompe Disease, Tay Sachs Disease, Sandhoff Disease, Metachromatic leukodystrophy, Mucolipidosis Type, I, II/III or IV, Hurler Disease, Hunter disease, Sanfilippo disease Types A,B,C,D, Morquio disease Types A and B, Maroteaux-Lamy disease, Sly disease, Niemann-Pick Disease Types A/B, C1 or C2, or Schindler Disease Types I or II.

[0122] In an embodiment, the autosomal recessive disorder is a human lysosomal storage disorder, such as Pompe disease.

[0123] A method of treating a patient with an autosomal recessive disorder with compound heterozygous mutations comprises transplanting the cell made by the methods described herein into the subject. Lysosomal storage diseases are caused by an inborn error of metabolism that results in the absence or deficiency of an enzyme, leading to the inappropriate storage of material in various cells of the body. Most lysosomal storage disorders are inherited in an autosomal recessive manner.

[0124] In an embodiment, modifying the target gene increases or decreases the expression of a gene product of the target gene.

[0125] In another embodiment, modifying the target gene comprises high-fidelity homology-directed repair (HDR).

[0126] In another embodiment, modifying the target gene comprises the addition of a genetic functionality, or the correction of a mutation.

[0127] In yet another embodiment, modifying the target gene creates a double strand break (DSB) which is repaired by a non-homologous end joining (NHEJ) cell repair mechanism generating indels thereby modifying the polynucleotide sequence of the target gene.

[0128] In a further embodiment, modifying the target gene creates a DSB which is repaired by a homologous recombination (HDR) cell repair mechanism incorporating a donor DNA sequence thereby modifying the polynucleotide sequence of the target gene.

[0129] In another aspect, the S1m-sgRNAs described herein can be used for the excision of genomic DNA. In an aspect, two S1m-sgRNAs can be employed simultaneously, wherein each S1m-sgRNA targets an end of the region to be excised. As shown in Example 12, human cells contain the properly excised region of genomic DNA

[0130] Delivery of polynucleotides and RNPs of the present disclosure to cells, in vitro, or in vivo, may be achieved by a number of methods known to one of skill in the art. These methods include lipofection, electroporation, nucleofection, microinjection, biolistics, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates. Lipofection is well known and lipofection reagents are sold commercially. Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides are described in the art.

[0131] Lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, and the preparation of such complexes is well known to one of skill in the art.

[0132] Electroporation can be used to deliver the polynucleotides and RNPs of the present disclosure. In these methods, the polynucleotides or RNPs are mixed in an electroporation buffer with the target cells to form a suspension. This suspension is then subjected to an electrical pulse at an optimized voltage, which creates temporary pores in the phospholipid bilayer of the cell membrane, permitting charged molecules like DNA and proteins to be driven through the pores and into the cell. Reagents and equipment to perform electroporation are sold commercially.

[0133] Biolistic, or microprojectile delivery, can be used to deliver the polynucleotides and RNPs of the present disclosure. In these methods, microprojectiles, such as gold or tungsten, are coated with the polynucleotide by precipitation with calcium chloride, spermidine or polyethylene glycol. The microprojectile particles are accelerated at high speed into a cell using a device such as the BIOLISTIC® PDS-1000/He Particle Delivery System (Bio-Rad; Hercules, Calif.).

[0134] In another embodiment, a viral vector expressing the modified guide RNA of the present disclosure, a viral vector expressing a Cas9 polypeptide and biotinylated donor DNA template (e.g., a biotinylated donor DNA template), can be transfected into a cell, such as a human cell. Human cells include human pluripotent stem cell lines and primary blood cell such as hematopoietic stem and progenitor cells and T-cells. Once editing has occurred in the cell line, the cells can be differentiated and transplanted into a subject, or used for drug development.

[0135] In some embodiments, the polynucleotides of the present disclosure may also comprise modifications that, for example, increase stability of the polynucleotide. Such modifications may include phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates such as 3'-alkylene phosphonates, 5'-alkylene phosphonates, chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and amino alkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates, and boranophosphates having normal 3'-5' linkages, 2-5' linked analogs, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', a 5' to 5' or a 2' to 2' linkage. Exemplary nucleic acid-targeting polynucleotides having inverted polarity can comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage (i.e. a single inverted nucleo-

side residue in which the nucleobase is missing or has a hydroxyl group in place thereof). Various salts (e.g., potassium chloride or sodium chloride), mixed salts, and free acid forms can also be included.

[0136] In some embodiments, the polynucleotides of the present disclosure may also contain other nucleic acids, or nucleic acid analogues. An example of a nucleic acid analogue is peptide nucleic acid (PNA).

[0137] In an embodiment, a method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has Pompe disease, comprises

[0138] delivering to the patient or cell a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide, wherein each modified guide RNA comprises,

[0139] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0140] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0141] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0142] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0143] wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

[0144] wherein the first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele,

[0145] wherein the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele,

[0146] wherein the single stranded protospacer region of the first modified guide RNA

[0147] comprises CTCGTTGTCCAGGTAGGCCC (SEQ ID NO: 1) and the single stranded

[0148] protospacer region of the second guide RNA comprises

[0149] TGGACCACCAGCTCCTGTA (SEQ ID NO: 2).

[0150] In another embodiment, a method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has Pompe disease, comprises

[0151] delivering to the patient or cell a first guide RNA, a second guide RNA, a Cas9 polypeptide, a first donor polynucleotide, and a second donor polynucleotide, wherein each guide RNA comprises,

[0152] a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0153] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0154] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0155] wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

[0156] wherein the first guide RNA and the first donor polynucleotide correct a first diseased allele,

[0157] wherein the second guide RNA and the second donor polynucleotide correct a second diseased allele,

[0158] wherein the single stranded protospacer region of the first modified guide RNA comprises CTCGTTGTCCAGGTAGGCCC (SEQ ID NO: 1) and the single stranded protospacer region of the second guide RNA comprises TGGACCACCAGCTCCTGTA (SEQ ID NO: 2).

[0159] Also included herein are methods of making RNP complexes, specifically first and second RNP complexes that provide biallelic correction. In an embodiment, a method of making an RNP complex, comprises

[0160] selecting a single stranded protospacer sequence by identifying a mutant allele to be corrected, wherein the mutant allele is within 0 to 100 bases of a protospacer adjacent motif,

[0161] producing a modified guide RNA comprising the selected single stranded protospacer region, wherein the modified guide RNA comprises

[0162] a crRNA comprising the single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

[0163] a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

[0164] wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

[0165] wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

[0166] selecting a donor polynucleotide to correct the mutant allele and complementary to an anti-sense strand of genomic DNA, wherein the donor polynucleotide comprises a silent mutation in a constant region of the protospacer adjacent motif, e.g., within the final two nucleotides of the protospacer adjacent motif for *Streptococcus pyogenes,*

[0167] producing a biotinylated donor polynucleotide, wherein the donor polynucleotide is biotinylated at the 5' end or the 3' end, and

[0168] assembling the modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, and the biotinylated donor polynucleotide.

[0169] In an embodiment, the donor polynucleotide is asymmetric around a cut site. In another embodiment, the method further comprises repeating the method and producing a second RNP complex, wherein the second RNP complex corrects a second mutant allele to result in a biallelic correction.

[0170] The invention is further illustrated by the following non-limiting examples.

EXAMPLES

[0171] Methods

[0172] Cell Culture:

[0173] All hPSCs were maintained in mTeSR1™ medium on Matrigel® (WiCell) coated tissue culture polystyrene

plates (BD Falcon). Cells were passaged every 4-5 days at a ratio of 1:8 using Versene™ solution (Life Technologies). Patient derived human induced pluripotent stem cell line Pompe GM04192 was a gift from the T. Kamp and M. Suzuki (UW-Madison) labs. Cardiomyocytes derived from hPSC and iPSC cultures were maintained in RPMI/B27 on Matrigel® (WiCell) coated polystyrene plates (BD Falcon). Patient derived fibroblast lines were obtained from Coriell Institute with different GAA mutations and cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. All cells were maintained at 37° C. in 5% CO_2, and tested monthly for possible *mycoplasma* contamination.

[0174] Cardiomyocyte Differentiation:

[0175] hPSCs and iPSCs were differentiated into cardiomyocytes using a small molecule-directed differentiation protocol in a 12-well plate format as previously described. Briefly, all adherent hPSCs and iPSCs were dissociated in TrypLE™ solution (Life Technologies), counted with a hemocytometer, and centrifuged at 200×g for 5 minutes. Cells were plated at a density between 0.5-1×10^6 cells/well depending on cell line. Once tissue culture plate wells reached 100% confluency (day 0), medium in each well was replaced with a solution containing ml RPMI/B27-Insulin (Life Technologies), 12 μM CHIR99021 (BioGems 25917), and 1 μg/ml Insulin solution (Sigma-Aldrich 19278). Exactly 24 hours later (day 1) medium in each well was removed and replaced with RPMI/B27-insulin. Exactly 48 hours after (day 3) half of the spent medium was collected. To this, an equal volume of fresh RPMI/B27-Insulin was mixed. This combined media was then supplemented with 7.5 μM IWP2 (BioGems 75844). Two days later (day 5) medium in each well was replaced with RPMI/B27-Insulin. Two days (day 7) later and every three days following, spent medium was replaced with RPMI/B27. Spontaneous contraction was generally observed between days 12-16 of differentiation.

[0176] Creation of S1m-sgRNAs:

[0177] S1m-sgRNAs were synthesized as previously described. S1m gBlocks were annealed with Phusion™ polymerase (New England Biolabs) under the following thermocycler conditions: 98° C. for 30 sec followed by 30 cycles at 98° C. for 10 s, and 72° C. for 15 s with a final extension at 72° C. for 10 minutes. S1m cDNA was annealed with Phusion™ polymerase (New England Biolabs) under the following thermocycler conditions: 98° C. for 30 sec followed by 30 cycles at 98° C. for 10 s, 60° C. for 10 s, and 72° C. for 15 s with a final extension at 72° C. for 10 minutes. In vitro transcription was performed with the MEGAShortscript™ T7 Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

[0178] For guides for fibroblast transfection, in vitro transcription was performed using HiScribe™ T7 RNA synthesis Kit (New England Biolabs).

[0179] Genome Editor Deliver:

[0180] All hPSC transfections were performed using the 4D-Nucleofector System™ (Lonza) in P3 solution using protocol CA-137. 50 pmol Cas9, 60 pmol sgRNA, 50 pmol streptavidin, and 60 pmol ssODN were used to form particles per ssODN-S1mplex as described above. Cells were then harvested using TrypLE™ (Life Technologies) and counted. 2×10^5 cells per transfection were then centrifuged at 100×g for 3 minutes Excess media was aspirated and cells were resuspended using 20 μL of RNP solution per condition. After nucleofection, samples were incubated in nucleocuvettes at room temperature for 15 minutes prior to plating into 2*10^4 cells per well on ArrayEdit in mTeSR™ media+10 μM ROCK inhibitor. Media was changed 24 hours post transfection and replaced with mTeSR™ medium. Fibroblast transfections were performed in 24 well plates using 50,000 cells/well using 2 μl Lipofectamine® 2000/well (0.5 μg Cas9/well and sgRNA, streptavidin and ssODN at a 1:1:1:1 molar ratio).

[0181] Synthesis of ArrayEdit Platform:

[0182] μCP was performed using previously described methods. The surface modification involved printing of an alkanethiol initiator to nucleate the polymerization of hydrophilic poly(ethylene glycol) (PEG) chains. Briefly, double sided-adhesive was attached to the bottom of a standard tissue culture plate, after which a laser cutter was used to cut out the well bottoms. Using previously described chemistry, patterns were transferred to gold-coated glass via a polydimethylsiloxane stamp after which the glass was submerged in a poly(ethylene glycol) (PEG) solution overnight to build hydrophilic PEG chains surrounding μFeatures. Standard tissue culture plates with well bottoms cut out were then fastened to processed sheets using a custom-made alignment device.

