(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 January 2021 (07.01.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/003337 A1

(51) International Patent Classification:
C12N 9/16 (2006.01) *C12N 9/78* (2006.01)
C12N 9/22 (2006.01) *C12N 9/99* (2006.01)

(21) International Application Number:
PCT/US2020/040608

(22) International Filing Date:
02 July 2020 (02.07.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/870,488 03 July 2019 (03.07.2019) US

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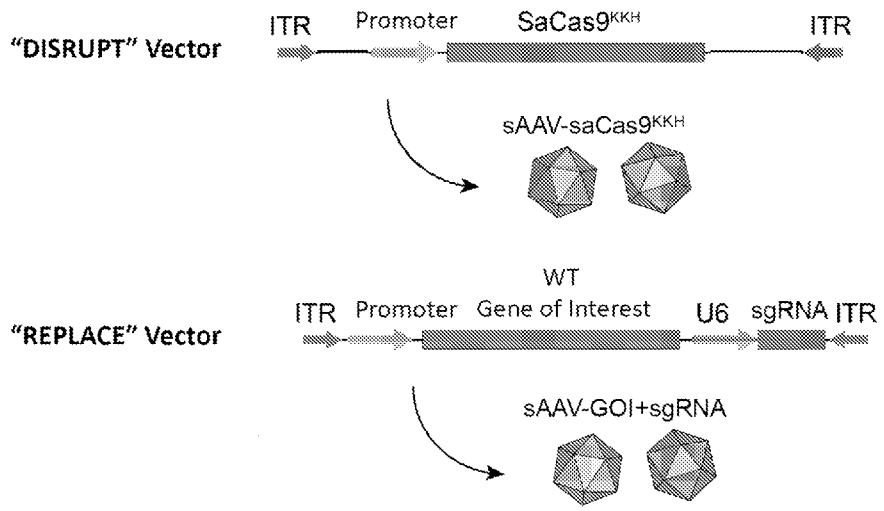
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: COMPOSITIONS AND METHODS FOR GENE REPLACEMENT

moter

FIG. 1



(57) Abstract: The present invention features a dual vector system for disrupting and replacing a target gene comprising a mutation (e.g., dominant, recessive mutation). Embodiments of the invention may also provide compositions comprising the dual vector system, and methods of using the dual vector system, including but not limited to methods of modifying the genome of a cell, methods of genomic editing, and methods of treating cells or a subject suffering from a genetic disease comprising a mutation.

[Continued on next page]

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

COMPOSITIONS AND METHODS FOR GENE REPLACEMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This PCT International application claims priority to and the benefit of U.S. Provisional Application No. 62/870,488, filed July 3, 2019, the disclosure of which is incorporated herein by reference in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant Nos. DC013521 and DC05439 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Gene editing is a promising method for the treatment of diseases and conditions associated with genetic alterations. However, methods for treating disease and disorders associated with dominant and recessive mutations remains challenging. Thus, there is a need for improved methods of gene editing.

SUMMARY OF THE INVENTION

As described below, the present invention features a dual vector system for disrupting and replacing a target gene comprising a mutation (e.g., dominant, recessive mutation).

In one aspect, the invention provides a dual vector system comprising a first vector comprising a polynucleotide encoding a Cas9-KKH polypeptide and a second vector comprising a polynucleotide encoding a guide RNA (gRNA) that binds a target gene comprising a mutation and a polynucleotide encoding a wild-type version of the target gene. One or both vectors may comprise at least one promoter selected from but not limited to: an Espin promoter, a protocadherin 15 (PCDH15) promoter, a protein tyrosine phosphatase receptor type Q (PTPRQ) promoter, a myosin VI (Myo6) promoter, a Potassium Voltage-Gated Channel Subfamily Q Member 4 (KCNQ4) promoter, a myosin VIIA (Myo7a) promoter, a synapsin promoter, a glial fibrillary acidic protein (GFAP) promoter, a cytomegalovirus (CMV) promoter, a CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site (CAG) promoter, a chicken β-actin (CBA) promoter, a CBH promoter, a U6 type III RNA polymerase promoter, and a tetraspan membrane protein of hair cell stereocilia

(TMHS) or lipoma HMGIC fusion partner-like 5 (LHFPL5) promoter. In some aspects, the promoter may be any one or more selected from: CMV and U6. One aspect provides the dual vector system, where the target gene comprises a mutation associated with a disease or condition. The target gene may be, for example, *TMC1*, and the mutation may be associated with hearing loss (e.g., progressive), such as those associated with a DFNA36 mutation. One vector of the dual vector system may comprise a Cas9-KKH polypeptide of SaCas9-KKH or SpCas9-KKH, where the Cas9-KKH is derived from *Staphylococcus aureus* or *Streptococcus pyogenes*, respectively. Another vector of the dual system may comprise a guide RNA selected from: gRNA 12, gRNA 15, and gRNA 16.

Another aspect of the invention may be directed to a dual vector system comprising: a) a first AAV9-PHP.B vector comprising a nucleotide sequence encoding Cas9-KKH; and b) a second AAV9-PHP.B vector comprising a nucleotide sequence encoding a guide RNA that binds a *TMC1* gene comprising a DFNA36 mutation and a polynucleotide encoding a wild-type *TMC1* gene. The guide RNA (gRNA) may be any one selected from: gRNA 12, gRNA 15, and gRNA 16.

A further aspect provides a composition comprising the dual vector system described herein. In some aspects, the composition may comprise a physiologically-acceptable carrier (including, e.g., diluent and/or excipient). Other aspects may be directed to a cell containing the dual vector system described here.

Yet another aspect of the invention provides a method of modifying the genome of a cell by contacting the cell with the dual vector system described here.

In one aspect of the invention, a method of genome editing may be provided, where the method comprising contacting a cell with the dual vector system described here.

Another aspect may be directed to a method of treating a subject suffering from a genetic disease by administering to the subject in need thereof, the dual vector system described here. The genetic disease may be an autosomal dominant disease, such as DFNA36 hearing loss, where the target gene is *TMC1*. The administering step may comprise contacting inner ear cells with the dual vector system, or composition or cells comprising the dual vector system described here. In some aspects, the administering may occur by injection. The method of treating a subject in need thereof with the dual vector system described here may result in the amelioration, reduction, or repair of the genetic disease suffered by the subject in need thereof.

Other features and advantages of the invention will be apparent from the detailed description and from the claims.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following

references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By "AAV9-PHP.B vector" is meant a viral vector comprising an AAV9-PHP.B polynucleotide or fragment. In one embodiment, the AAV9-PHP.B vector transfects at least 70% of inner hair cells and 70% of outer hair cells following administration to the inner ear of a subject or contact with a cell derived from an inner ear *in vitro*. In other embodiments, at least 85%, 90%, 95% or virtually 100% of inner hair cells and/or 85%, 90%, 95% or virtually 100% of outer hair cells are transfected. The transfection efficiency may be assessed using a gene encoding green fluorescent protein (GFP) in a mouse model. The sequence of an exemplary AAV9-PHP.B vector is provided below.

AAV9-PHP.B

CCAATGATACGCGTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATT
AAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTC
ACTATAGGGCGAATTGGGTACATCGACGGTATCGGGGGAGCTCGCAGGGTCTCCATTTTGAAGCGGGAGGTTTGA
ACGCGCAGCCGCCATGCCGGGGTTTTACGAGATTGTGATTAAGGTCCCCAGCGACCTTGACGAGCATCTGCCCGG
CATTTCTGACAGCTTTGTGAACTGGGTGGCCGAGAAGGAATGGGAGTTGCCGCCAGATTCTGACATGGATCTGAA
TCTGATTGAGCAGGCACCCCTGACCGTGGCCGAGAAGCTGCAGCGCGACTTTCTGACGGAATGGCGCCGTGTGAG
TAAGGCCCCGGAGGCTCTTTTCTTTGTGCAATTTGAGAAGGGAGAGAGCTACTTCCACATGCACGTGCTCGTGGA
AACCACCGGGGTGAAATCCATGGTTTTGGGACGTTTCCTGAGTCAGATTCGCGAAAAACTGATTCAGAGAATTTA
CCGCGGGATCGAGCCGACTTTGCCAAACTGGTTCGCGGTCACAAAGACCAGAAATGGCGCCGGAGGCGGGAACAA
GGTGGTGGATGAGTGCTACATCCCCAATTACTTGCTCCCCAAAACCCAGCCTGAGCTCCAGTGGGCGTGGACTAA
TATGGAACAGTATTTAAGCGCCTGTTTGAATCTCACGGAGCGTAAACGGTTGGTGGCGCAGCATCTGACGCACGT
GTCGCAGACGCAGGAGCAGAACAAAGAGAATCAGAATCCCAATTCTGATGCGCCGGTGATCAGATCAAAAACTTC
AGCCAGGTACATGGAGCTGGTCGGGTGGCTCGTGGACAAGGGGATTACCTCGGAGAAGCAGTGGATCCAGGAGGA
CCAGGCCTCATACATCTCCTTCAATGCGGCCTCCAACTCGCGGTCCCAAATCAAGGCTGCCTTGGACAATGCGGG
AAAGATTATGAGCCTGACTAAAACCGCCCCCGACTACCTGGTGGGCCAGCAGCCCGTGGAGGACATTTCCAGCAA
TCGGATTTATAAAATTTTGGAACTAAACGGGTACGATCCCCAATATGCGGCTTCCGTCTTTCTGGGATGGGCCAC
GAAAAAGTTCGGCAAGAGGAACACCATCTGGCTGTTTGGGCCTGCAACTACCGGGAAGACCAACATCGCGGAGGC
CATAGCCCACACTGTGCCCTTCTACGGGTGCGTAAACTGGACCAATGAGAACTTTCCCTTCAACGACTGTGTGGA
CAAGATGGTGATCTGGTGGGAGGAGGGGAAGATGACCGCCAAGGTCGTGGAGTCGGCCAAAGCCATTCTCGGAGG
AAGCAAGGTGCGCGTGGACCAGAAATGCAAGTCCTCGGCCCAGATAGACCCGACTCCCGTGATCGTCACCTCCAA
CACCAATATGTGCGCCGTGATTGACGGGAACTCAACGACCTTCGAACACCAGCAGCCGTTGCAAGACCGGATGTT
CAAATTTGAACTCACCCGCCGTCTGGATCATGACTTTGGGAAGGTCACCAAGCAGGAAGTCAAAGACTTTTTCCG