[0183] High-Content Analysis:

[0184] Automated microscopy was performed using a Nikon Eclipse TI epifluorescent scope. A 20×20 grid with one μFeature per image was established and maintained so that each feature imaged was consistent each day. Nikon Perfect Focus was used to ensure that all colonies were in the same Z-plane and LysoSensor™ intensity was measured accurately. Images were processed using CellProfiler™ to count the number of nuclei and quantify LysoSensor™ intensity.

[0185] Genomic Sequencing:

[0186] DNA was isolated from cells using DNA QuickExtract™ (Epicentre) following TrypLE™ treatment and centrifugation. Extracted DNA was incubated at 65° C. for 15 min, 68° C. for 15 min, and 98° C. for 10 min. Genomic PCR was performed using AccuPrime™ HiFi Taq (Life Technologies) and 500 ng of genomic DNA according to manufacturer's instructions. Long (8 kb) PCR reactions were thermocycled using an extension step of 10 minutes. All genomic PCR products were then submitted to the University of Wisconsin-Madison Biotechnology Center for DNA sequencing.

[0187] RT- and qPCR:

[0188] RNA was isolated from cells using RNA QuickExtract™ (Epicentre) following the manufacturer's protocol. 100 ng of extracted RNA was reverse transcribed using Superscript® IV Reverse Transcriptase (Invitrogen). Endpoint PCR amplification of the cDNA product was performed following the manufacturer's instructions using AccuPrime™ HiFi Taq (Life Technologies) and 1 μl of cDNA Product. Efficacy of the endpoint PCR was performed via gel electrophoresis of the PCR product in a 1% agarose gel.

[0189] The qPCR reaction was set up in triplicate for each cell line and sequence (GAPDH, dT, 746, and GAA), by mixing 10 μl iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.5 μl sequence specific forward primer, 0.5 μl sequence specific reverse primer, 1 μl cDNA product, and 8 μl water. qPCR analysis was performed in a CFX96 Real Time PCR System under the following thermocycling con-

ditions: 95° C. for 30 s followed by 35 cycles of 95° C. for 5 s, and 60° C. for 30 s, with a melt curve analysis increasing stepwise from 65° C. to 95° C. in increments of 0.5° C.

[0190] Next Generation Sequencing Analysis:

[0191] A custom python script was developed to perform sequence analysis. For each sample, sequences with frequency of less than 1000 were filtered from the data. Sequences in which the reads matched with primer and reverse complement subsequences classified as "target sequences". Target sequences were aligned with corresponding wildtype sequence using global pairwise sequence alignment. The frequency, length, and position of matches, insertions, deletions, and mismatches were all tracked in the resulting aligned sequences.

[0192] Western Blotting:

[0193] Protein expression of GAA and β-Actin was determined in each cell line. Following cell lysis in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors and EDTA (5 mM), protein concentration was determined (DC Protein Assay, BioRad). Forty μg of protein from each cell line was loaded into a 4-12% Bis-Tris precast gel (Criterion XT, BioRad) and gel electrophoresis performed. Proteins were then transferred to a nitrocellulose membrane and blocked in filtered 5% nonfat dry milk in TBS-T (Tris buffered saline, 0.15% Tween20) for 1 hour at room temperature. The membrane was then incubated overnight at 4° C. with GAA (Abcam ab137068, 1:1000) and β-Actin (Millipore, MAB1501, 1:40,000) primary antibodies. Following the incubation period, the membrane was washed in TBS-T and incubated with appropriate horseradish peroxidase secondary antibodies (Goat Anti-Rabbit IgG, Abcam ab205718, 1:2000; Anti-Mouse IgG, Cell Signaling Technologies 7076 1:20,000) for 1 hour. The membrane was washed again in TBS-T, and then developed (SuperSignal™ West Pico Plus Chemiluminescent Substrate, Thermo Scientific) for 5 minutes using a ChemiDoc-It™2 Imaging System (UVP) and imaged.

[0194] GAA Activity Assay:

[0195] Acid glucosidase activity was measured by hydrolysis of 4-methylumbelliferyl—D-glucoside (4-MUG, Sigma M-9766) at pH 4 to release the fluorophore 4-methylumbelliferone (4-MU) as previously described. Briefly, 4-MUG was incubated with 10 μL protein lystate in 0.2M sodium acetate for one hour at 37° C. Fluoresence was then measured using a Glomax® plate reader (Progmega) and activity was calculated using a standard curve.

[0196] Immunocytochemistry:

[0197] Live cell imaging of lysosome intensity was done using LysoSensor™ Green (Life Technologies L7535). Dye was mixed in culture media at a 1:1000 dilution prior to adding media to wells. Cells were then incubated for 5 minutes in LysoSensor™ solution. Media was then aspirated and cells were washed 2× with PBS. All imaging was done within one hour of staining.

[0198] To assay for pluripotency markers, hPSC cultures were fixed using 4% PFA and incubated at room temperature for 10 minutes. Cells were then permeabilized using 0.05% Triton™ X-100 and incubated for 10 minutes. Following two washes with 5% goat serum, NANOG antibody (R&D Systems AF1997, 1:200) and TRA-1-60 antibody (Millipore MAB5360, 1:150), was added to cells and incubated overnight at 4° C. The next day, cells were rinsed twice with 5% goat serum and then incubated with a donkey anti-goat secondary antibody (Life Technologies A11055 1:500) for one hour at room temperature. Cells were then washed twice with PBS and mounted for imaging.

[0199] Cardiomyocyte cultures were processed in the same manner as above. After permeabilization, cells were incubated with anti-sarcomeric alpha-actinin (Abcam ab68167 1:250) overnight at 4° C. The next day, cells were rinsed twice with 5% goat serum and then incubated with a goat anti-rabbit secondary antibody (Santa Cruz Biotech sc-362262, 1:500.

[0200] Media Exchange:

[0201] Cardiomyocytes were cultured in RPMI/B27+insulin and media was exchanged every two days. As a normal media exchange, diseased and corrected cells were introduced to RPMI/B27+insulin/−glucose. 24 hours post change, cells were stained with LysoSensor™ as described above to determine a baseline fluorescent intensity. After staining, media was replaced with media from either corrected or healthy lines and cultured for an additional 24 hours. After incubation, cells were again stained with LysoSensor™ and imaged using confocal microscopy.

[0202] Isolation of Corrected iPSCs:

[0203] On day one post plating, we measured the presence of S1mplex within the nucleus as well as identified μFeatures that contained only one cell to ensure clonal populations. On days two through six, we measured the number of cells to obtain a growth rate via day over day change. Finally, on day 7, we measured the number of cells as well as stained cells with LysoSensor™ to identify populations that may have been edited using phenotypic recovery as a marker. We also mock transfected WA09 and Pompe iPSCs and plated them on ArrayEdit and subjected them to the same high content analysis as a control. After 7 days we plotted each individual μFeature by its LysoSensor™ intensity and growth rate and color coded each feature by the presence of S1mplexes on day 1. We observed a large population of clones that grew slower than the slowest mock transfected Pompe colonies suggesting that that population may undergo editing events. By comparing LysoSensor™ intensity we also observed that many of the μFeatures within the wells had higher, and therefore more acidic organelles than mock transfected Pompe iPSCs. In fact, many of the μFeatures had similar intensities to control WA09 colonies. By combining these data with the presence of S1mplexes we were able to select colonies that were potentially preferably edited at either loci individually or both simultaneously. Using this knowledge, we selected colonies of interest for expansion and analysis.

[0204] Following expansion of selected clones, we analyzed each one at both loci for the correction of mutations. We also designed the introduction of a PAM codon wobble to ensure that supplied donor DNA was the source for repair. When we looked at colonies that only had the presence of one S1mplex on day 1 we obtained clones that were edited at the specified allele. Interestingly we did not isolate any clones that had indel mutations caused by NHEJ. Further, colonies that were positive for one S1mplex were not observed to be edited at the other locus. We next analyzed clones positive for both S1mplexes and managed to obtain a clone that was edited at both alleles and also contained the PAM wobble. There was also one colony that contained one PAM mutation while the other allele was repaired but did not introduce the novel mutation. Importantly, across all screened clones we did not obtain any that contained indel

products. We then selected one clone from each population (edited at either allele individually, or both) to assay for phenotypic recovery.

Example 1: Correction of Two Diseased Alleles within Pompe iPSCs

[0205] To explore whether two corrected endogenous alleles within the same cell is possible, several clonal isogenic iPSC lines were generated by CRISPR-Cas9 gene editing of an iPSC line derived from a patient with infantile-onset Pompe disease. In this line, compound heterozygous GAA mutations responsible for the disease phenotype are a deletion of a thymidine nucleotide at position 1441 (GAA: c.[1441delT], "1441delT") causing a frameshift, and premature stop codon on one allele, and a G>A conversion at nucleotide 2237 (GAA:c.[2237G>A], "2237G>A") forming an immediate stop codon on the other (FIG. **1***b*). The mutations within GAA in this patient are ~6.1 kb apart, and hence using a single double strand break (DSB) with homology directed repair from a long plasmid or viral donor would likely be inefficient. We therefore used a strategy utilizing two distinct Cas9 ribonucleoproteins (RNPs) with accompanying single stranded oligonucleotide (ssODN) templates encoding the gene correction. (FIG. **1***b*, Tables 1-2)

TABLE 1

Protospacer and respective PAMs used for genomic targeting.

Name	Protospacer	SEQ ID NO:	PAM
1441delT sgRNA	CTCGTTGTCCAGGTAGGCCC	7	GGG
2237G>A sgRNA	TGGACCACCAGCTCCTGTAG	8	GGG
W746X sgRNA	GGACCACCAGCTCCTGTAGG	60	GGG
D645N sgRNA	GCCCAGGAAGCCGCAGACGT	61	TGG
R660H sgRNA	CAGAGGAGCTGTGTGTGCAC	62	TGG

TABLE 2

ssODNs used to direct HDR after DSB Formation.

Name	Sequence (5'-3')	SEQ ID NO:
1441insT ssODN	CTTCCATGCAGGCCCTGGGTGGGGCCGGGTCTCCCCA CTGCAGCCTCTCGTTGTCCAGGTATGGCCCGGATCCAC TGCCTTCCCCGACTTCACCAACCCC	9

TABLE 2-continued

ssODNs used to direct HDR after DSB Formation.

Name	Sequence (5'-3')	SEQ ID NO:
2237A>G ssODN	TGCCCATCCCCCTTGCAGGTTCCCCAAGGACTCTAGCA CCTGGACTGTGGACCACCAGCTCCTGTGGGGAGAGGC CCTGCTCATCACCCCAGTGCTCCAG	10
W746X ssODN	TGCCCATCCCCCTTGCAGGTTCCCCAAGGACTCTAGCA CCTGGACTGTGGACCACCAGCTCCTGTGGGGAGAGGC CCTGCTCATCACCCCAGTGCTCCAG	63
D645N ssODN	AGAAATCCTGCAGTTTAACCTGCTGGGGGTGCCTCTG GTCGGGGCAGACGTCTGTGGCTTCCTGGGCAACACCT CAGAGGAGCTGTGTGTGCACTGGACC	64
R660H ssODN	GGGCCAACGTCTGCGGCTTCCTGGGCAACACCTCAGA GGAGCTATGTGTGCGCTGGACCCAGCTGGGGGCCTTC TACCCCTTCATGCGGAACCACAACAG	65

[0206] Specifically, using a combination of S1mplex and ArrayEdit technologies developed by our lab, we enriched for properly-edited iPSCs after delivery of the two genome editors by tracking the presence of genome editors within the nucleus (FIG. **1***c*). Using high-content analysis imaging of the iPSC clones during culture post delivery of the editors, we tracked the growth rate of clones, as well as screening the pH of the lysosome using a Lysosensor™ dye. Lysosensor™ is sensitive to the buildup of glycogen in the diseased lysosome of mutant GAA cells, as high glycogen neutralizes this otherwise acidic organelle (FIG. **1***c*; FIGS. **5-6**). We isolated cell lines that were corrected at the 1441delT allele and the 2237G>A allele individually (FIG. **1***d*, termed 'single corrected'). We also isolated a clone corrected at both GAA:c.[1441delT];[2237G>A] alleles (FIG. **1***d*, termed 'double corrected').