GTGGGCAAAGGATCACGTGGTTGAGGTGGAGCATGAATTCTACGTCAAAAAGGGTGGAGCCAAGAAAAGACCCGC
CCCCAGTGACGCAGATATAAGTGAGCCCAAACGGGTGCGCGAGTCAGTTGCGCAGCCATCGACGTCAGACGCGGA
AGCTTCGATCAACTACGCGGACAGGTACCAAAACAAATGTTCTCGTCACGTGGGCATGAATCTGATGCTGTTTCC
CTGCAGACAATGCGAGAGACTGAATCAGAATTCAAATATCTGCTTCACTCACGGTGTCAAAGACTGTTTAGAGTG
CTTTCCCGTGTCAGAATCTCAACCCGTTTCTGTCGTCAAAAAGGCGTATCAGAAACTGTGCTACATTCATCACAT
CATGGGAAAGGTGCCAGACGCTTGCACTGCTTGCGACCTGGTCAATGTGGACTTGGATGACTGTGTTTCTGAACA
ATAAATGACTTAAACCAGGTATGAGTCGGCTGGATAAATCTAAAGTCATAAACGGCGCTCTGGAATTACTCAATG
AAGTCGGTATCGAAGGCCTGACGACAAGGAAACTCGCTCAAAAGCTGGGAGTTGAGCAGCCTACCCTGTACTGGC
ACGTGAAGAACAAGCGGGCCCTGCTCGATGCCCTGGCCATCGAGATGCTGGACAGGCATCATACCCACTTCTGCC
CCCTGGAAGGCGAGTCATGGCAAGACTTTCTGCGGAACAACGCCAAGTCATTCCGCTGTGCTCTCCTCTCACATC
GCGACGGGGCTAAAGTGCATCTCGGCACCCGCCCAACAGAGAAACAGTACGAAACCCTGGAAAATCAGCTCGCGT
TCCTGTGTCAGCAAGGCTTCTCCCTGGAGAACGCACTGTACGCTCTGTCCGCCGTGGGCCACTTTACACTGGGCT
GCGTATTGGAGGAACAGGAGCATCAAGTAGCAAAAGAGGAAAGAGAGACACCTACCACCGATTCTATGCCCCCAC
TTCTGAGACAAGCAATTGAGCTGTTCGACCGGCAGGGAGCCGAACCTGCCTTCCTTTTCGGCCTGGAACTAATCA
TATGTGGCCTGGAGAAACAGCTAAAGTGCGAAAGCGGCGGGCCGGCCGACGCCCTTGACGATTTTGACTTAGACA
TGCTCCCAGCCGATGCCCTTGACGACTTTGACCTTGATATGCTGCCTGCTGACGCTCTTGACGATTTTGACCTTG
ACATGCTCCCCGGGTAAATGCATGAATTCGATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTC
GCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCC
TGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATT
CTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATG
CGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCGAATCAAGCTATCAAGTGCCACCTGACGT
CTCCCTATCAGTGATAGAGAAGTCGACACGTCTCGAGCTCCCTATCAGTGATAGAGAAGGTACGTCTAGAACGTC
TCCCTATCAGTGATAGAGAAGTCGACACGTCTCGAGCTCCCTATCAGTGATAGAGAAGGTACGTCTAGAACGTCT
CCCTATCAGTGATAGAGAAGTCGACACGTCTCGAGCTCCCTATCAGTGATAGAGAAGGTACGTCTAGAACGTCTC
CCTATCAGTGATAGAGAAGTCGACACGTCTCGAGCTCCCTATCAGTGATAGAGAAGGTACCCCCTATATAAGCAG
AGAGATCTGTTCAAATTTGAACTGACTAAGCGGCTCCCGCCAGATTTTGGCAAGATTACTAAGCAGGAAGTCAAG
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ATCATGGGAAAGGTGCCAGACGCTTGCACTGCCTGCGATCTGGTCAATGTGGATTTGGATGACTGCATCTTTGAA
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AATTCGCGAGTGGTGGGCTTTGAAACCTGGAGCCCCTCAACCCAAGGCAAATCAACAACATCAAGACAACGCTAG
AGGTCTTGTGCTTCCGGGTTACAAATACCTTGGACCCGGCAACGGACTCGACAAGGGGGAGCCGGTCAACGCAGC
AGACGCGGCGGCCCTCGAGCACGACAAAGCCTACGACCAGCAGCTCAAGGCCGGAGACAACCCGTACCTCAAGTA
CAACCACGCCGACGCCGAGTTCCAGGAGCGGCTCAAAGAAGATACGTCTTTTGGGGGCAACCTCGGGCGAGCAGT
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GAGGCCTGTAGAGCAGTCTCCTCAGGAACCGGACTCCTCCGCGGGTATTGGCAAATCGGGTGCACAGCCCGCTAA
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CACCCGAACCTGGGCCCTGCCCACCTACAACAATCACCTCTACAAGCAAATCTCCAACAGCACATCTGGAGGATC
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CTCACCACGTGACTGGCAGCGACTCATCAACAACAACTGGGGATTCCGGCCTAAGCGACTCAACTTCAAGCTCTT
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GGTCTTCACGGACTCAGACTATCAGCTCCCGTACGTGCTCGGGTCGGCTCACGAGGGCTGCCTCCCGCCGTTCCC
AGCGGACGTTTTCATGATTCCTCAGTACGGGTATCTGACGCTTAATGATGGAAGCCAGGCCGTGGGTCGTTCGTC
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CTTGTACTATCTCTCTAGAACTATTAACGGTTCTGGACAGAATCAACAAACGCTAAAATTCAGTGTGGCCGGACC
CAGCAACATGGCTGTCCAGGGAAGAAACTACATACCTGGACCCAGCTACCGACAACAACGTGTCTCAACCACTGT
GACTCAAAACAACAACAGCGAATTTGCTTGGCCTGGAGCTTCTTCTTGGGCTCTCAATGGACGTAATAGCTTGAT
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TGGCAAACAAGGTACTGGCAGAGACAACGTGGATGCGGACAAAGTCATGATAACCAACGAAGAAGAAATTAAAAC
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CTTCAACAAGGACAAGCTGAACTCTTTCATCACCCAGTATTCTACTGGTCAAGTCAGCGTGGAGATCGAGTGGGA
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TGAATTTGCTGTTAATACTGAAGGTGTATATAGTGAACCCCGCCCCATTGGCACCAGATACCTGACTCGTAATCT
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TCGTTTAAACCTGCAGGACTAGAGGTCCTGTATTAGAGGTCACGTGAGTGTTTTGCGACATTTTGCGACACCATG
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ATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCC
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TTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAAC
CCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTAT
CGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGT
GGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAA
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TTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAA
ACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAA

GTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTA
TCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGG
GCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAA
ACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTT
GCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGG
TGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCA
TGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCAC
TCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGT
ACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATA
CCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCT
TACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCA
GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAA
TACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTG
AATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC.

By "administer" is meant providing one or more compositions (e.g., viral vectors) described herein to a subject in need thereof. Non-limiting routes of administration may include systemic administration (e.g., intravenous intraperitoneal, intramuscular, subdermal, or intracranial infusion), topical, injection, infusion, electroporation, or *ex vivo*, where cells (e.g., tissues, bone marrow aspirates, lymphocytes, stem cells) from a subject in need thereof are removed and returned to the subject after incorporation of the viral vectors. By way of example and without limitation, the composition (e.g., viral vectors) of the disclosure may be administed by injection, for example, into the cochlea. Other routes that deliver the composition to cells affected by a mutation can be employed. Administration can be, for example, by bolus injection or by gradual perfusion over time.

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% or greater change (e.g., a 25% change, a 40% change, a 50% or greater change) in expression levels.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease. Exemplary diseases associated with a dominant or a recessive mutation.

By "Anc80 polypeptide" is meant a capsid polypeptide having at least about85% or greater (e.g., 90%, 95%, 97%, 98%, 99%) amino acid identity to the following polypeptide sequence:

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLDKGEPVNAADAAALEH
DKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQAKKRVLEPLGLVEEGAKTAPGKKRPVEQSP

QEPDSSSGIGKKGQQPARKRLNFGQTGDSESVPDPQPLGEPPAAPSGVGSNTMAAGGGAPMADNNEGADGVGNAS
GNWHCDSTWLGDRVITTSTRTALPTYNNHLYKQISSQSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLI
NNNWGFRPKKLNFKLFNIQVKEVTTNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMIPQY
GYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQT
TSGTAGNRTLQFSQAGPSSMANQAKNWLPGPCYRQQRVSKTTNQNNNSNFAWTGATKYHLNGRDSLVNPGPAMAT
HKDDEDKFFPMSGVLIFGKQGAGNSNVDLDNVMITNEEEIKTTNPVATEEYGTVATNLQSANTAPATGTVNSQGA
LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPPTTFSPAKFASFITQYST
GQVSVEIEELQKENSKRWNPEIQ YTSNYNKSTNVDFAVDTNGVYSEPRPIGTRYLTRNL.

By "Anc80 polynucleotide" is meant a nucleic acid molecule encoding an Anc80 polypeptide.

By "Cas9 (CRISPR associated protein 9) polypeptide" is meant a polypeptide or fragment thereof having at least about85% (e.g., 90%, 95%, 97%, 98%, 99%) amino acid identity to NCBI Accession No. NP_269215 and having RNA binding activity, DNA binding activity, and/or DNA cleavage activity (e.g., endonuclease or nickase activity). The Cas9 enzyme may be selected from *S. aureus*, *S. pneumoniae, S. pyogenes,* or *S. thermophilus* Cas9. In some embodiments, the enzyme may be a Cas9 homolog or include mutated Cas9 from any of the aforementioned organisms.. An exemplary Cas9 polypeptide sequence is provided below.

Cas9: 1 MDKKYSIGLD IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE
61 ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG
121 NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD
181 VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN
241 LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI
301 LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA
361 GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH
421 AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE
481 VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL
541 SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI
601 IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG
661 RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL
721 HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER
781 MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH
841 IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL
901 TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS
961 KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK
1021 MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF
1081 ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA
1141 YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK
1201 YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE
1261 QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA

1321 PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD.

By "Cas 9 nucleic acid molecule" is meant a polynucleotide encoding a Cas9 polypeptide or fragment thereof. An exemplary *S. pyogenes* Cas9 nucleic acid molecule sequence is provided at NCBI Accession No. NC_002737 and is shown below.

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   1 ATGGATAAGA AATACTCAAT AGGCTTAGAT ATCGGCACAA ATAGCGTCGG ATGGGCGGTG
  61 ATCACTGATG AATATAAGGT TCCGTCTAAA AAGTTCAAGG TTCTGGGAAA TACAGACCGC
 121 CACAGTATCA AAAAAAATCT TATAGGGGCT CTTTTATTTG ACAGTGGAGA GACAGCGGAA
 181 GCGACTCGTC TCAAACGGAC AGCTCGTAGA AGGTATACAC GTCGGAAGAA TCGTATTTGT
 241 TATCTACAGG AGATTTTTTC AAATGAGATG GCGAAAGTAG ATGATAGTTT CTTTCATCGA
 301 CTTGAAGAGT CTTTTTTGGT GGAAGAAGAC AAGAAGCATG AACGTCATCC TATTTTTGGA
 361 AATATAGTAG ATGAAGTTGC TTATCATGAG AAATATCCAA CTATCTATCA TCTGCGAAAA
 421 AAATTGGTAG ATTCTACTGA TAAAGCGGAT TTGCGCTTAA TCTATTTGGC CTTAGCGCAT
 481 ATGATTAAGT TTCGTGGTCA TTTTTTGATT GAGGGAGATT TAAATCCTGA TAATAGTGAT
 541 GTGGACAAAC TATTTATCCA GTTGGTACAA ACCTACAATC AATTATTTGA AGAAAACCCT
 601 ATTAACGCAA GTGGAGTAGA TGCTAAAGCG ATTCTTTCTG CACGATTGAG TAAATCAAGA
 661 CGATTAGAAA ATCTCATTGC TCAGCTCCCC GGTGAGAAGA AAAATGGCTT ATTTGGGAAT
 721 CTCATTGCTT TGTCATTGGG TTTGACCCCT AATTTTAAAT CAAATTTTGA TTTGGCAGAA
 781 GATGCTAAAT TACAGCTTTC AAAAGATACT TACGATGATG ATTTAGATAA TTTATTGGCG
 841 CAAATTGGAG ATCAATATGC TGATTTGTTT TTGGCAGCTA AGAATTTATC AGATGCTATT
 901 TTACTTTCAG ATATCCTAAG AGTAAATACT GAAATAACTA AGGCTCCCCT ATCAGCTTCA
 961 ATGATTAAAC GCTACGATGA ACATCATCAA GACTTGACTC TTTTAAAAGC TTTAGTTCGA
1021 CAACAACTTC CAGAAAAGTA TAAAGAAATC TTTTTTGATC AATCAAAAAA CGGATATGCA
1081 GGTTATATTG ATGGGGGAGC TAGCCAAGAA GAATTTTATA AATTTATCAA ACCAATTTTA
1141 GAAAAAATGG ATGGTACTGA GGAATTATTG GTGAAACTAA ATCGTGAAGA TTTGCTGCGC
1201 AAGCAACGGA CCTTTGACAA CGGCTCTATT CCCCATCAAA TTCACTTGGG TGAGCTGCAT
1261 GCTATTTTGA GAAGACAAGA AGACTTTTAT CCATTTTTAA AAGACAATCG TGAGAAGATT
1321 GAAAAAATCT TGACTTTTCG AATTCCTTAT TATGTTGGTC CATTGGCGCG TGGCAATAGT
1381 CGTTTTGCAT GGATGACTCG GAAGTCTGAA GAAACAATTA CCCCATGGAA TTTTGAAGAA
1441 GTTGTCGATA AAGGTGCTTC AGCTCAATCA TTTATTGAAC GCATGACAAA CTTTGATAAA
1501 AATCTTCCAA ATGAAAAAGT ACTACCAAAA CATAGTTTGC TTTATGAGTA TTTTACGGTT
1561 TATAACGAAT TGACAAAGGT CAAATATGTT ACTGAAGGAA TGCGAAAACC AGCATTTCTT
1621 TCAGGTGAAC AGAAGAAAGC CATTGTTGAT TTACTCTTCA AAACAAATCG AAAAGTAACC
1681 GTTAAGCAAT TAAAAGAAGA TTATTTCAAA AAAATAGAAT GTTTTGATAG TGTTGAAATT
1741 TCAGGAGTTG AAGATAGATT TAATGCTTCA TTAGGTACCT ACCATGATTT GCTAAAAATT
1801 ATTAAAGATA AAGATTTTTT GGATAATGAA GAAAATGAAG ATATCTTAGA GGATATTGTT
1861 TTAACATTGA CCTTATTTGA AGATAGGGAG ATGATTGAGG AAAGACTTAA AACATATGCT
1921 CACCTCTTTG ATGATAAGGT GATGAAACAG CTTAAACGTC GCCGTTATAC TGGTTGGGGA
1981 CGTTTGTCTC GAAAATTGAT TAATGGTATT AGGGATAAGC AATCTGGCAA AACAATATTA
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2041 GATTTTTTGA AATCAGATGG TTTTGCCAAT CGCAATTTTA TGCAGCTGAT CCATGATGAT
2101 AGTTTGACAT TTAAAGAAGA CATTCAAAAA GCACAAGTGT CTGGACAAGG CGATAGTTTA
2161 CATGAACATA TTGCAAATTT AGCTGGTAGC CCTGCTATTA AAAAAGGTAT TTTACAGACT
2221 GTAAAAGTTG TTGATGAATT GGTCAAAGTA ATGGGGCGGC ATAAGCCAGA AAATATCGTT
2281 ATTGAAATGG CACGTGAAAA TCAGACAACT CAAAAGGGCC AGAAAAATTC GCGAGAGCGT
2341 ATGAAACGAA TCGAAGAAGG TATCAAAGAA TTAGGAAGTC AGATTCTTAA AGAGCATCCT
2401 GTTGAAAATA CTCAATTGCA AAATGAAAAG CTCTATCTCT ATTATCTCCA AAATGGAAGA
2461 GACATGTATG TGGACCAAGA ATTAGATATT AATCGTTTAA GTGATTATGA TGTCGATCAC
2521 ATTGTTCCAC AAAGTTTCCT TAAAGACGAT TCAATAGACA ATAAGGTCTT AACGCGTTCT
2581 GATAAAAATC GTGGTAAATC GGATAACGTT CCAAGTGAAG AAGTAGTCAA AAAGATGAAA
2641 AACTATTGGA GACAACTTCT AAACGCCAAG TTAATCACTC AACGTAAGTT TGATAATTTA
2701 ACGAAAGCTG AACGTGGAGG TTTGAGTGAA CTTGATAAAG CTGGTTTTAT CAAACGCCAA
2761 TTGGTTGAAA CTCGCCAAAT CACTAAGCAT GTGGCACAAA TTTTGGATAG TCGCATGAAT
2821 ACTAAATACG ATGAAAATGA TAAACTTATT CGAGAGGTTA AAGTGATTAC CTTAAAATCT
2881 AAATTAGTTT CTGACTTCCG AAAAGATTTC CAATTCTATA AAGTACGTGA GATTAACAAT
2941 TACCATCATG CCCATGATGC GTATCTAAAT GCCGTCGTTG GAACTGCTTT GATTAAGAAA
3001 TATCCAAAAC TTGAATCGGA GTTTGTCTAT GGTGATTATA AAGTTTATGA TGTTCGTAAA
3061 ATGATTGCTA AGTCTGAGCA AGAAATAGGC AAAGCAACCG CAAAATATTT CTTTTACTCT
3121 AATATCATGA ACTTCTTCAA AACAGAAATT ACACTTGCAA ATGGAGAGAT TCGCAAACGC
3181 CCTCTAATCG AAACTAATGG GGAAACTGGA GAAATTGTCT GGGATAAAGG GCGAGATTTT
3241 GCCACAGTGC GCAAAGTATT GTCCATGCCC CAAGTCAATA TTGTCAAGAA AACAGAAGTA
3301 CAGACAGGCG GATTCTCCAA GGAGTCAATT TTACCAAAAA GAAATTCGGA CAAGCTTATT
3361 GCTCGTAAAA AAGACTGGGA TCCAAAAAAA TATGGTGGTT TTGATAGTCC AACGGTAGCT
3421 TATTCAGTCC TAGTGGTTGC TAAGGTGGAA AAAGGGAAAT CGAAGAAGTT AAAATCCGTT
3481 AAAGAGTTAC TAGGGATCAC AATTATGGAA AGAAGTTCCT TTGAAAAAAA TCCGATTGAC
3541 TTTTTAGAAG CTAAAGGATA TAAGGAAGTT AAAAAAGACT TAATCATTAA ACTACCTAAA
3601 TATAGTCTTT TTGAGTTAGA AAACGGTCGT AAACGGATGC TGGCTAGTGC CGGAGAATTA
3661 CAAAAAGGAA ATGAGCTGGC TCTGCCAAGC AAATATGTGA ATTTTTTATA TTTAGCTAGT
3721 CATTATGAAA AGTTGAAGGG TAGTCCAGAA GATAACGAAC AAAAACAATT GTTTGTGGAG
3781 CAGCATAAGC ATTATTTAGA TGAGATTATT GAGCAAATCA GTGAATTTTC TAAGCGTGTT
3841 ATTTTAGCAG ATGCCAATTT AGATAAAGTT CTTAGTGCAT ATAACAAACA TAGAGACAAA
3901 CCAATACGTG AACAAGCAGA AAATATTATT CATTTATTTA CGTTGACGAA TCTTGGAGCT
3961 CCCGCTGCTT TTAAATATTT TGATACAACA ATTGATCGTA AACGATATAC GTCTACAAAA
4021 GAAGTTTTAG ATGCCACTCT TATCCATCAA TCCATCACTG GTCTTTATGA AACACGCATT
4081 GATTTGAGTC AGCTAGGAGG TGACTGA