[0207] All gene-corrected lines remained pluripotent, and after karyotyping each of the isolated lines, we observed no large transversions or inversions (FIG. **7**). Because genome editing can create large indel mutations, we also conducted an 8 kb PCR on GAA that included both sgRNA target sites and observed no genomic deletions between the sgRNA target sites. (FIG. **8**, **9**) Sequencing of these large PCR amplicons confirmed that both alleles were present and no other sequence abnormalities were detected at the edited loci. (FIG. **10**) Finally, chromatograms from Sanger sequencing at the top three off-target sites for each sgRNA matched the untransfected, patient-derived cell line, indicating that none of the top off-target regions were modified by our editing strategy (Table 3). Similar S1mplex strategies generated edits at 84-93% precision at the sgRNA target in primary fibroblasts derived from other Pompe diseased patients (FIG. **4**).

TABLE 3

Off-target sequencing results

Off-target site	Correction	Sequence	SEQ ID NO:
2237 O1-1	Unedited	CCTCCCTTCCTAGACCACCAGCTCCTGCAGGAG GGCTTGG	72
	1441dclT Corrected	CCTCCCTTCCTAGACCACCAGCTCCTGCAGGAG GGCTTGG	72
	2237G>A Corrected	CCTCCCTTCCTAGACCACCAGCTCCTGCAGGAG GGCTTGG	72

TABLE 3-continued

Off-target sequencing results

Off-target site	Correction	Sequence	SEQ ID NO:
	Double Corrected	CCTCCCTTCCTAGACCACCAGCTCCTGCAGGAG GGCTTGG	72
2237 OT-2	Unedited	GCCCCTGCCTCTACAGGAGCAGGTGGTGAGGAT GGCTCCG	73
	1441delT Corrected	GCCCCTGCCTCTACAGGAGCAGGTGGTGAGGAT GGCTCCG	73
	2237G>A Corrected	GCCCCTGCCTCTACAGGAGCAGGTGGTGAGGAT GGCTCCG	73
	Double Corrected	GCCCCTGCCTCTACAGGAGCAGGTGGTGAGGAT GGCTCCG	73
2237 OT-3	Unedited	CACGCTGCCACGACAGGAGCTGCTGGTCAACTC CTCCTCT	74
	1441delT Corrected	CACGCTGCCACGACAGGAGCTGCTGGTCAACTC CTCCTCT	74
	2237G>A Corrected	CACGCTGCCACGACAGGAGCTGCTGGTCAACTC CTCCTCT	74
	Double Corrected	CACGCTGCCACGACAGGAGCTGCTGGTCAACTC CTCCTCT	74
1441 OT-1	Unedited	CCCCGTATCCCTGGTTGTCCAGGTGGGCCCTGG GAGAACA	75
	1441delT Corrected	CCCCGTATCCCTGGTTGTCCAGGTGGGCCCTGG GAGAACA	75
	2237G>A Corrected	CCCCGTATCCCTGGTTGTCCAGGTGGGCCCTGG GAGAACA	75
	Double Corrected	CCCCGTATCCCTGGTTGTCCAGGTGGGCCCTGG GAGAACA	75
1441 OT-2	Unedited	CAGCTGCCGTCTCGATGGCCAGGTAGGCCTGGG CAAGGAC	76
	1441del1 Corrected	CAGCTGCCGTCTCGATGGCCAGGTAGGCCTGGG CAAGGAC	76
	2237G>A Corrected	CAGCTGCCGTCTCGATGGCCAGGTAGGCCTGGG CAAGGAC	76
	Double Corrected	CAGCTGCCGTCTCGATGGCCAGGTAGGCCTGGG CAAGGAC	76
1441 OT-3	Unedited	AATTAGGCTAGGGCCTACCTGGTCAATAATGAAATAAT TG	77
	1441delT Corrected	AATTAGGCTAGGGCCTACCTGGTCAATAATGAAATAAT TG	77
	2237G>A Corrected	AATTAGGCTAGGGCCTACCTGGTCAATAATGAAATAAT TG	77
	Double Corrected	AATTAGGCTAGGGCCTACCTGGTCAATAATGAAATAAT TG	77

[0208] Quantitative RT-PCR (qRT-PCR) at the 3' end of the GAA mRNA transcript as well as around each edited locus (FIG. **2***a*) indicated that the corrected loci were correctly expressed. We observed that the unedited line expressed the lowest levels of GAA when compared to internal GAPDH levels (FIG. **2***a*), despite the presence of full-length, mature mRNA that could be used to express protein (FIG. **2***a*). The single corrected lines also expressed mature mRNA, while the double corrected line contained approximately a 2-fold increase in GAA transcripts. We confirmed that double-corrected cell lines consistently produced greater amounts of mature mRNA than any other condition (FIG. **2***a*) by conducting qPCR at both edited loci.

[0209] By looking for the presence of disease variants and protoadjacent motif (PAM) wobbles introduced by the ssODN (FIG. **1***b*) via deep sequencing on endpoint PCR samples of mRNA, we observe that both alleles are expressed individually at higher levels (3-5 fold increase) than unedited cells (FIG. **2***a*). Each allele is expressed similarly to the corresponding single corrected line (FIG. **2***a*). These findings suggest nonsense mediated decay of the mutant transcript or cellular compensation to overcome the mutant allele within the single corrected lines. We detected active GAA protein using a Western blot (FIG. **2***b*) at levels comparable to a control hPSC line. We were also able to identify precursor polypeptides, which are important to protein secretion, showing the GAA transcripts from the edited alleles are being correctly translated and processed within cells. Notably, we are able to detect only small amounts of GAA protein and precursor polypeptides in the unedited iPSCs. All edited cell lines were able to secrete active GAA (FIG. **2***c*).

Example 2: Enzymatic Cross-Correction by Gene-Corrected Cells

[0210] Detection of active GAA secretion led us to test the potential of edited cells to enzymatically cross-correct dis-

eased cells (FIG. **3***a*). Because Pompe disease has a significant effect on cardiac tissue in infants, we differentiated all iPSC lines to cardiomyocytes (Pompe iPSC-CMs) using a previously described small-molecule inhibitor protocol (FIG. **3***a*). For all differentiations, we observed spontaneous contraction and confirmed the expression of α-actinin, a marker of cardiac lineage commitment. Similar to results seen in the iPSC state, differentiated corrected lines still expressed and secreted active GAA, as indicated in a 4-MUG cleavage assay on cardiomyocyte protein lysates and spent culture media. It has previously been demonstrated that by culturing in medium devoid of glucose, Pompe iPSC-CMs display an accumulation of glycogen within the lysosome. We performed a medium exchange experiment wherein we took partially spent, glucose-free medium from each corrected line (putatively containing secreted active GAA) and used it to replace glucose-free medium on unedited Pompe iPSC-CMs (FIG. **3***a*). One day after this media exchange, cells were stained with LysoSensor and subsequent confocal microscopy was used to measure lysosome acidity as a proxy for glycogen clearance. As a control we added rhGAA to unedited Pompe iPSC-CMs to simulate ERT. When unedited cardiomyocytes were supplemented with 10 nM rhGAA (ERT), LysoSensor™ intensity increased, indicating a clearance of glycogen from the lysosome. Media from all edited cells were able to recover the lysosomal pH at 96 hr (FIG. **3***b*), and this clearance is expected to continue until normal levels of glycogen were reached. Within these cultures, lysosomal size of unedited Pompe iPSC-CMs in GAA-positive media was profiled through visualization of Lysosomal Associated Membrane Protein 1, (LAMP-1). In media from unedited cells, lysosomes were enlarged, consistent with buildup of glycogen (FIG. **3***c*). In comparison, when media was taken from double-corrected cells or supplemented with rhGAA, lysosomes appeared as punctae. Samples from single corrected cells fell between these two extremes. Taken together, the single- and double-corrected cells enzymatically cross-correct diseased cardiomyocytes quickly and effectively.

[0211] Based on our experimental results, cells corrected at both alleles a1 and a2 have been modeled to secrete 3-fold more GAA than those edited at a single allele. Gene correction rates relative to other potential editing outcomes come from experiments with patient-derived fibroblasts. After delivery of the S1mplex genome editors, approximately 80-90% of all edits achieved gene correction. We utilize the nomenclature of Shen et al to describe this ratio of gene correction to other editing outcome. The remaining 10-20% of edits are imprecise at the on-target site, which could destroy the PAM or modify the on-target site for subsequent editing of these alleles, and these alleles are tracked in silico

[0212] Tables 4-6 provide the forward and reverse primer sequences (Table 4), the off-target sequences and corresponding genomic locus for each sgRNA used (Table 5), and the forward and reverse primers used to amplify off-target genomic loci (Table 6).

TABLE 4

Forward and reverse primers for genomic loci.

Name	Primer F (5'-3')	SEQ ID NO:	Primer R (5'-3')	SEQ ID NO:
1441delT genomic	AGCTGCTCATTGACCT CCAG	11	CAATCCACATGCCGTCG AAG	12
2237G>A genomic	AATTCAGCCTCTTCCT GTGC	13	CATACGTTCCTCTTTCCG CC	14
Full length genomic	TGACAGGTTTCCCTCT TCCCAG	15	TTGATAACCTACACTGCG GGGG	16
1441delT qPCR/NGS	AGTGGGGCTTCCATGC AG	17	GGTTGGTGAAGTCGGGG AAG	18
2237G>A qPCR/NGS	CCAAGGACTCTAGCAC CTGGAC	19	GGGAAGTAGCCAGTCAC TTCGG	20
W746X NGS	TCCCATTCATCACCCG TATGC	66	AGGTCGTACCATGTGCCC AA	67
D645N R660H NGS	CTGAGGACCAGCCTG ACTCT	68	CCACCCTACCAGACTGA GCA	69

TABLE 5

Off-target sequences and corresponding genomic locus for each sgRNA used. Mismatches from protospacer are bolded.

sgRNA Target Sequence	Off-target sequence		SEQ ID NO:	Chromosome location
2237G>A	OT1	T**A**GACCACCAGCTCCTG**C**AG	22	chr8:-42696136
TGGACCACCAGCTCCTGTAG	OT2	**CTC**ACCACC**T**GCTCCTGTAG	23	chr9:-123379574
SEQ ID NO: 21	OT3	T**T**GACCA**G**CAGCTCCTGTCG	24	chr15:-77699091
1441delT	OT1	CT**G**GTTGTCCAGGT**G**GGCCC	26	chr19:+9976610
TGGACCACCAGCTCCTGTAG	OT2	CTCG**A**TGGCCAGGTAGGCC**T**	27	chr9:+113788274
SEQ ID NO: 25	OT3	**ATTA**TTG**A**CCAGGTAGGCCC	28	chr20:-42195228

TABLE 6

Forward and reverse primers used to amplify off-target genomic loci.

Off-Target Primer	Primer F (5'-3')	SE QID NO.	Primer R (5'-3')	SEQ ID NO:
2237-OT1	CCCTCCTCTGTGTGCCATTA	29	GTGCCATATTTTGGGGACCAC	30
2237-OT2	GGGGCATGGTCAGATGATGG	31	CACAGAAATTCCTGAGGCCAAC	32
2237-OT3	GGAGAGGCTGACCTTCATGG	33	TCGTGCTTTCCTGACCATCG	34
1441-OT1	AGTGTGCTTCCACTGTCGTT	35	GTGCGGGTAACCTTCTCCAT	36
1441-OT2	TTCCTCTGCTGCTGAGTTGG	37	GCCGATTAAAAGGCTGTCGC	38
1441-OT3	AGAGCCCTGGAGGTCATTGT	39	CTGTCTGGCCTCTGAATCGG	40

DISCUSSION

[0213] While potential off-target effects and other safety concerns have been extensively studied, the efficacy of genome editing strategies has yet to be quantitatively analyzed, especially in polygenic cases. Emerging human cell based and in silico models have been used to facilitate translation of gene augmentation therapy, but have yet to be applied to genome editing. Our in vitro model constitutes a novel generalized framework to quantitatively understand the efficacy and potency of various genome editing strategies, and other gene and cell therapies.