SaCas9:

MAPKKKRKVGIHGVPAAKRNYILGLDIGITSVGYGIIDYETRDVIDAGVRLFKEANVENNEGRRSKRGARRLKRR
RRHRIQRVKKLLFDYNLLTDHSELSGINPYEARVKGLSQKLSEEEFSAALLHLAKRRGVHNVNEVEEDTGNELST

KEQISRNSKALEEKYVAELQLERLKKDGEVRGSINRFKTSDYVKEAKQLLKVQKAYHQLDQSFIDTYIDLLETRR
TYYEGPGEGSPFGWKDIKEWYEMLMGHCTYFPEELRSVKYAYNADLYNALNDLNNLVITRDENEKLEYYEKFQII
ENVFKQKKKPTLKQIAKEILVNEEDIKGYRVTSTGKPEFTNLKVYHDIKDITARKEIIENAELLDQIAKILTIYQ
SSEDIQEELTNLNSELTQEEIEQISNLKGYTGTHNLSLKAINLILDELWHTNDNQIAIFNRLKLVPKKVDLSQQK
EIPTTLVDDFILSPVVKRSFIQSIKVINAIIKKYGLPNDIIIELAREKNSKDAQKMINEMQKRNRQTNERIEEII
RTTGKENAKYLIEKIKLHDMQEGKCLYSLEAIPLEDLLNNPFNYEVDHIIPRSVSFDNSFNNKVLVKQEENSKKG
NRTPFQYLSSSDSKISYETFKKHILNLAKGKGRISKTKKEYLLEERDINRFSVQKDFINRNLVDTRYATRGLMNL
LRSYFRVNNLDVKVKSINGGFTSFLRRKWKFKKERNKGYKHHAEDALIIANADFIFKEWKKLDKAKKVMENQMFE
EKQAESMPEIETEQEYKEIFITPHQIKHIKDFKDYKYSHRVDKKPNRELINDTLYSTRKDDKGNTLIVNNLNGLY
DKDNDKLKKLINKSPEKLLMYHHDPQTYQKLKLIMEQYGDEKNPLYKYYEETGNYLTKYSKKDNGPVIKKIKYYG
NKLNAHLDITDDYPNSRNKVVKLSLKPYRFDVYLDNGVYKFVTVKNLDVIKKENYYEVNSKCYEEAKKLKKISNQ
AEFIASFYNNDLIKINGELYRVIGVNNDLLNRIEVNMIDITYREYLENMNDKRPPRIIKTIASKTQSIKKYSTDI
LGNLYEVKSKKHPQIIKKGKRPAATKKAGQAKKKKGS

SaCas9-KKH:

A PAM variant of *Staphylococcus aureus* Cas9 (SaCas9-KKH) selectively and efficiently disrupts the mutant allele, but not the wild-type *Tmc1/TMC1* allele, in Beethoven mice and in a DFNA36 human cell line. AAV-mediated SaCas9-KKH delivery prevented deafness in Beethoven mice up to one year post transduction. The SaCas9-KKH amino acid sequence is provided below. Bold and underlined text in the amino acid sequence denotes variation from SaCas9.

MAPKKKRKVGIHGVPAAKRNYILGLDIGITSVGYGIIDYETRDVIDAGVRLFKEANVENNEGRRSKRGARRLKRR
RRHRIQRVKKLLFDYNLLTDHSELSGINPYEARVKGLSQKLSEEEFSAALLHLAKRRGVHNVNEVEEDTGNELST
KEQISRNSKALEEKYVAELQLERLKKDGEVRGSINRFKTSDYVKEAKQLLKVQKAYHQLDQSFIDTYIDLLETRR
TYYEGPGEGSPFGWKDIKEWYEMLMGHCTYFPEELRSVKYAYNADLYNALNDLNNLVITRDENEKLEYYEKFQII
ENVFKQKKKPTLKQIAKEILVNEEDIKGYRVTSTGKPEFTNLKVYHDIKDITARKEIIENAELLDQIAKILTIYQ
SSEDIQEELTNLNSELTQEEIEQISNLKGYTGTHNLSLKAINLILDELWHTNDNQIAIFNRLKLVPKKVDLSQQK
EIPTTLVDDFILSPVVKRSFIQSIKVINAIIKKYGLPNDIIIELAREKNSKDAQKMINEMQKRNRQTNERIEEII
RTTGKENAKYLIEKIKLHDMQEGKCLYSLEAIPLEDLLNNPFNYEVDHIIPRSVSFDNSFNNKVLVKQEENSKKG
NRTPFQYLSSSDSKISYETFKKHILNLAKGKGRISKTKKEYLLEERDINRFSVQKDFINRNLVDTRYATRGLMNL
LRSYFRVNNLDVKVKSINGGFTSFLRRKWKFKKERNKGYKHHAEDALIIANADFIFKEWKKLDKAKKVMENQMFE
EKQAESMPEIETEQEYKEIFITPHQIKHIKDFKDYKYSHRVDKKPNR**K**LINDTLYSTRKDDKGNTLIVNNLNGLY
DKDNDKLKKLINKSPEKLLMYHHDPQTYQKLKLIMEQYGDEKNPLYKYYEETGNYLTKYSKKDNGPVIKKIKYYG
NKLNAHLDITDDYPNSRNKVVKLSLKPYRFDVYLDNGVYKFVTVKNLDVIKKENYYEVNSKCYEEAKKLKKISNQ
AEFIASFY**K**NDLIKINGELYRVIGVNNDLLNRIEVNMIDITYREYLENMNDKRPP**H**IIKTIASKTQSIKKYSTDI
LGNLYEVKSKKHPQIIKKGKRPAATKKAGQAKKKKGS

Kleinstiver et al. (Nat Biotechnol. 2015 Dec;33(12):1293-1298. doi: 10.1038/nbt.3404. Epub 2015 Nov 2) describes SaCas9-KKH.

"Detect" refers to identifying the presence, absence or amount of the analyte to be detected.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include any pathology, such as a hearing disorder, associated with genetic variation (e.g., a mutation).

By "effective amount" is meant the amount of an agent required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, at least 10% (e.g., 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%) of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10 or greater (e.g., 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000) nucleotides or amino acids.

"Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

By "identity" is meant the amino acid or nucleic acid sequence identity between a sequence of interest and a reference sequence. Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e−3 and e−100 indicating a closely related sequence.

The term "indel" refers to the insertion or deletion of at least one nucleotide at a locus in a nucleic acid molecule. An indel present in the coding region of a gene may result in a frameshift mutation resulting in a premature stop codon or other signal for the expressed protein to be degraded.

The terms "isolated," "purified," or "biologically pure" refer to material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. A "purified" or "biologically pure" protein is sufficiently free of other

materials such that any impurities do not materially affect the biological properties of the protein or cause other adverse consequences. That is, a nucleic acid or peptide of this invention is purified if it is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Purity and homogeneity are typically determined using analytical chemistry techniques, for example, polyacrylamide gel electrophoresis or high-performance liquid chromatography. The term "purified" can denote that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. For a protein that can be subjected to modifications, for example, phosphorylation or glycosylation, different modifications may give rise to different isolated proteins, which can be separately purified.

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. In some embodiments, the preparation is at least 75% or greater (e.g., at least 90%, at least 99%), by weight, a polypeptide of the invention. An isolated polypeptide of the invention may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "marker" is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

By "mechanosensation" is meant a response to a mechanical stimulus. Touch, hearing, and balance of examples of the conversion of a mechanical stimulus into a neuronal signal. Mechanosensory input is converted into a response to a mechanical stimulus through a process termed "mechanotransduction."

As used herein, “obtaining” as in “obtaining an agent” includes synthesizing, purchasing, or otherwise acquiring the agent.

As used herein, the terms “prevent,” “preventing,” “prevention,” “prophylactic treatment” and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

By "promoter" is meant a polynucleotide sufficient to direct transcription of a downstream polynucleotide.

By “Espin promoter” is meant a regulatory polynucleotide sequence derived from NCBI Reference Sequence: NG_015866.1 that is sufficient to direct expression of a downstream polynucleotide in an outer or inner hair cell, vestibular hair cell, a spiral ganglion, or a vestibular ganglion. In one embodiment, the Espin promoter comprises or consists of at least about350 base pairs (e.g., 500, 1000, 2000, 3000, 4000, 5000), or more base pairs upstream of an Espin coding sequence.

By “protocadherin related 15 (PCDH15) promoter” is meant a regulatory polynucleotide sequence derived from NCBI Reference Sequence: NG_009191 that is sufficient to direct expression of a downstream polynucleotide in an outer or inner hair cell, vestibular hair cell, a spiral ganglion, or a vestibular ganglion. In one embodiment, the PCDH15 promoter comprises at least about 350 base pairs (e.g., 500, 1000, 2000, 3000, 4000, 5000), or more base pairs upstream of an PCDH15 coding sequence. In some embodiments, the PCDH15 promoter comprises or consists of a nucleic acid sequence having at least about 85% (e.g., 90%, 95%, 97%, 98%, 99%) sequence identity to the following nucleotide sequence:

TCTTCACCTGTCATTTTCAACCAGCCTCAGCCTATCTGCTCTGTCACAATCACTACTAAAATATGTTCCTAAATT
GCTTGTTTCTAGATCCTTCCTTCTCATATGCTCAGGTGAACACATGGGTGAAATTTAATATGGAATTGAAATATG
TACTATGCAAGATAGATTCCTTAAGAAATGTTTCTCTGATTTATATGACATAATTGTATTTTACTAGTTTACCTG
TCCATCTGTAAAACTTTGTTTTGGAGATTTCATATATTACAATGTTTAAGAAATATGCTATAATGTTTTGTATAG
TATATTTCTTCGTGATAACCTTATATACTACCAGTCACACGTGTTTGTAAAAATCTAAAGAGTACTTTTGGCTCC
TACAGAATGTGTGAAGTTGTGAAATTGTTTTTTTGTTTTGTTTTGTTTTGTTTTTATGCCCCAAAGATGTGGAGG
GCTTCATATAAGAGGGTAGATTTAATGAGAGAGAGAGGGAGAGACAGAGAGAATGATAAAAGAAGCTTAAGAGAT
TATTTTATCTTGTCAACGACATTGTTATTGAATGTAAGCTGCTAAACTTCTTAGATAAAGTAAAACAGTAAAAAC
AAACACACAAAACAGAACAGAGAATCATCAGACAGGCTGACGAACACAGTACAATAAAGCAGCCAGTACCGATGA
TCAGTGGACATCAATTTGTCTTTTGGGCTGTAGCACCTGCTACTAATTGGTGCAAAGCGCTCACCAGTCAGTGCG
TGGTTTAGCGCACTCAGCTGTCTCCTGTATGTGCTGCGAGAAGCAAGATAGCTAATTGCTGTTGCTTCAGTGCCA
GTGAAATCAACGTGCTGAGCTAATAGCGACAGATAGAGGGCAGACAGATTCCTGCTAGCAGCTTAGTGTTAGTTG
CTTGTGGTAACTAAGGCAGGTGGCATACATCTCAGAACGTGGAGAATGATGGTATGCTTTCTGA

By "protein tyrosine phosphatase, receptor type Q (PTPRQ) promoter" is meant a regulatory polynucleotide sequence derived from GeneID: 374462 that is sufficient to direct expression of a downstream polynucleotide in an outer or inner hair cell, vestibular hair cell, a spiral ganglion, or a vestibular ganglion. In one embodiment, the PTPRQ promoter comprises at least about 350 (e.g., 500, 1000, 2000, 3000, 4000, 5000), or more base pairs upstream of an PTPRQ coding sequence. In some embodiments, the PTPRQ promoter comprises or consists of a nucleic acid sequence having at least about 85% (e.g., 90%, 95%, 97%, 98%, 99%) sequence identity to the following nucleotide sequence:

TGGTAGCCTCCCTAGAGACACAGAGCTGGGCCGGATGAGTCCAGGCACTGACGTGATCCATTATCTTTCACCTTA
AAGAGTAAAAGGGAAACTAAAGTTAATTACCTCCACGAAACAAAAAGGTGCCTTCTTGTGCTTCAATTACATGGA
TATATTCTACTAGTCTAAAAGTATCTTCTCACTTCTTTCTGTCACTGTGAGGACTTGAGTCAGAAGAAAGTTTAA
ATACAGTCATTGAGCTGGAAAGAGTGGAAAGAGAAGCAAAGAGGGGGAAGCTGTAGGAAGGACGAAGTCACCCCC
AAGATACATGGTTACTGCTTACACCAAGCAAGCTGCCTTGGGAACGCTTCCCCCGAGCAGCCAGAATGCTCAGCA
GTGGAAGACACCTCTATTCCTGTAGGCGAGTCCTGGGAAGCTGGTCAATCTGCAAATGCCAATTCCCAGCAGTGA
GCTCGGTCCACGTGTAAATCAAGATTTGGGGAAAGAGTAGGGTGGGTGGCATGGTTGACAATGTCATCAGCTCCC
TCCTCTGACTCCTGTGGTCGTGCCCCCATCTACTCTCACTCAGCTACACCCCACCTTCGGATTTGTGATGGACGC
TGGGTCCCTAGTAACCACAGCAAGTGTCTCCCCCGCACTTCCCCCTTCCCCACCCCCACCCCCACCCCCAACCAC
CACCCCAGCGATGGAGCCTACTCTGCTCCAAGCCGCCGCTAAGACCCGGAGAAGCGGAATTTCACTTTGAAATTC
CCTTGCCTCGTGAGGGCCGGCGCTGGGCATGCTCAGTAGCCGCGGCGCTGCTGCTGGGCTGCTGGGCTGGCGCGG
AGTCCACCCTGCCGTCTCCGCCTTGGCTTCTGGGCGTCCAGAAGGCCAGGCATTTGCCGCCTCTGAGCGCTTCTG
TTCCCCTTACCCGCAACCTCCTACTGCTCTTCCTCTCTCCCTCTCTTAGGGAGGTTGAAGCTGGTGCTGGTTTCT
GTCGGCGCCACAGACTGACTGCTCTGCAAACCCCAGCCGAGGACCTGAATCCCGGAGACTAGAAG

By "lipoma HMGIC fusion partner-like 5 (LHFPL5) promoter" also termed "TMHS promoter" is meant a regulatory polynucleotide sequence derived from NCBI Reference Sequence: GeneID: 222662 that is sufficient to direct expression of a downstream polynucleotide in an outer or inner hair cell, vestibular hair cell, a spiral ganglion, or a vestibular ganglion. In one embodiment, the TMHS promoter comprises at least about 350 base pairs (e.g., 500, 1000, 2000, 3000, 4000, 5000), or more base pairs upstream of an PCDH15 coding sequence. In some embodiments, the TMHS promoter comprises or consists of a nucleic acid sequence having at least about 85% (90%, 95%, 97%, 98%, 99%) sequence identity to the following nucleotide sequence:

GCCCAGTGGAATTTTCCTAGTTCTTTACACTAGCCATGTATTTACCTATAAAATCAGGAGAAATATGTATATATA
TAATATATTAAAACATATATATATTTAAATGGGGAAATATGTAACAAACAAATAGAAACAAGGGGAGAAAGGCAT
TGTATTTGACAAAACACATATGTTCAGGTCTGAGAAGGCTCATAAAGAATGTTGTCTGCTATACTTTGTAGTTGC
TTCTGTTATCACACAATCAGTCTGCATATACAGGCGTTTTATATATATATTTATATAGACTACATATATACGTAT
ATTATATATGTAAATATTTCACTGTCTTTGAGGACGGGGGCCCTGTCTTTTTTATCTGTGGTTTTGCTTAGATGT

CCTCCAACATAATCTTAACACATAGTATGCTTTTAGAAATCGTTGACTGAATGCTAAGGACGAAAAACCGGTGAC
CAGAAGGCAACCAGGAAAGGCTTTGCTGACCTCCGGAGTGGTGGAGTTGGAGGTTCTGGGAAGGCGACTAGGGAG
CCAGGCAGGGGCGGGGTGGGATGGGATGTGGACAGCGCTTTTGCGGGGGGAAAGCGTTTTTGCTGCTGGAATTGA
GCAGTAGGAATGTGTCAGTCACATCCCCACCTTCCCAATTCTTGTCATCTCGGTTCAGGAAGGTGAACGGTGTTC
CGATTCCCCGCGGCGGGGGCCTGTAGTGGGAGCTCTGCCCCTTCCCCGCCTCTGCTGCAGGCCCCGCCCCTCGCC
CGGAACCCCGGGGCGCTGGCCGCGGTGCTGAAACGGCGCCCTCCGCGGACGGAGGAGGGGGCGGGGCTCTCGGGA
GCCGTGAGCCGGGAAGAGGGAGACGGGCAGGGCGGCGCCAGCAGGCCCTGGTGGGCTTGGGAGGAGGCAGGAGAC
TGGAGACAGCCTCGGCTAGAGCGGACACAGGCACCTGGCAAGCTTTCCTTGACCAAATCAAGGT.

By “synapsin promoter” also termed “Syn promoter” is meant a regulatory polynucleotide sequence comprising or consisting of a nucleic acid sequence sufficient to direct expression of a downstream polynucleotide in an outer or inner hair cell, a vestibular hair cell, a spiral ganglion, or a vestibular ganglion and having at least about85% (90%, 95%, 97%, 98%, 99%) sequence identity to the following nucleotide sequence:

TCTAGACTGCAGAGGGCCCTGCGTATGAGTGCAAGTGGGTTTTAGGACCAGGATGAGGCGGGGTGGGGGTGCCTA
CCTGACGACCGACCCCGACCCACTGGACAAGCACCCAACCCCCATTCCCCAAATTGCGCATCCCCTATCAGAGAG
GGGGAGGGGAAACAGGATGCGGCGAGGCGCGTGCGCACTGCCAGCTTCAGCACCGCGGACAGTGCCTTCGCCCCC
GCCTGGCGGCGCGCGCCACCGCCGCCTCAGCACTGAAGGCGCGCTGACGTCACTCGCCGGTCCCCCGCAAACTCC
CCTTCCCGGCCACCTTGGTCGCGTCCGCGCCGCCGCCGGCCCAGCCGGACCGCACCACGCGAGGCGCGAGATAGG
GGGGCACGGGCGCGACCATCTGCGCTGCGGCGCCGGCGACTCAGCGCTGCCTCAGTCTGCGGTGGGCAGCGGAGG
AGTCGTGTCGTGCCTGAGAGCGCAGTC.

By “reduces” is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

By “reference” is meant a standard or control condition.

A “reference sequence” is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, at least about 20 amino acids, at least about 25 amino acids, and in some embodiments, at least about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, at least about 60 nucleotides, at least about 75 nucleotides, and at least about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence but will typically

exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) Methods Enzymol. 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, less than about 500 mM NaCl and 50 mM trisodium citrate, or less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, or at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, at least about 37° C, or at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In an embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In another embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In yet a further embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will, in some instances, be less than about 30 mM NaCl and 3 mM trisodium citrate, or less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C (e.g., at least about 42° C, at least about 68° C). In one embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In another embodiment, wash steps will occur at 42 C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In yet a

further embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). In some embodiments, such a sequence is at least 60% (e.g., 80%, 85%, 90%, 95%, 99%) identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e-3 and e-100 indicating a closely related sequence.

By "subject" is meant any organism to which a composition and/or compound in accordance with the disclosure may be administered, e.g., for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Generally, subjects may include any animal (e.g., a mammal, including, but not limited to, a human or non-human mammal, such as a rodent, primate, bovine, equine, canine, ovine, or feline. A "subject in need thereof" is typically a subject for whom it is desirable to treat a disease as described herein. For example, a subject in need thereof may seek or be in need of treatment, require treatment, be receiving treatment, may be receiving treatment in the future, or a human or non-human animal that is under care by a trained professional for a particular disease.