[0214] We demonstrate biallelic gene correction with no detected off-target effects, and many of the common Pompe disease mutations can be targeted in an allele-specific manner using Sp.Cas9 strategies (Table 7). We observe that transcriptional regulation is driven by the endogenous promoter, potentially correcting a number of different isoforms for GAA. The targets for somatic cell genome editors therefore could expand from the traditional foci of liver and muscle to other tissues that may use alternate GAA isoforms. In contrast, in the gene augmentation approach, all cells must process a single isoform. Further, silencing from synthetic or viral elements has been observed for gene therapies, and in our hands with targeted knockin strategies that overexpress a transgene via a synthetic promoter. Transgene silencing raises concerns about the durability of viral gene therapies and proposed cell therapies where GAA is overexpressed from a safe harbor locus. In our strategy, post-translational processing of the enzyme also appears to be intact, as the distribution of processed GAA is identical to healthy controls. In contrast, GAA overexpression in mammalian cells can cause cellular stress, leading to differential trafficking and processing of the nascent translated peptide. The RNP-based correction strategy also avoids insertional oncogenesis by using a non-viral approach for delivery of the genome editor.

TABLE 7

Subset of mutations in GAA and accompanying allele-specific sgRNAs.
Bold denotes location of mutations relative to wildtype for allele-specific sgRNAs.
Italics denotes the PAM sequence.

Mutation	sgRNA Sequence	SEQ ID NO:	Allele Frequency ($\times 10^{-5}$)
c.118C>T	GAGGAGCCACTCAGCTCTC**A***GGG*	41	0.86
c.258dupC	ATCGAAGCGGCTGTT**G**GGGG*GGG*	42	2.65
c.525delT	CTGGACGTGATGATGGAGAC-*GAG*	43	7.04
c.1822C>T	AGTGGCCGGCGTATC**A**GCCG*TGG*	44	2.76
c.1827delC	TGCTGGCCACGGCCGATA-GC**CGG**	45	3.75
c.1930_1936dupGCCGACG	AAGCCGCAGA**CGTCGGC**CGT*CGG*	46	1.17
c.2242dupG	CACCAGCTCCTGTAGGGGG**G***AGG*	47	1.66
c.2.560C>T	ACCAAGGGTGGGGAGGCC**T**G*AGG*	48	21.5
c.2662G>T	TAACACGATCGTGAAT**T**AGC*TGG*	49	1.65

[0215] The use of the terms "a" and "an" and "the" and similar referents (especially in the context of the following claims) are to be construed to cover both the singular and the

plural, unless otherwise indicated herein or clearly contradicted by context. The terms first, second etc. as used herein are not meant to denote any particular ordering, but simply for convenience to denote a plurality of, for example, layers. The terms "comprising", "having", "including", and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The endpoints of all ranges are included within the range and independently combinable. All methods described herein can be performed in a suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as"), is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention as used herein.

[0216] While the invention has been described with reference to an exemplary embodiment, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to the particular embodiment disclosed as the best mode contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the appended claims. Any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 77

<210> SEQ ID NO 1
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protospacer region

<400> SEQUENCE: 1

ctcgttgtcc aggtaggccc 20

<210> SEQ ID NO 2
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protospacer region

<400> SEQUENCE: 2

tggaccacca gctcctgtag 20

<210> SEQ ID NO 3
<211> LENGTH: 1368
<212> TYPE: PRT
<213> ORGANISM: Streptococcus pyogenes

<400> SEQUENCE: 3

Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val
1 5 10 15

Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
20 25 30

Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
35 40 45

Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
50 55 60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65 70 75 80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser

-continued

```
                85                  90                  95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
            100                 105                 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
        115                 120                 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
    130                 135                 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
            180                 185                 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
        195                 200                 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225                 230                 235                 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270

Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300

Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350

Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445

Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495
```

-continued

```
Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560

Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670

Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685

Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
705                 710                 715                 720

His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln
        755                 760                 765

Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
    770                 775                 780

Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785                 790                 795                 800

Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
                805                 810                 815

Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
            820                 825                 830

Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys
        835                 840                 845

Asp Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg
    850                 855                 860

Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
865                 870                 875                 880

Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
                885                 890                 895
```

-continued

```
Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
            900                 905                 910

Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
        915                 920                 925

Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
    930                 935                 940

Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945                 950                 955                 960

Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
                965                 970                 975

Glu Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val
            980                 985                 990

Val Gly Thr Ala Leu Ile Lys Lys  Tyr Pro Lys Leu Glu  Ser Glu Phe
        995                 1000                1005

Val Tyr  Gly Asp Tyr Lys Val  Tyr Asp Val Arg Lys  Met Ile Ala
    1010                1015                1020

Lys Ser  Glu Gln Glu Ile Gly  Lys Ala Thr Ala Lys  Tyr Phe Phe
    1025                1030                1035

Tyr Ser  Asn Ile Met Asn Phe  Phe Lys Thr Glu Ile  Thr Leu Ala
    1040                1045                1050

Asn Gly  Glu Ile Arg Lys Arg  Pro Leu Ile Glu Thr  Asn Gly Glu
    1055                1060                1065

Thr Gly  Glu Ile Val Trp Asp  Lys Gly Arg Asp Phe  Ala Thr Val
    1070                1075                1080

Arg Lys  Val Leu Ser Met Pro  Gln Val Asn Ile Val  Lys Lys Thr
    1085                1090                1095

Glu Val  Gln Thr Gly Gly Phe  Ser Lys Glu Ser Ile  Leu Pro Lys
    1100                1105                1110

Arg Asn  Ser Asp Lys Leu Ile  Ala Arg Lys Lys Asp  Trp Asp Pro
    1115                1120                1125

Lys Lys  Tyr Gly Gly Phe Asp  Ser Pro Thr Val Ala  Tyr Ser Val
    1130                1135                1140

Leu Val  Val Ala Lys Val Glu  Lys Gly Lys Ser Lys  Lys Leu Lys
    1145                1150                1155

Ser Val  Lys Glu Leu Leu Gly  Ile Thr Ile Met Glu  Arg Ser Ser
    1160                1165                1170

Phe Glu  Lys Asn Pro Ile Asp  Phe Leu Glu Ala Lys  Gly Tyr Lys
    1175                1180                1185

Glu Val  Lys Lys Asp Leu Ile  Ile Lys Leu Pro Lys  Tyr Ser Leu
    1190                1195                1200

Phe Glu  Leu Glu Asn Gly Arg  Lys Arg Met Leu Ala  Ser Ala Gly
    1205                1210                1215

Glu Leu  Gln Lys Gly Asn Glu  Leu Ala Leu Pro Ser  Lys Tyr Val
    1220                1225                1230

Asn Phe  Leu Tyr Leu Ala Ser  His Tyr Glu Lys Leu  Lys Gly Ser
    1235                1240                1245

Pro Glu  Asp Asn Glu Gln Lys  Gln Leu Phe Val Glu  Gln His Lys
    1250                1255                1260

His Tyr  Leu Asp Glu Ile Ile  Glu Gln Ile Ser Glu  Phe Ser Lys
    1265                1270                1275

Arg Val  Ile Leu Ala Asp Ala  Asn Leu Asp Lys Val  Leu Ser Ala
```

-continued

```
    1280                1285                1290

Tyr Asn  Lys His Arg Asp Lys  Pro Ile Arg Glu Gln  Ala Glu Asn
    1295                1300                1305

Ile Ile  His Leu Phe Thr Leu  Thr Asn Leu Gly Ala  Pro Ala Ala
    1310                1315                1320

Phe Lys  Tyr Phe Asp Thr Thr  Ile Asp Arg Lys Arg  Tyr Thr Ser
    1325                1330                1335

Thr Lys  Glu Val Leu Asp Ala  Thr Leu Ile His Gln  Ser Ile Thr
    1340                1345                1350

Gly Leu  Tyr Glu Thr Arg Ile  Asp Leu Ser Gln Leu  Gly Gly Asp
    1355                1360                1365


<210> SEQ ID NO 4
<211> LENGTH: 1409
<212> TYPE: PRT
<213> ORGANISM: Streptococcus thermophilus

<400> SEQUENCE: 4

Met Leu Phe Asn Lys Cys Ile Ile Ile Ser Ile Asn Leu Asp Phe Ser
1               5                   10                  15

Asn Lys Glu Lys Cys Met Thr Lys Pro Tyr Ser Ile Gly Leu Asp Ile
            20                  25                  30

Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Asn Tyr Lys Val
        35                  40                  45

Pro Ser Lys Lys Met Lys Val Leu Gly Asn Thr Ser Lys Lys Tyr Ile
    50                  55                  60

Lys Lys Asn Leu Leu Gly Val Leu Leu Phe Asp Ser Gly Ile Thr Ala
65                  70                  75                  80

Glu Gly Arg Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg
                85                  90                  95

Arg Asn Arg Ile Leu Tyr Leu Gln Glu Ile Phe Ser Thr Glu Met Ala
            100                 105                 110

Thr Leu Asp Asp Ala Phe Phe Gln Arg Leu Asp Asp Ser Phe Leu Val
        115                 120                 125

Pro Asp Asp Lys Arg Asp Ser Lys Tyr Pro Ile Phe Gly Asn Leu Val
    130                 135                 140

Glu Glu Lys Val Tyr His Asp Glu Phe Pro Thr Ile Tyr His Leu Arg
145                 150                 155                 160

Lys Tyr Leu Ala Asp Ser Thr Lys Lys Ala Asp Leu Arg Leu Val Tyr
                165                 170                 175

Leu Ala Leu Ala His Met Ile Lys Tyr Arg Gly His Phe Leu Ile Glu
            180                 185                 190

Gly Glu Phe Asn Ser Lys Asn Asn Asp Ile Gln Lys Asn Phe Gln Asp
        195                 200                 205

Phe Leu Asp Thr Tyr Asn Ala Ile Phe Glu Ser Asp Leu Ser Leu Glu
    210                 215                 220

Asn Ser Lys Gln Leu Glu Glu Ile Val Lys Asp Lys Ile Ser Lys Leu
225                 230                 235                 240

Glu Lys Lys Asp Arg Ile Leu Lys Leu Phe Pro Gly Glu Lys Asn Ser
                245                 250                 255

Gly Ile Phe Ser Glu Phe Leu Lys Leu Ile Val Gly Asn Gln Ala Asp
            260                 265                 270
```

-continued

```
Phe Arg Lys Cys Phe Asn Leu Asp Glu Lys Ala Ser Leu His Phe Ser
        275                 280                 285

Lys Glu Ser Tyr Asp Glu Asp Leu Glu Thr Leu Leu Gly Tyr Ile Gly
    290                 295                 300

Asp Asp Tyr Ser Asp Val Phe Leu Lys Ala Lys Lys Leu Tyr Asp Ala
305                 310                 315                 320

Ile Leu Leu Ser Gly Phe Leu Thr Val Thr Asp Asn Glu Thr Glu Ala
                325                 330                 335

Pro Leu Ser Ser Ala Met Ile Lys Arg Tyr Asn Glu His Lys Glu Asp
            340                 345                 350

Leu Ala Leu Leu Lys Glu Tyr Ile Arg Asn Ile Ser Leu Lys Thr Tyr
        355                 360                 365

Asn Glu Val Phe Lys Asp Asp Thr Lys Asn Gly Tyr Ala Gly Tyr Ile
    370                 375                 380

Asp Gly Lys Thr Asn Gln Glu Asp Phe Tyr Val Tyr Leu Lys Asn Leu
385                 390                 395                 400

Leu Ala Glu Phe Glu Gly Ala Asp Tyr Phe Leu Glu Lys Ile Asp Arg
                405                 410                 415

Glu Asp Phe Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro
            420                 425                 430

Tyr Gln Ile His Leu Gln Glu Met Arg Ala Ile Leu Asp Lys Gln Ala
        435                 440                 445

Lys Phe Tyr Pro Phe Leu Ala Lys Asn Lys Glu Arg Ile Glu Lys Ile
    450                 455                 460

Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn
465                 470                 475                 480

Ser Asp Phe Ala Trp Ser Ile Arg Lys Arg Asn Glu Lys Ile Thr Pro
                485                 490                 495

Trp Asn Phe Glu Asp Val Ile Asp Lys Glu Ser Ser Ala Glu Ala Phe
            500                 505                 510

Ile Asn Arg Met Thr Ser Phe Asp Leu Tyr Leu Pro Glu Glu Lys Val
        515                 520                 525

Leu Pro Lys His Ser Leu Leu Tyr Glu Thr Phe Asn Val Tyr Asn Glu
    530                 535                 540

Leu Thr Lys Val Arg Phe Ile Ala Glu Ser Met Arg Asp Tyr Gln Phe
545                 550                 555                 560

Leu Asp Ser Lys Gln Lys Lys Asp Ile Val Arg Leu Tyr Phe Lys Asp
                565                 570                 575

Lys Arg Lys Val Thr Asp Lys Asp Ile Ile Glu Tyr Leu His Ala Ile
            580                 585                 590

Tyr Gly Tyr Asp Gly Ile Glu Leu Lys Gly Ile Glu Lys Gln Phe Asn
        595                 600                 605

Ser Ser Leu Ser Thr Tyr His Asp Leu Leu Asn Ile Ile Asn Asp Lys
    610                 615                 620

Glu Phe Leu Asp Asp Ser Ser Asn Glu Ala Ile Ile Glu Glu Ile Ile
625                 630                 635                 640

His Thr Leu Thr Ile Phe Glu Asp Arg Glu Met Ile Lys Gln Arg Leu
                645                 650                 655

Ser Lys Phe Glu Asn Ile Phe Asp Lys Ser Val Leu Lys Lys Leu Ser
            660                 665                 670

Arg Arg His Tyr Thr Gly Trp Gly Lys Leu Ser Ala Lys Leu Ile Asn
```

-continued

```
        675                 680                 685

Gly Ile Arg Asp Glu Lys Ser Gly Asn Thr Ile Leu Asp Tyr Leu Ile
    690                 695                 700

Asp Asp Gly Ile Ser Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp
705                 710                 715                 720

Ala Leu Ser Phe Lys Lys Lys Ile Gln Lys Ala Gln Ile Ile Gly Asp
                725                 730                 735

Glu Asp Lys Gly Asn Ile Lys Glu Val Val Lys Ser Leu Pro Gly Ser
            740                 745                 750

Pro Ala Ile Lys Lys Gly Ile Leu Gln Ser Ile Lys Ile Val Asp Glu
        755                 760                 765

Leu Val Lys Val Met Gly Gly Arg Lys Pro Glu Ser Ile Val Val Glu
    770                 775                 780

Met Ala Arg Glu Asn Gln Tyr Thr Asn Gln Gly Lys Ser Asn Ser Gln
785                 790                 795                 800

Gln Arg Leu Lys Arg Leu Glu Lys Ser Leu Lys Glu Leu Gly Ser Lys
                805                 810                 815

Ile Leu Lys Glu Asn Ile Pro Ala Lys Leu Ser Lys Ile Asp Asn Asn
            820                 825                 830

Ala Leu Gln Asn Asp Arg Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Lys
        835                 840                 845

Asp Met Tyr Thr Gly Asp Asp Leu Asp Ile Asp Arg Leu Ser Asn Tyr
    850                 855                 860

Asp Ile Asp His Ile Ile Pro Gln Ala Phe Leu Lys Asp Asn Ser Ile
865                 870                 875                 880

Asp Asn Lys Val Leu Val Ser Ser Ala Ser Asn Arg Gly Lys Ser Asp
                885                 890                 895

Asp Phe Pro Ser Leu Glu Val Val Lys Lys Arg Lys Thr Phe Trp Tyr
            900                 905                 910

Gln Leu Leu Lys Ser Lys Leu Ile Ser Gln Arg Lys Phe Asp Asn Leu
        915                 920                 925

Thr Lys Ala Glu Arg Gly Gly Leu Leu Pro Glu Asp Lys Ala Gly Phe
    930                 935                 940

Ile Gln Arg Gln Leu Val Glu Thr Arg Gln Ile Thr Lys His Val Ala
945                 950                 955                 960

Arg Leu Leu Asp Glu Lys Phe Asn Asn Lys Lys Asp Glu Asn Asn Arg
                965                 970                 975

Ala Val Arg Thr Val Lys Ile Ile Thr Leu Lys Ser Thr Leu Val Ser
            980                 985                 990

Gln Phe Arg Lys Asp Phe Glu Leu  Tyr Lys Val Arg Glu  Ile Asn Asp
        995                 1000                1005

Phe His  His Ala His Asp Ala  Tyr Leu Asn Ala Val  Ile Ala Ser
    1010                1015                1020

Ala Leu  Leu Lys Lys Tyr Pro  Lys Leu Glu Pro Glu  Phe Val Tyr
    1025                1030                1035

Gly Asp  Tyr Pro Lys Tyr Asn  Ser Phe Arg Glu Arg  Lys Ser Ala
    1040                1045                1050

Thr Glu  Lys Val Tyr Phe Tyr  Ser Asn Ile Met Asn  Ile Phe Lys
    1055                1060                1065

Lys Ser  Ile Ser Leu Ala Asp  Gly Arg Val Ile Glu  Arg Pro Leu
    1070                1075                1080
```

-continued

```
Ile Glu  Val Asn Glu Glu Thr  Gly Glu Ser Val Trp  Asn Lys Glu
    1085                 1090                 1095

Ser Asp  Leu Ala Thr Val Arg  Arg Val Leu Ser Tyr  Pro Gln Val
    1100                 1105                 1110

Asn Val  Val Lys Lys Val Glu  Glu Gln Asn His Gly  Leu Asp Arg
    1115                 1120                 1125

Gly Lys  Pro Lys Gly Leu Phe  Asn Ala Asn Leu Ser  Ser Lys Pro
    1130                 1135                 1140

Lys Pro  Asn Ser Asn Glu Asn  Leu Val Gly Ala Lys  Glu Tyr Leu
    1145                 1150                 1155

Asp Pro  Lys Lys Tyr Gly Gly  Tyr Ala Gly Ile Ser  Asn Ser Phe
    1160                 1165                 1170

Ala Val  Leu Val Lys Gly Thr  Ile Glu Lys Gly Ala  Lys Lys Lys
    1175                 1180                 1185

Ile Thr  Asn Val Leu Glu Phe  Gln Gly Ile Ser Ile  Leu Asp Arg
    1190                 1195                 1200

Ile Asn  Tyr Arg Lys Asp Lys  Leu Asn Phe Leu Leu  Glu Lys Gly
    1205                 1210                 1215

Tyr Lys  Asp Ile Glu Leu Ile  Ile Glu Leu Pro Lys  Tyr Ser Leu
    1220                 1225                 1230

Phe Glu  Leu Ser Asp Gly Ser  Arg Arg Met Leu Ala  Ser Ile Leu
    1235                 1240                 1245

Ser Thr  Asn Asn Lys Arg Gly  Glu Ile His Lys Gly  Asn Gln Ile
    1250                 1255                 1260

Phe Leu  Ser Gln Lys Phe Val  Lys Leu Leu Tyr His  Ala Lys Arg
    1265                 1270                 1275

Ile Ser  Asn Thr Ile Asn Glu  Asn His Arg Lys Tyr  Val Glu Asn
    1280                 1285                 1290

His Lys  Lys Glu Phe Glu Glu  Leu Phe Tyr Tyr Ile  Leu Glu Phe
    1295                 1300                 1305

Asn Glu  Asn Tyr Val Gly Ala  Lys Lys Asn Gly Lys  Leu Leu Asn
    1310                 1315                 1320

Ser Ala  Phe Gln Ser Trp Gln  Asn His Ser Ile Asp  Glu Leu Cys
    1325                 1330                 1335

Ser Ser  Phe Ile Gly Pro Thr  Gly Ser Glu Arg Lys  Gly Leu Phe
    1340                 1345                 1350

Glu Leu  Thr Ser Arg Gly Ser  Ala Ala Asp Phe Glu  Phe Leu Gly
    1355                 1360                 1365

Val Lys  Ile Pro Arg Tyr Arg  Asp Tyr Thr Pro Ser  Ser Leu Leu
    1370                 1375                 1380

Lys Asp  Ala Thr Leu Ile His  Gln Ser Val Thr Gly  Leu Tyr Glu
    1385                 1390                 1395

Thr Arg  Ile Asp Leu Ala Lys  Leu Gly Glu Gly
    1400                 1405


<210> SEQ ID NO 5
<211> LENGTH: 1082
<212> TYPE: PRT
<213> ORGANISM: Neisseria

<400> SEQUENCE: 5

Met Ala Ala Phe Lys Pro Asn Pro Ile Asn Tyr Ile Leu Gly Leu Asp
```

-continued

```
1               5                   10                  15

Ile Gly Ile Ala Ser Val Gly Trp Ala Met Val Glu Ile Asp Glu Glu
            20                  25                  30

Glu Asn Pro Ile Arg Leu Ile Asp Leu Gly Val Arg Val Phe Glu Arg
        35                  40                  45

Ala Glu Val Pro Lys Thr Gly Asp Ser Leu Ala Met Val Arg Arg Leu
    50                  55                  60

Ala Arg Ser Val Arg Arg Leu Thr Arg Arg Arg Ala His Arg Leu Leu
65                  70                  75                  80

Arg Ala Arg Arg Leu Leu Lys Arg Glu Gly Val Leu Gln Ala Ala Asp
                85                  90                  95

Phe Asp Glu Asn Gly Leu Ile Lys Ser Leu Pro Asn Thr Pro Trp Gln
            100                 105                 110

Leu Arg Ala Ala Ala Leu Asp Arg Lys Leu Thr Pro Leu Glu Trp Ser
        115                 120                 125

Ala Val Leu Leu His Leu Ile Lys His Arg Gly Tyr Leu Ser Gln Arg
    130                 135                 140

Lys Asn Glu Gly Glu Thr Ala Asp Lys Glu Leu Gly Ala Leu Leu Lys
145                 150                 155                 160

Gly Val Ala Asp Asn Ala His Ala Leu Gln Thr Gly Asp Phe Arg Thr
                165                 170                 175

Pro Ala Glu Leu Ala Leu Asn Lys Phe Glu Lys Glu Ser Gly His Ile
            180                 185                 190

Arg Asn Gln Arg Gly Asp Tyr Ser His Thr Phe Ser Arg Lys Asp Leu
        195                 200                 205

Gln Ala Glu Leu Ile Leu Leu Phe Glu Lys Gln Lys Glu Phe Gly Asn
    210                 215                 220

Pro His Ile Ser Gly Gly Leu Lys Glu Gly Ile Glu Thr Leu Leu Met
225                 230                 235                 240

Thr Gln Arg Pro Ala Leu Ser Gly Asp Ala Val Gln Lys Met Leu Gly
                245                 250                 255

His Cys Thr Phe Glu Pro Ala Glu Pro Lys Ala Ala Lys Asn Thr Tyr
            260                 265                 270

Thr Ala Glu Arg Phe Ile Trp Leu Thr Lys Leu Asn Asn Leu Arg Ile
        275                 280                 285

Leu Glu Gln Gly Ser Glu Arg Pro Leu Thr Asp Thr Glu Arg Ala Thr
    290                 295                 300

Leu Met Asp Glu Pro Tyr Arg Lys Ser Lys Leu Thr Tyr Ala Gln Ala
305                 310                 315                 320

Arg Lys Leu Leu Gly Leu Glu Asp Thr Ala Phe Phe Lys Gly Leu Arg
                325                 330                 335

Tyr Gly Lys Asp Asn Ala Glu Ala Ser Thr Leu Met Glu Met Lys Ala
            340                 345                 350

Tyr His Ala Ile Ser Arg Ala Leu Glu Lys Glu Gly Leu Lys Asp Lys
        355                 360                 365

Lys Ser Pro Leu Asn Leu Ser Pro Glu Leu Gln Asp Glu Ile Gly Thr
    370                 375                 380

Ala Phe Ser Leu Phe Lys Thr Asp Glu Asp Ile Thr Gly Arg Leu Lys
385                 390                 395                 400

Asp Arg Ile Gln Pro Glu Ile Leu Glu Ala Leu Leu Lys His Ile Ser
                405                 410                 415
```