By "TMC1 polypeptide" is meant a Transmembrane Channel-Like 1 polypeptide having at least about85% or greater (e.g., 90%, 95%, 97%, 98%, 99%) amino acid sequence identity to NCBI Reference Sequence: NP_619636.2 or a fragment thereof having mechanotransduction channel activity. An exemplary amino acid sequence of TMC1 is provided below:

```
   1 MSPKKVQIKV EEKEDETEES SSEEEEEVED KLPRRESLRP KRKRTRDVIN EDDPEPEPED
  61 EETRKAREKE RRRRLKRGAE EEEIDEEELE RLKAELDEKR QIIATVKCKP WKMEKKIEVL
 121 KEAKKFVSEN EGALGKGKGK RWFAFKMMMA KKWAKFLRDF ENFKAACVPW ENKIKAIESQ
 181 FGSSVASYFL FLRWMYGVNM VLFILTFSLI MLPEYLWGLP YGSLPRKTVP RAEEASAANF
 241 GVLYDFNGLA QYSVLFYGYY DNKRTIGWMN FRLPLSYFLV GIMCIGYSFL VVLKAMTKNI
 301 GDDGGGDDNT FNFSWKVFTS WDYLIGNPET ADNKFNSITM NFKEAITEEK AAQVEENVHL
 361 IRFLRFLANF FVFLTLGGSG YLIFWAVKRS QEFAQQDPDT LGWWEKNEMN MVMSLLGMFC
 421 PTLFDLFAEL EDYHPLIALK WLLGRIFALL LGNLYVFILA LMDEINNKIE EEKLVKANIT
 481 LWEANMIKAY NASFSENSTG PPFFVHPADV PRGPCWETMV GQEFVRLTVS DVLTTYVTIL
 541 IGDFLRACFV RFCNYCWCWD LEYGYPSYTE FDISGNVLAL IFNQGMIWMG SFFAPSLPGI
 601 NILRLHTSMY FQCWAVMCCN VPEARVFKAS RSNNFYLGML LLILFLSTMP VLYMIVSLPP
 661 SFDCGPFSGK NRMFEVIGET LEHDFPSWMA KILRQLSNPG LVIAVILVMV LAIYYLNATA
 721 KGQKAANLDL KKKMKMQALE NKMRNKKMAA ARAAAAAGRQ.
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By "TMC1 polynucleotide" is meant a polynucleotide encoding a TMC1 polypeptide. The sequence of an exemplary TMC1 polynucleotide is provided at NCBI Reference Sequence: NM_138691.2, which is reproduced below:

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   1 CAGAAACTAT GAGGGCAGAA CCCAGCAATC TGTGCTTTCT TTCACAAGCC CTCCAGGAGT
  61 TGCTGAAATT TAGGAATCAT TGCCCCAAAA AGTGGCCCTC ATAATGATGC CAGATGGGAT
 121 CTTACTCTGT TGCCCAGGCT GGAGTGCAGT GGTGCGATCT CGGCTCTCTG CAACCTCCGC
 181 CTCCCAGGTT CAAGTGATTC TCCTGCCTCG GCCTCCTGAG TAGCTGGGAT TTCAGGCCAT
 241 GAAAGATCAC TGTTTTAGTC TGCGTGGTGC AGTGGAACAG ATAGACCTCG GTTTGAATCT
 301 CAGCTCTACT GTTTACTAGA CATGAAATGG GGAAATCTAA AATGAGATGC CAGAAGCCTC
 361 AAAAATGGAA AACCCCCTGT GCTTCACATC TGAAAATCTC TGCTGGGGGC AGCAACTTTG
 421 AGCCTGTGGG GAAGGAACTG TCCACGTGGA GTGGTCTGGT GAATGCTTAA GGAGCTGCAG
 481 AAGGGAAGTC CCTCTCCAAA CTAGCCAGCC ACTGAGACCT TCTGACAGGA CACCCCCAGG
 541 ATGTCACCCA AAAAAGTACA AATCAAAGTG GAGGAAAAAG AAGACGAGAC TGAGGAAAGC
 601 TCAAGTGAAG AGGAAGAGGA GGTGGAAGAT AAGCTACCTC GAAGAGAGAG CTTGAGACCA
 661 AAGAGGAAAC GGACCAGAGA TGTTATCAAT GAGGATGACC CAGAACCTGA ACCAGAGGAT
 721 GAAGAAACAA GGAAGGCAAG AGAAAAAGAG AGGAGGAGGA GGCTAAAGAG AGGAGCAGAA
 781 GAAGAAGAAA TTGATGAAGA GGAATTGGAA AGATTGAAGG CAGAGTTAGA TGAGAAAAGA
 841 CAAATAATTG CTACTGTCAA ATGCAAACCA TGGAAGATGG AGAAGAAAAT TGAAGTTCTC
 901 AAGGAGGCAA AAAAATTTGT GAGTGAAAAT GAAGGGGCTC TTGGGAAAGG AAAAGGAAAA
 961 CGGTGGTTTG CATTTAAGAT GATGATGGCC AAGAAATGGG CAAAATTCCT CCGTGATTTT
1021 GAGAACTTCA AAGCTGCGTG TGTCCCATGG GAAAATAAAA TCAAGGCTAT TGAAAGTCAG
1081 TTTGGCTCCT CAGTGGCCTC ATACTTCCTC TTCTTGAGAT GGATGTATGG AGTCAATATG
1141 GTTCTCTTTA TCCTGACATT TAGCCTCATC ATGTTGCCAG AGTACCTCTG GGGTTTGCCA
1201 TATGGCAGTT TACCTAGGAA AACCGTTCCC AGAGCCGAAG AGGCATCGGC AGCAAACTTT
1261 GGTGTGTTGT ACGACTTCAA TGGTTTGGCA CAATATTCCG TTCTCTTTTA TGGCTATTAT
1321 GACAATAAAC GAACAATTGG ATGGATGAAT TTCAGGTTGC CGCTCTCCTA TTTTCTAGTG
1381 GGGATTATGT GCATTGGATA CAGCTTTCTG GTTGTCCTCA AAGCAATGAC CAAAAACATT
1441 GGTGATGATG GAGGTGGAGA TGACAACACT TTCAATTTCA GCTGGAAGGT CTTTACCAGC
1501 TGGGACTACC TGATCGGCAA TCCTGAAACA GCAGACAACA AATTTAATTC TATCACAATG
1561 AACTTTAAGG AAGCTATCAC AGAAGAAAAA GCAGCCCAAG TAGAAGAAAA CGTCCACTTG
1621 ATCAGATTCC TGAGGTTTCT GGCTAACTTC TTCGTGTTTC TAACACTTGG AGGGAGTGGA
1681 TACCTCATCT TTTGGGCTGT GAAGCGATCC CAGGAATTTG CACAGCAAGA TCCTGACACC
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1741 CTTGGGTGGT GGGAAAAAAA TGAAATGAAC ATGGTTATGT CCCTCCTAGG GATGTTCTGT
1801 CCAACATTGT TTGACTTATT TGCTGAATTA GAAGACTACC ATCCTCTCAT CGCTTTGAAA
1861 TGGCTACTGG GACGCATTTT TGCTCTTCTT TTAGGCAATT TATACGTATT TATTCTTGCA
1921 TTAATGGATG AGATTAACAA CAAGATTGAA GAGGAGAAGC TAGTAAAGGC CAATATTACC
1981 CTTTGGGAAG CCAATATGAT CAAGGCCTAC AATGCATCAT TCTCTGAAAA TAGCACTGGA
2041 CCACCCTTTT TTGTTCACCC TGCAGATGTA CCTCGAGGAC CTTGCTGGGA AACAATGGTG
2101 GGACAGGAGT TTGTGAGGCT GACAGTCTCT GATGTTCTGA CCACCTACGT CACAATCCTC
2161 ATTGGGGACT TTCTAAGGGC ATGTTTTGTG AGGTTTTGCA ATTATTGCTG GTGCTGGGAC
2221 TTGGAGTATG GATATCCTTC ATACACCGAA TTCGACATCA GTGGCAACGT CCTCGCTCTG
2281 ATCTTCAACC AAGGCATGAT CTGGATGGGC TCCTTCTTTG CTCCCAGCCT CCCAGGCATC
2341 AATATCCTTC GACTCCATAC ATCCATGTAC TTCCAGTGCT GGGCCGTTAT GTGCTGCAAT
2401 GTTCCTGAGG CCAGGGTCTT CAAAGCTTCC AGATCAAATA ACTTCTACCT GGGCATGCTA
2461 CTGCTCATCC TCTTCCTGTC CACAATGCCT GTCTTGTACA TGATCGTGTC CCTCCCACCA
2521 TCTTTTGATT GTGGTCCATT CAGTGGCAAA AATAGAATGT TTGAAGTCAT TGGAGAGACC
2581 CTGGAGCACG ATTTCCCAAG CTGGATGGCG AAGATCTTGA GACAGCTTTC AAACCCTGGG
2641 CTGGTCATTG CTGTCATTTT GGTGATGGTT TTGGCCATCT ATTATCTCAA TGCTACTGCC
2701 AAGGGCCAGA AGGCAGCGAA TCTGGATCTC AAAAAGAAGA TGAAAATGCA AGCTTTGGAG
2761 AACAAAATGC GAAACAAGAA AATGGCAGCT GCACGAGCAG CTGCAGCTGC TGGTCGCCAG
2821 TAATAAGTAT CCTGAGAGCC CAGAAAAGGT ACACTTTGCC TTGCTGTTTA AAAGTAATGC
2881 AATATGTGAA CGCCCAGAGA ACAAGCACTG TGGAACTGCT ATTTTCCTGT TCTACCCTTG
2941 ATGGATTTTC AAGGTCATGC TGGCCAATTA AGGCATCATC AGTCCTACCT GAGCAACAAG
3001 AATCTAAACT TTATTCCAAG TCAGAAACTG TTTCTGCAGA GCCACTCTCT CCCCTGCTCC
3061 ATTTCGTGAC TTTTTTTTTT TTTTTAACAA ATTGAGTTTA GAAGTGAGTG TAATCCAGCA
3121 ATACAGTTTA CTGGTTTAGT TGGTGGGTTA ATTAAAAAAA ATTTGCTCAT ATGAACTTTC
3181 ATTTTATATG TTTCTTTTGC C.
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As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disease (including a disorder or condition) and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so

long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of a dual vector gene editing and replacement system. Shown are two vectors, a first vector encoding a the SaCas9-KKH nuclease and a second vector encoding a wild type (WT) gene of interest. This second vector also encodes U6 promoter that drives expression of a single guide RNA (sgRNA). Inverted tandem repeats (ITRs) are at the termini of both vectors.

FIG. 2 provides an exemplary schematic of the dual vector gene editing and replacement system. Vector 1 carries a CMV promoter and the coding sequence for SaCas9-KKH. Vector 2 carries a CMV promoter driving the WT *TMC1* sequence with a mutation that does not affect the amino acid sequence and is not recognized by the SaCas9-KKH coding sequence. Vector 2 also contains the U6 promoter and guide RNA sequence that recognizes WT and mutant *TMC1* alleles. Both vectors are packaged into AAV9-PHP.B.

FIG. 3 is an illustration of human dominant mutations in the ClinVar database (accessed 2019.03.25) and mutations targetable with SaCas9 and SaCas9-KKH.

FIG. 4 shows a single vector to replace with wild type (WT) Tmc1. Using a single vector, with WT *TMC1* encoded in either Anc80 or AAV9-PHP.B to replace mutant Tmc1 in mouse inner ears, auditory function was recovered. Auditory brainstem responses (ABR) were recorded from WT, Tmc1-/-, or Tmc1-/- mice injected with Anc80-Tmc1 or AAV9-PHP.B-Tmc1. Distortion Product Otoaccoustic Emissions (DPOAE) were recorded and indicate function of outer hair cells.

FIG. 5A shows dual AAV vector constructs for expression of the gRNA and SpCas9 nuclease.

FIG. 5B illustrates a design of gRNA sequences targeting the Bth mutation. Aligned sequences of the mouse Tmc1 and human TMC1 genes are shown in the lower boxes. The protospacer adjacent motif (PAM) sequence is depicted in underlined blue text, the nucleotide corresponding to the Bth mutation in red (* over T nucleotides of gRNA 11-20; A nucleotide of gRNA 21-22), and green # over the G nucleotides of gRNA 16 and gRNA 22 from the WT.

FIG. 5C provides representative sequencing chromatograms of the *TMC1* gene in mouse embryonic fibroblast (MEF) cells. * indicates the position corresponding to the T-to-A Bth point mutation. The predominant T in this position in Bth/+ cells transfected with gRNA 15 indicates selective SpCas9-mediated cleavage of the Bth allele. The arrowhead shows the SpCas9 cleavage site.

FIG. 5D presents TIDE quantification of gRNA-induced cleavage efficiency based on the relative presence of the + or Bth sequence in Bth/+ mouse embryonic fibroblast (MEF) cells transfected with each of the gRNA-expressing constructs. For each grouping left to right, gRNA 11 (blue circle), gRNA 12 (red square), gRNA 13 (green triangle), gRNA 14 (burgundy inverted triangle), gRNA 15 (orange diamond), gRNA 21 (PAM) (black circle). Note the significant selectivity for the Bth allele of gRNA 12 and 15 (**$\leq$0.01, ***$\leq$0.001; multiple t-tests with Holm-Sidak method). ns, not significant.

FIG. 5E shows representative sequencing chromatograms of the *TMC1* gene in near-haploid human cell line (HAP) TMC1+ and TMC1Bth cells. Note that indels (arrowhead) are present only in the SpCas9-expressing HAP TMC1Bth cells transfected with gRNA 16. * indicates the position corresponding to the T-to-A Bth point mutation.