-continued

```
Phe Asp Lys Phe Val Gln Ile Ser Leu Lys Ala Leu Arg Arg Ile Val
            420                 425                 430

Pro Leu Met Glu Gln Gly Lys Arg Tyr Asp Glu Ala Cys Ala Glu Ile
        435                 440                 445

Tyr Gly Asp His Tyr Gly Lys Lys Asn Thr Glu Glu Lys Ile Tyr Leu
    450                 455                 460

Pro Pro Ile Pro Ala Asp Glu Ile Arg Asn Pro Val Val Leu Arg Ala
465                 470                 475                 480

Leu Ser Gln Ala Arg Lys Val Ile Asn Gly Val Val Arg Arg Tyr Gly
                485                 490                 495

Ser Pro Ala Arg Ile His Ile Glu Thr Ala Arg Glu Val Gly Lys Ser
            500                 505                 510

Phe Lys Asp Arg Lys Glu Ile Glu Lys Arg Gln Glu Glu Asn Arg Lys
        515                 520                 525

Asp Arg Glu Lys Ala Ala Ala Lys Phe Arg Glu Tyr Phe Pro Asn Phe
    530                 535                 540

Val Gly Glu Pro Lys Ser Lys Asp Ile Leu Lys Leu Arg Leu Tyr Glu
545                 550                 555                 560

Gln Gln His Gly Lys Cys Leu Tyr Ser Gly Lys Glu Ile Asn Leu Gly
                565                 570                 575

Arg Leu Asn Glu Lys Gly Tyr Val Glu Ile Asp His Ala Leu Pro Phe
            580                 585                 590

Ser Arg Thr Trp Asp Asp Ser Phe Asn Asn Lys Val Leu Val Leu Gly
        595                 600                 605

Ser Glu Asn Gln Asn Lys Gly Asn Gln Thr Pro Tyr Glu Tyr Phe Asn
    610                 615                 620

Gly Lys Asp Asn Ser Arg Glu Trp Gln Glu Phe Lys Ala Arg Val Glu
625                 630                 635                 640

Thr Ser Arg Phe Pro Arg Ser Lys Lys Gln Arg Ile Leu Leu Gln Lys
                645                 650                 655

Phe Asp Glu Asp Gly Phe Lys Glu Arg Asn Leu Asn Asp Thr Arg Tyr
            660                 665                 670

Val Asn Arg Phe Leu Cys Gln Phe Val Ala Asp Arg Met Arg Leu Thr
        675                 680                 685

Gly Lys Gly Lys Lys Arg Val Phe Ala Ser Asn Gly Gln Ile Thr Asn
    690                 695                 700

Leu Leu Arg Gly Phe Trp Gly Leu Arg Lys Val Arg Ala Glu Asn Asp
705                 710                 715                 720

Arg His His Ala Leu Asp Ala Val Val Val Ala Cys Ser Thr Val Ala
                725                 730                 735

Met Gln Gln Lys Ile Thr Arg Phe Val Arg Tyr Lys Glu Met Asn Ala
            740                 745                 750

Phe Asp Gly Lys Thr Ile Asp Lys Glu Thr Gly Glu Val Leu His Gln
        755                 760                 765

Lys Thr His Phe Pro Gln Pro Trp Glu Phe Phe Ala Gln Glu Val Met
    770                 775                 780

Ile Arg Val Phe Gly Lys Pro Asp Gly Lys Pro Glu Phe Glu Glu Ala
785                 790                 795                 800

Asp Thr Pro Glu Lys Leu Arg Thr Leu Leu Ala Glu Lys Leu Ser Ser
                805                 810                 815
```

-continued

```
Arg Pro Glu Ala Val His Glu Tyr Val Thr Pro Leu Phe Val Ser Arg
            820                 825                 830

Ala Pro Asn Arg Lys Met Ser Gly Gln Gly His Met Glu Thr Val Lys
        835                 840                 845

Ser Ala Lys Arg Leu Asp Glu Gly Val Ser Val Leu Arg Val Pro Leu
    850                 855                 860

Thr Gln Leu Lys Leu Lys Asp Leu Glu Lys Met Val Asn Arg Glu Arg
865                 870                 875                 880

Glu Pro Lys Leu Tyr Glu Ala Leu Lys Ala Arg Leu Glu Ala His Lys
                885                 890                 895

Asp Asp Pro Ala Lys Ala Phe Ala Glu Pro Phe Tyr Lys Tyr Asp Lys
            900                 905                 910

Ala Gly Asn Arg Thr Gln Gln Val Lys Ala Val Arg Val Glu Gln Val
        915                 920                 925

Gln Lys Thr Gly Val Trp Val Arg Asn His Asn Gly Ile Ala Asp Asn
    930                 935                 940

Ala Thr Met Val Arg Val Asp Val Phe Glu Lys Gly Asp Lys Tyr Tyr
945                 950                 955                 960

Leu Val Pro Ile Tyr Ser Trp Gln Val Ala Lys Gly Ile Leu Pro Asp
                965                 970                 975

Arg Ala Val Val Gln Gly Lys Asp Glu Glu Asp Trp Gln Leu Ile Asp
            980                 985                 990

Asp Ser Phe Asn Phe Lys Phe Ser  Leu His Pro Asn Asp  Leu Val Glu
        995                 1000                1005

Val Ile  Thr Lys Lys Ala Arg  Met Phe Gly Tyr Phe  Ala Ser Cys
    1010                1015                1020

His Arg  Gly Thr Gly Asn Ile  Asn Ile Arg Ile His  Asp Leu Asp
    1025                1030                1035

His Lys  Ile Gly Lys Asn Gly  Ile Leu Glu Gly Ile  Gly Val Lys
    1040                1045                1050

Thr Ala  Leu Ser Phe Gln Lys  Tyr Gln Ile Asp Glu  Leu Gly Lys
    1055                1060                1065

Glu Ile  Arg Pro Cys Arg Leu  Lys Lys Arg Pro Pro  Val Arg
    1070                1075                1080


<210> SEQ ID NO 6
<211> LENGTH: 1395
<212> TYPE: PRT
<213> ORGANISM: Treponema

<400> SEQUENCE: 6

Met Lys Lys Glu Ile Lys Asp Tyr Phe Leu Gly Leu Asp Val Gly Thr
1               5                   10                  15

Gly Ser Val Gly Trp Ala Val Thr Asp Thr Asp Tyr Lys Leu Leu Lys
            20                  25                  30

Ala Asn Arg Lys Asp Leu Trp Gly Met Arg Cys Phe Glu Thr Ala Glu
        35                  40                  45

Thr Ala Glu Val Arg Arg Leu His Arg Gly Ala Arg Arg Arg Ile Glu
    50                  55                  60

Arg Arg Lys Lys Arg Ile Lys Leu Leu Gln Glu Leu Phe Ser Gln Glu
65                  70                  75                  80

Ile Ala Lys Thr Asp Glu Gly Phe Phe Gln Arg Met Lys Glu Ser Pro
                85                  90                  95
```

-continued

```
Phe Tyr Ala Glu Asp Lys Thr Ile Leu Gln Glu Asn Thr Leu Phe Asn
            100                 105                 110

Asp Lys Asp Phe Ala Asp Lys Thr Tyr His Lys Ala Tyr Pro Thr Ile
        115                 120                 125

Asn His Leu Ile Lys Ala Trp Ile Glu Asn Lys Val Lys Pro Asp Pro
    130                 135                 140

Arg Leu Leu Tyr Leu Ala Cys His Asn Ile Ile Lys Lys Arg Gly His
145                 150                 155                 160

Phe Leu Phe Glu Gly Asp Phe Asp Ser Glu Asn Gln Phe Asp Thr Ser
                165                 170                 175

Ile Gln Ala Leu Phe Glu Tyr Leu Arg Glu Asp Met Glu Val Asp Ile
            180                 185                 190

Asp Ala Asp Ser Gln Lys Val Lys Glu Ile Leu Lys Asp Ser Ser Leu
        195                 200                 205

Lys Asn Ser Glu Lys Gln Ser Arg Leu Asn Lys Ile Leu Gly Leu Lys
    210                 215                 220

Pro Ser Asp Lys Gln Lys Lys Ala Ile Thr Asn Leu Ile Ser Gly Asn
225                 230                 235                 240

Lys Ile Asn Phe Ala Asp Leu Tyr Asp Asn Pro Asp Leu Lys Asp Ala
                245                 250                 255

Glu Lys Asn Ser Ile Ser Phe Ser Lys Asp Asp Phe Asp Ala Leu Ser
            260                 265                 270

Asp Asp Leu Ala Ser Ile Leu Gly Asp Ser Phe Glu Leu Leu Leu Lys
        275                 280                 285

Ala Lys Ala Val Tyr Asn Cys Ser Val Leu Ser Lys Val Ile Gly Asp
    290                 295                 300

Glu Gln Tyr Leu Ser Phe Ala Lys Val Lys Ile Tyr Glu Lys His Lys
305                 310                 315                 320

Thr Asp Leu Thr Lys Leu Lys Asn Val Ile Lys Lys His Phe Pro Lys
                325                 330                 335

Asp Tyr Lys Lys Val Phe Gly Tyr Asn Lys Asn Glu Lys Asn Asn Asn
            340                 345                 350

Asn Tyr Ser Gly Tyr Val Gly Val Cys Lys Thr Lys Ser Lys Lys Leu
        355                 360                 365

Ile Ile Asn Asn Ser Val Asn Gln Glu Asp Phe Tyr Lys Phe Leu Lys
    370                 375                 380

Thr Ile Leu Ser Ala Lys Ser Glu Ile Lys Glu Val Asn Asp Ile Leu
385                 390                 395                 400

Thr Glu Ile Glu Thr Gly Thr Phe Leu Pro Lys Gln Ile Ser Lys Ser
                405                 410                 415

Asn Ala Glu Ile Pro Tyr Gln Leu Arg Lys Met Glu Leu Glu Lys Ile
            420                 425                 430

Leu Ser Asn Ala Glu Lys His Phe Ser Phe Leu Lys Gln Lys Asp Glu
        435                 440                 445

Lys Gly Leu Ser His Ser Glu Lys Ile Ile Met Leu Leu Thr Phe Lys
    450                 455                 460

Ile Pro Tyr Tyr Ile Gly Pro Ile Asn Asp Asn His Lys Lys Phe Phe
465                 470                 475                 480

Pro Asp Arg Cys Trp Val Val Lys Lys Glu Lys Ser Pro Ser Gly Lys
                485                 490                 495
```

-continued

```
Thr Thr Pro Trp Asn Phe Phe Asp His Ile Asp Lys Glu Lys Thr Ala
            500                 505                 510

Glu Ala Phe Ile Thr Ser Arg Thr Asn Phe Cys Thr Tyr Leu Val Gly
        515                 520                 525

Glu Ser Val Leu Pro Lys Ser Ser Leu Leu Tyr Ser Glu Tyr Thr Val
    530                 535                 540

Leu Asn Glu Ile Asn Asn Leu Gln Ile Ile Ile Asp Gly Lys Asn Ile
545                 550                 555                 560

Cys Asp Ile Lys Leu Lys Gln Lys Ile Tyr Glu Asp Leu Phe Lys Lys
                565                 570                 575

Tyr Lys Lys Ile Thr Gln Lys Gln Ile Ser Thr Phe Ile Lys His Glu
            580                 585                 590

Gly Ile Cys Asn Lys Thr Asp Glu Val Ile Ile Leu Gly Ile Asp Lys
        595                 600                 605

Glu Cys Thr Ser Ser Leu Lys Ser Tyr Ile Glu Leu Lys Asn Ile Phe
    610                 615                 620

Gly Lys Gln Val Asp Glu Ile Ser Thr Lys Asn Met Leu Glu Glu Ile
625                 630                 635                 640

Ile Arg Trp Ala Thr Ile Tyr Asp Glu Gly Glu Gly Lys Thr Ile Leu
                645                 650                 655

Lys Thr Lys Ile Lys Ala Glu Tyr Gly Lys Tyr Cys Ser Asp Glu Gln
            660                 665                 670

Ile Lys Lys Ile Leu Asn Leu Lys Phe Ser Gly Trp Gly Arg Leu Ser
        675                 680                 685

Arg Lys Phe Leu Glu Thr Val Thr Ser Glu Met Pro Gly Phe Ser Glu
    690                 695                 700

Pro Val Asn Ile Ile Thr Ala Met Arg Glu Thr Gln Asn Asn Leu Met
705                 710                 715                 720

Glu Leu Leu Ser Ser Glu Phe Thr Phe Thr Glu Asn Ile Lys Lys Ile
                725                 730                 735

Asn Ser Gly Phe Glu Asp Ala Glu Lys Gln Phe Ser Tyr Asp Gly Leu
            740                 745                 750

Val Lys Pro Leu Phe Leu Ser Pro Ser Val Lys Lys Met Leu Trp Gln
        755                 760                 765

Thr Leu Lys Leu Val Lys Glu Ile Ser His Ile Thr Gln Ala Pro Pro
    770                 775                 780

Lys Lys Ile Phe Ile Glu Met Ala Lys Gly Ala Glu Leu Glu Pro Ala
785                 790                 795                 800

Arg Thr Lys Thr Arg Leu Lys Ile Leu Gln Asp Leu Tyr Asn Asn Cys
                805                 810                 815

Lys Asn Asp Ala Asp Ala Phe Ser Ser Glu Ile Lys Asp Leu Ser Gly
            820                 825                 830

Lys Ile Glu Asn Glu Asp Asn Leu Arg Leu Arg Ser Asp Lys Leu Tyr
        835                 840                 845

Leu Tyr Tyr Thr Gln Leu Gly Lys Cys Met Tyr Cys Gly Lys Pro Ile
    850                 855                 860

Glu Ile Gly His Val Phe Asp Thr Ser Asn Tyr Asp Ile Asp His Ile
865                 870                 875                 880

Tyr Pro Gln Ser Lys Ile Lys Asp Asp Ser Ile Ser Asn Arg Val Leu
                885                 890                 895

Val Cys Ser Ser Cys Asn Lys Asn Lys Glu Asp Lys Tyr Pro Leu Lys
```

-continued

```
           900                 905                 910

Ser Glu Ile Gln Ser Lys Gln Arg Gly Phe Trp Asn Phe Leu Gln Arg
       915                 920                 925

Asn Asn Phe Ile Ser Leu Glu Lys Leu Asn Arg Leu Thr Arg Ala Thr
   930                 935                 940

Pro Ile Ser Asp Asp Glu Thr Ala Lys Phe Ile Ala Arg Gln Leu Val
945                 950                 955                 960

Glu Thr Arg Gln Ala Thr Lys Val Ala Ala Lys Val Leu Glu Lys Met
               965                 970                 975

Phe Pro Glu Thr Lys Ile Val Tyr Ser Lys Ala Glu Thr Val Ser Met
           980                 985                 990

Phe Arg Asn Lys Phe Asp Ile Val  Lys Cys Arg Glu Ile  Asn Asp Phe
       995                 1000                1005

His His  Ala His Asp Ala Tyr  Leu Asn Ile Val Val  Gly Asn Val
   1010                1015                1020

Tyr Asn  Thr Lys Phe Thr Asn  Asn Pro Trp Asn Phe  Ile Lys Glu
   1025                1030                1035

Lys Arg  Asp Asn Pro Lys Ile  Ala Asp Thr Tyr Asn  Tyr Tyr Lys
   1040                1045                1050

Val Phe  Asp Tyr Asp Val Lys  Arg Asn Asn Ile Thr  Ala Trp Glu
   1055                1060                1065

Lys Gly  Lys Thr Ile Ile Thr  Val Lys Asp Met Leu  Lys Arg Asn
   1070                1075                1080

Thr Pro  Ile Tyr Thr Arg Gln  Ala Ala Cys Lys Lys  Gly Glu Leu
   1085                1090                1095

Phe Asn  Gln Thr Ile Met Lys  Lys Gly Leu Gly Gln  His Pro Leu
   1100                1105                1110

Lys Lys  Glu Gly Pro Phe Ser  Asn Ile Ser Lys Tyr  Gly Gly Tyr
   1115                1120                1125

Asn Lys  Val Ser Ala Ala Tyr  Tyr Thr Leu Ile Glu  Tyr Glu Glu
   1130                1135                1140

Lys Gly  Asn Lys Ile Arg Ser  Leu Glu Thr Ile Pro  Leu Tyr Leu
   1145                1150                1155

Val Lys  Asp Ile Gln Lys Asp  Gln Asp Val Leu Lys  Ser Tyr Leu
   1160                1165                1170

Thr Asp  Leu Leu Gly Lys Lys  Glu Phe Lys Ile Leu  Val Pro Lys
   1175                1180                1185

Ile Lys  Ile Asn Ser Leu Leu  Lys Ile Asn Gly Phe  Pro Cys His
   1190                1195                1200

Ile Thr  Gly Lys Thr Asn Asp  Ser Phe Leu Leu Arg  Pro Ala Val
   1205                1210                1215

Gln Phe  Cys Cys Ser Asn Asn  Glu Val Leu Tyr Phe  Lys Lys Ile
   1220                1225                1230

Ile Arg  Phe Ser Glu Ile Arg  Ser Gln Arg Glu Lys  Ile Gly Lys
   1235                1240                1245

Thr Ile  Ser Pro Tyr Glu Asp  Leu Ser Phe Arg Ser  Tyr Ile Lys
   1250                1255                1260

Glu Asn  Leu Trp Lys Lys Thr  Lys Asn Asp Glu Ile  Gly Glu Lys
   1265                1270                1275

Glu Phe  Tyr Asp Leu Leu Gln  Lys Lys Asn Leu Glu  Ile Tyr Asp
   1280                1285                1290
```

-continued

```
Met Leu  Leu Thr Lys His Lys  Asp Thr Ile Tyr Lys  Lys Arg Pro
    1295                 1300                 1305

Asn Ser  Ala Thr Ile Asp Ile  Leu Val Lys Gly Lys  Glu Lys Phe
    1310                 1315                 1320

Lys Ser  Leu Ile Ile Glu Asn  Gln Phe Glu Val Ile  Leu Glu Ile
    1325                 1330                 1335

Leu Lys  Leu Phe Ser Ala Thr  Arg Asn Val Ser Asp  Leu Gln His
    1340                 1345                 1350

Ile Gly  Gly Ser Lys Tyr Ser  Gly Val Ala Lys Ile  Gly Asn Lys
    1355                 1360                 1365

Ile Ser  Ser Leu Asp Asn Cys  Ile Leu Ile Tyr Gln  Ser Ile Thr
    1370                 1375                 1380

Gly Ile  Phe Glu Lys Arg Ile  Asp Leu Leu Lys Val
    1385                 1390                 1395


<210> SEQ ID NO 7
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protospacer

<400> SEQUENCE: 7

ctcgttgtcc aggtaggccc                                              20


<210> SEQ ID NO 8
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protospacer

<400> SEQUENCE: 8

tggaccacca gctcctgtag                                              20


<210> SEQ ID NO 9
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ssODN

<400> SEQUENCE: 9

cttccatgca ggccctgggt ggggccgggt ctccccactg cagcctctcg ttgtccaggt     60

atggcccgga tccactgcct tccccgactt caccaacccc                          100


<210> SEQ ID NO 10
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ssODN

<400> SEQUENCE: 10

tgcccatccc ccttgcaggt tccccaagga ctctagcacc tggactgtgg accaccagct     60

cctgtgggga gaggccctgc tcatcacccc agtgctccag                          100


<210> SEQ ID NO 11
```

-continued

<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 11

agctgctcat tgacctccag 20

<210> SEQ ID NO 12
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 12

caatccacat gccgtcgaag 20

<210> SEQ ID NO 13
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 13

aattcagcct cttcctgtgc 20

<210> SEQ ID NO 14
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 14

catacgttcc tctttccgcc 20

<210> SEQ ID NO 15
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 15

tgacaggttt ccctcttccc ag 22

<210> SEQ ID NO 16
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 16

ttgataacct acactgcggg gg 22

<210> SEQ ID NO 17
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 17

agtggggctt ccatgcag 18

<210> SEQ ID NO 18
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protein

<400> SEQUENCE: 18

ggttggtgaa gtcggggaag 20

<210> SEQ ID NO 19
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 19

ccaaggactc tagcacctgg ac 22

<210> SEQ ID NO 20
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 20

gggaagtagc cagtcacttc gg 22

<210> SEQ ID NO 21
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA Target Sequence

<400> SEQUENCE: 21

tggaccacca gctcctgtag 20

<210> SEQ ID NO 22
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Off-target sequence

<400> SEQUENCE: 22

tagaccacca gctcctgcag 20

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Off-target sequence

<400> SEQUENCE: 23

-continued

ctcaccacct gctcctgtag 20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Off-target sequence

<400> SEQUENCE: 24

ttgaccagca gctcctgtcg 20

<210> SEQ ID NO 25
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA Target Sequence

<400> SEQUENCE: 25

tggaccacca gctcctgtag 20

<210> SEQ ID NO 26
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Off-target sequence

<400> SEQUENCE: 26

ctggttgtcc aggtgggccc 20

<210> SEQ ID NO 27
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Off-target sequence

<400> SEQUENCE: 27

ctcgatggcc aggtaggcct 20

<210> SEQ ID NO 28
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Off-target sequence

<400> SEQUENCE: 28

attattgacc aggtaggccc 20

<210> SEQ ID NO 29
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 29

ccctcctctg tgtgccatta 20

<210> SEQ ID NO 30

-continued

<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 30

gtgccatatt ttggggacca c 21

<210> SEQ ID NO 31
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 31

ggggcatggt cagatgatgg 20

<210> SEQ ID NO 32
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 32

cacagaaatt cctgaggcca ac 22

<210> SEQ ID NO 33
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 33

ggagaggctg accttcatgg 20

<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 34

tcgtgctttc ctgaccatcg 20

<210> SEQ ID NO 35
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 35

agtgtgcttc cactgtcgtt 20

<210> SEQ ID NO 36
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 36

gtgcgggtaa ccttctccat 20

<210> SEQ ID NO 37
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 37

ttcctctgct gctgagttgg 20

<210> SEQ ID NO 38
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 38

gccgattaaa aggctgtcgc 20

<210> SEQ ID NO 39
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 39

agagccctgg aggtcattgt 20

<210> SEQ ID NO 40
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 40

ctgtctggcc tctgaatcgg 20

<210> SEQ ID NO 41
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 41

gaggagccac tcagctctca ggg 23

<210> SEQ ID NO 42
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 42

-continued

atcgaagcgg ctgttggggg ggg 23

<210> SEQ ID NO 43
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 43

ctggacgtga tgatggagac gag 23

<210> SEQ ID NO 44
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 44

agtggccggc gtatcagccg tgg 23

<210> SEQ ID NO 45
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 45

tgctggccac ggccgatagc cgg 23

<210> SEQ ID NO 46
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 46

aagccgcaga cgtcggccgt cgg 23

<210> SEQ ID NO 47
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 47

caccagctcc tgtaggggg agg 23

<210> SEQ ID NO 48
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 48

accaagggtg gggaggcctg agg 23

<210> SEQ ID NO 49

-continued

<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: sgRNA sequence

<400> SEQUENCE: 49

taacacgatc gtgaattagc tgg 23

<210> SEQ ID NO 50
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 2237G repair ssODN

<400> SEQUENCE: 50

gactgtggac caccagctcc tgtggggaga 30

<210> SEQ ID NO 51
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: allele 1

<400> SEQUENCE: 51

gactgtggac caccagctcc tgtagggaga 30

<210> SEQ ID NO 52
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: allele 2

<400> SEQUENCE: 52

gactgtggac caccagctcc tgtgggggga 30

<210> SEQ ID NO 53
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 1441 insT repair ssODN

<400> SEQUENCE: 53

gcctctcgtt gtccaggtat ggcccggatc cac 33

<210> SEQ ID NO 54
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: allele 2

<400> SEQUENCE: 54

gcctctcgtt gtccaggtat ggcccgggtc cac 33

<210> SEQ ID NO 55
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: allele 1

<400> SEQUENCE: 55

gcctctcgtt gtccaggtat ggcccgggtc cac 33

<210> SEQ ID NO 56
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: unedited allele
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(25)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (28)..(28)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 56

gcctctcgtt gtccaggtan gnccnggntc 30

<210> SEQ ID NO 57
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 1441 delT corrected

<400> SEQUENCE: 57

gcctctcgtt gtccaggtat ggcccggatc 30

<210> SEQ ID NO 58
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: unedited allele
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (24)..(24)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 58

gactgtggac caccagctcc tgtngggg 28

<210> SEQ ID NO 59
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 2337G>A corrected

<400> SEQUENCE: 59

gactgtggac caccagctcc tgtgggag 28

<210> SEQ ID NO 60

-continued

<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protospacer

<400> SEQUENCE: 60

ggaccaccag ctcctgtagg 20

<210> SEQ ID NO 61
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: protospacer

<400> SEQUENCE: 61

gcccaggaag ccgcagacgt 20

<210> SEQ ID NO 62
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Protospacer

<400> SEQUENCE: 62

cagaggagct gtgtgtgcac 20

<210> SEQ ID NO 63
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ssODN

<400> SEQUENCE: 63

tgcccatccc ccttgcaggt tccccaagga ctctagcacc tggactgtgg accaccagct 60

cctgtgggga gaggccctgc tcatcacccc agtgctccag 100

<210> SEQ ID NO 64
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ssODN

<400> SEQUENCE: 64

agaaatcctg cagtttaacc tgctgggggt gcctctggtc ggggcagacg tctgtggctt 60

cctgggcaac acctcagagg agctgtgtgt gcactggacc 100

<210> SEQ ID NO 65
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: ssODN

<400> SEQUENCE: 65

gggccaacgt ctgcggcttc ctgggcaaca cctcagagga gctatgtgtg cgctggaccc 60

agctgggggc cttctacccc ttcatgcgga accacaacag 100

-continued

<210> SEQ ID NO 66
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 66

tcccattcat cacccgtatg c 21

<210> SEQ ID NO 67
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 67

aggtcgtacc atgtgcccaa 20

<210> SEQ ID NO 68
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 68

ctgaggacca gcctgactct 20

<210> SEQ ID NO 69
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 69

ccaccctacc agactgagca 20

<210> SEQ ID NO 70
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Unedited sequence, 1441delT corrected
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (24)..(24)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 70

gactgtggac caccagctcc tgtnggggg agg 33

<210> SEQ ID NO 71
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 2237G>A corrected, double corrected

<400> SEQUENCE: 71

gactgtggac caccagctcc tgtggggaga gg 32

-continued

<210> SEQ ID NO 72
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 2237 OT-1

<400> SEQUENCE: 72

cctcccttcc tagaccacca gctcctgcag gagggcttgg 40

<210> SEQ ID NO 73
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 2237 OT-2

<400> SEQUENCE: 73

gcccctgcct ctacaggagc aggtggtgag gatggctccg 40

<210> SEQ ID NO 74
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 2237 OT-3

<400> SEQUENCE: 74

cacgctgcca cgacaggagc tgctggtcaa ctcctcctct 40

<210> SEQ ID NO 75
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 1441 OT-1

<400> SEQUENCE: 75

ccccgtatcc ctggttgtcc aggtgggccc tgggagaaca 40

<210> SEQ ID NO 76
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 1441 OT-2

<400> SEQUENCE: 76

cagctgccgt ctcgatggcc aggtaggcct gggcaaggac 40

<210> SEQ ID NO 77
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 1441 OT-3

<400> SEQUENCE: 77

aattaggcta gggcctacct ggtcaataat gaaataattg 40

1. A modified guide RNA, comprising

a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

wherein the single stranded protospacer region comprises

(SEQ ID NO: 1)
CTCGTTGTCCAGGTAGGCCC,

(SEQ ID NO: 2)
TGGACCACCAGCTCCTGTAG,

(SEQ ID NO: 60)
GGACCACCAGCTCCTGTAGG,

(SEQ ID NO: 61)
GCCCAGGAAGCCGCAGACGT,
or

(SEQ ID NO: 62)
CAGAGGAGCTGTGTGTGCAC.

2. The modified guide RNA of claim **1**, wherein the crRNA and the tracrRNA form an sgRNA, the sgRNA comprising, from 5' to 3',

the single-stranded protospacer sequence,

the first complementary strand of a binding region for the Cas9 polypeptide,

the aptamer that binds a biotin-binding molecule, and

the second complementary strand of the binding region for the Cas9 polypeptide.

3. The modified guide RNA of claim **2**, wherein, in the secondary structure of the modified sgRNA, the binding region for the Cas9 polypeptide and the aptamer that binds the biotin-binding molecule forms a stem-loop structure.

4. A guide RNA, comprising

a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

wherein the single stranded protospacer region comprises

(SEQ ID NO: 1)
CTCGTTGTCCAGGTAGGCCC,

(SEQ ID NO: 2)
TGGACCACCAGCTCCTGTAG,

(SEQ ID NO: 60)
GGACCACCAGCTCCTGTAGG,

(SEQ ID NO: 61)
GCCCAGGAAGCCGCAGACGT,
or

(SEQ ID NO: 62)
CAGAGGAGCTGTGTGTGCAC.

5. An RNP complex, comprising the modified guide RNA of claim **1**, and a Cas9 polypeptide or active fragment thereof.

6. A method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has an autosomal recessive disorder with compound heterozygous mutations, the method comprising

delivering to the patient or cell a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide, wherein each modified guide RNA comprises,

a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

wherein the first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele,

wherein the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele.

7. The method of claim **6**, wherein the patient-derived cell comprises an induced pluripotent stem cell, a progenitor cell, a mesenchymal stem cell, or a tissue-specific stem cell.

8. The method of claim **7**, wherein the tissue-specific stem cell comprises a skeletal stem cell, a hematopoietic stem cell, an epithelial stem cell, or a neural stem cell.

9. The method of claim **6**, wherein a first RNP complex comprises the first modified guide RNA, the Cas9 polypeptide, the biotin-binding molecule and the first biotinylated donor polynucleotide; and a second RNP complex comprises the second modified guide RNA, the Cas9 polypeptide, the biotin-binding molecule and the second biotinylated donor polynucleotide.

10. The method of claim **6**, wherein the first modified guide RNA, the second modified guide RNA and the Cas9 polypeptide are expressed from one or more viral vectors.

11. The method of claim **10**, wherein a first viral vector expresses the first modified guide RNA, and a second viral vector expresses the second modified guide RNA, and a third viral vector expresses the Cas9 polypeptide.

12. The method of claim **6**, wherein the patient is a human patient and the autosomal recessive disorder is aspartylglucosaminuria, Batten disease, cystinosis, Fabry disease, Gaucher Disease Type I, II or III, Pompe Disease, Tay Sachs Disease, Sandhoff Disease, Metachromatic leukodystrophy, Mucolipidosis Type, I, II/III or IV, Hurler Disease, Hunter disease, Sanfilippo disease Types A,B,C,D, Morquio disease Types A and B, Maroteaux-Lamy disease, Sly disease, Niemann-Pick Disease Types A/B, C1 or C2, or Schindler Disease Types I or II.

13. The method of claim **6**, wherein the autosomal recessive disorder is a human lysosomal storage disorder.

14. The method of claim **13**, wherein the autosomal recessive disorder is Pompe Disease.

15. The method of claim **6**, wherein the wherein the biotin-binding molecule is covalently linked to a donor polynucleotide, either directly or via a linker molecule.

16. The method of claim **6**, wherein the biotinylated donor polynucleotide comprises a biotinylated nanoparticle, a dye, a contrast agent, a cell or tissue targeting ligand, or a peptide.

17. The method of claim **16**, wherein the nanoparticle is a quantum dot, a gold particle, a magnetic particle, or a polymeric nanoparticle.

18. The method of claim **6**, wherein the donor polynucleotide comprises single-stranded DNA, double-stranded DNA, RNA, or a duplex of RNA and DNA.

19. The method of claim **6**, wherein the biotin-binding molecule has one, two, three or four biotin binding sites, wherein the biotin-binding molecule optionally comprises a fluorescent label.

20. A method of treating a patient with an autosomal recessive disorder with compound heterozygous mutations, comprising transplanting the cell made by the method of claim **6** into the subject.

21. A method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has Pompe disease, the method comprising

delivering to the patient or cell a first modified guide RNA, a second modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, a first biotinylated donor polynucleotide, and a second biotinylated donor polynucleotide, wherein each modified guide RNA comprises,

a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

wherein the first modified guide RNA and the first biotinylated donor polynucleotide correct a first diseased allele,

wherein the second modified guide RNA and the second biotinylated donor polynucleotide correct a second diseased allele,

wherein the single stranded protospacer region of the first modified guide RNA comprises CTCGTTGTCCAGGTAGGCCC (SEQ ID NO: 1) and the single stranded protospacer region of the second guide RNA comprises TGGACCACCAGCTCCTGTA (SEQ ID NO: 2).

22. A method of modifying a target gene in a patient or in a patient-derived cell, wherein the patient has Pompe disease, the method comprising

delivering to the patient or cell a first guide RNA, a second guide RNA, a Cas9 polypeptide, a first donor polynucleotide, and a second donor polynucleotide, wherein each guide RNA comprises,

a crRNA comprising, a single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

wherein the single-stranded protospacer sequence of the modified guide RNA hybridizes to a sequence in the target gene to be modified.

wherein the first guide RNA and the first donor polynucleotide correct a first diseased allele,

wherein the second guide RNA and the second donor polynucleotide correct a second diseased allele,

wherein the single stranded protospacer region of the first modified guide RNA comprises CTCGTTGTCCAGGTAGGCCC (SEQ ID NO: 1) and the single stranded protospacer region of the second guide RNA comprises TGGACCACCAGCTCCTGTA (SEQ ID NO: 2).

23. A method of making an RNP complex, comprising

selecting a single stranded protospacer sequence by identifying a mutant allele to be corrected, wherein the mutant allele is within 0 to 100 bases of a protospacer adjacent motif,

producing a modified guide RNA comprising the selected single stranded protospacer region, wherein the modified guide RNA comprises

a crRNA comprising the single-stranded protospacer sequence and a first complementary strand of a binding region for the Cas9 polypeptide, and

a tracrRNA comprising a second complementary strand of the binding region for the Cas9 polypeptide,

wherein the crRNA or the tracrRNA comprises an aptamer that binds a biotin-binding molecule,

wherein the crRNA and the tracrRNA hybridize through the first and second complementary strands of the binding region for the Cas9 polypeptide,

selecting a donor polynucleotide to correct the mutant allele and complementary to an anti-sense strand of genomic DNA, wherein the donor polynucleotide comprises a silent mutation in a constant region of the protospacer adjacent motif, e.g., within the final two nucleotides of the protospacer adjacent motif for *Streptococcus pyogenes,*

producing a biotinylated donor polynucleotide, wherein the donor polynucleotide is biotinylated at the 5' end or the 3' end, and

assembling the modified guide RNA, a Cas9 polypeptide, a biotin-binding molecule, and the biotinylated donor polynucleotide.

24. The method of claim **21**, further comprising repeating the method and producing a second RNP complex, wherein the second RNP complex corrects a second mutant allele to result in a biallelic correction.

* * * * *