FIG. 5F illustrates tracking of indels by decomposition (TIDE) analysis of gRNA-induced cleavage efficiency based on indel frequency in human haploid (HAP) cells transfected with each of the gRNA-expressing constructs. For each grouping, gRNA 16 (blue circle), gRNA 17 (red square), gRNA 18 (green triangle), gRNA 19 (burgundy inverted triangle), gRNA 20 (orange diamond), gRNA 22 (PAM) (black circle). Note the significant selectivity of gRNA 16 for the Bth allele (*$\leq$0.05; Multiple t tests with Holm-Sidak method). ns, not significant.

FIG. 6 presents representative 10x confocal images (left and middle images) from apical-mid cochlear sections showing GFP (green) and RFP (red) co-expression in inner and outer hair cells. Magnification (63x) of 100 μm sections (right images) illustrate GFP and RFP expression in individual hair cells, merged, and stained against MyoVIIa (blue). Scale bars 100 μm at 10x and 20 μm at 63x.

FIG. 7A provides representative ABR waveform families recorded from mice at 24 weeks for indicated conditions, using 11.3-kHz tone bursts at incrementally increasing sound pressure levels.

Thresholds were determined by the presence of peak 1 and is indicated by colored traces pointed to by arrows. Scale bar applies to all families.

FIG. 7B shows mean ABR (left) and DPOAE (right) thresholds mice plotted as a function of stimulus frequency for $TMC1^{Bth/+}$ un-injected controls (bold line beneath "Red", n = 6 at four weeks, n = 3 at six weeks, n = 9 at twelve weeks, n = 9 at twenty four weeks old) and $TMC1^{Bth/+}$ mice dual injected with AAV9-PHP.B-spCas9 and AAV9-PHP.B-gRNA15 (bold line above "Blue", n = 9 at four weeks, n = 13 at six weeks, n = 9 at twelve weeks, n = 8 at twenty four weeks old). Lighter traces show individual responses. Error bars are SEM.

FIG. 7C illustrates DPOAE thresholds at 11.3 kHz measured in **(FIG. 6B)** plotted as a function of age from 4, 6, 12 and 24 weeks. For each timepoint of 4, 6, 12 and 24 weeks, WT or $TMC1^{+/+}$, n = 8, 9, 9, 6 (Left); $TMC1^{Bth/+}$, n = 6, 3, 9, 9 (Center); Dual injection with AAV9-PHP.B-SpCas9 and AAV9-PHP.B-gRNA15, n = 9, 13, 9, 8 (Right)). Error bars are SEM.

FIG. 8A shows representative 63x confocal images of 100 μm sections from the apex, middle, and basal cochlear turns of $TMC1^{+/+}$ wild-type C57BL/6 and un-injected $TMC1^{Bth/+}$ mice (left two columns) or $TMC1^{Bth/+}$ mice dual injected with PHP.B-spCas9 and PHP.B-gRNA15.GFP (green) immunostained against MyoVIIa (red) at 24 weeks of age (right three columns). Scale bar is 20 μm.

FIG. 8B provides mean cell counts of inner (left panel) and outer (right panel) hair cells for wild-type C57BL/6 ($TMC1^{+/+}$)(n = 3) (Left grouping), TMC1Bth/+ un-injected controls (n = 3) (Center grouping), and TMC1Bth/+ mice dual injected with PHP.B-spCas9 and PHP.B-gRNA15.GFP (n = 6) (Right grouping) at 24 weeks of age measured from 100 μm sections of the apex, middle, and basal cochlear turns. Individual samples are shown as scatter plot.

FIG. 8C presents the ABR threshold (dB) as compared to the percentage of surviving hair cells (left panel) and the percentage of Green Fluorescent Protein (GFP) stained hair cells (right panel) at varying kilohertz (kHz).

FIG. 9A shows the edited reads in an AAV9-PHP.B-SpCas9 + AAV9-PHP.B-gRNA15 injected $Tmc1^{Bth/+}$ mouse from the apical and basal halves of the cochlea. Reads are shown separately for Bth and WT alleles.

FIG. 9B presents a representative indel profile from an AAV9-PHP.B-SpCas9 + AAV9-PHP.B-gRNA15 injected $Tmc1^{Bth/+}$ mouse on the Bth (Left panel) and WT (Right panel) allele. Minus numbers represent nucleotide deletions, positive numbers are insertions, no indels have a value of 0.

DETAILED DESCRIPTION OF THE INVENTION

As described below, the present invention features a dual vector system for disrupting and replacing a target gene comprising a mutation.

The invention is based, at least in part, on the discovery that administration of a dual vector system comprising a first vector ("disrupting vector") encoding a Cas9 protein (e.g., SaCas9-KKH, SpCas9-KKH) and a second vector ("replacing vector") encoding a wild-type gene that replaces a mutant target gene and a guide RNA (gRNA) that directs the Cas9 protein to the target gene. Importantly, Cas9-mediated gene editing does not occur in the absence of a gRNA. Because the first vector encodes the Cas9 protein, it is not sufficient to disrupt the endogenous target gene. Only when the first vector and the second vector (i.e., containing the gRNA and the wild-type replacement gene) are present together in a cell will gene editing occur. This eliminates the possibility of a Cas9 protein inactivating a gene when a replacement gene is not present.

Dual Vector System

The invention provides a dual vector system for gene editing comprising a first vector encoding a Cas9 polypeptide, such as an SaCas9-KKH or SpCas9-KKH polypeptide, or a fragment thereof, that disrupts a target gene; and a second vector that encodes a guide RNA (gRNA) and a wild-type version of the target gene that replaces the disrupted target gene (**FIGs. 1-2**). Advantageously, the presence of both vectors in a single cell is required for activity.

Cas9

Cas9 proteins are known in the art, such as *Streptococcus pyogenes* Cas9 (SpCas9), *Staphylococcus aureus* Cas9 (SaCas9), and *Francisella novicida* Cas9 (FnCas9). Cas9 is a nuclease, an enzyme specialized for cutting DNA, with two active cutting sites, one for each strand of the double helix. In general, Cas9 proteins preferentially interrogate and act upon DNA sequences containing a protospacer adjacent motif (PAM) sequence, and different Cas9 proteins have affinities for different PAMs. The canonical PAM sequence is 5'-NGG-3', which is recognized by multiple Cas9 proteins, where N can be any nucleotide. For example, SpCas9 and FnCas9 recognize the canonical NGG PAM sequence. *Streptococcus thermophilus* Cas9 recognizes a 5'-NGA-3' PAM sequence, and SaCas9 recognizes a 5'-NNGRR(N)-3' PAM sequence. Additionally, Cas9 proteins can be modified to recognize PAM sequences that are distinct from the PAM sequences recognized by the unmodified Cas9 protein. For example, SaCas9-KKH recognizes a 5'-RRT-3', where R denotes an adenosine or guanine nucleotide.

To effectively direct a Cas9 polypeptide to a target nucleic acid that contains a PAM sequence, a guide RNA can be designed that has a sequence complementary to a nucleic acid sequence in the target nucleic acid molecule. It has been proposed that such synthetic guide RNAs might be able to be used for gene editing (Jinek et al., Science. 2012 Aug 17;337(6096):816-21).

Guide RNA (gRNA)

A Cas9 protein, having an affinity for a particular PAM sequence can be directed to a particular locus in a genome by a guide RNA (gRNA). In some embodiments, the guide RNA is a "single guide RNA" (sgRNA) which comprises a *trans*-activating CRISPR RNA (crRNA) (tracrRNA) and a spacer RNA, where the guide RNA can bind to both the Cas9 protein and the target DNA sequence. The tracrRNA provides a scaffold that can interact with a Cas9 protein. The short spacer RNA, comprising a nucleic acid sequence that specifically binds to the target genomic locus, directs the Cas9 protein to the target, which is then cleaved by the Cas9 protein's nuclease activity. In some embodiments, synthetic gRNAs are about 18 basepairs (bp) or greater (e.g., 19, 20, 21, 22, 23, 24, 25, 30, 40, 50, 60, 70, 80, 90, 100, over 100 bp) and comprise a nucleic acid sequence complementary to protospacer nucleotides near the PAM sequence

A spacer RNA, tracrRNA, or sgRNA can comprise a nucleotide analog or other modification. In some embodiments, the modification can be a nucleotide analog that is lacking the 3' OH group on the ribose sugar. Nucleotide analogs are known in the art. Additionally, the RNA molecule may comprise a modified backbone. For example, rather than having the canonical sugar-phosphate backbone of a naturally occurring RNA molecule, the molecule may have a sugar thiophosphate backbone. In some embodiments, incorporating a modified nucleotide, nucleotide analog, or modified backbone (and like modifications) can decrease a guide RNA's susceptibility to degradation.

In some embodiments, the guide RNA will bind a nucleic acid sequence comprising a PAM sequence that is present in one or more alleles. In some embodiments, the guide RNA binds a nucleic acid sequence that is in close proximity to a PAM sequence. For example, the PAM sequence may be 1 or greater (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10) nucleotides upstream or downstream of the sequence to which the guide RNA binds. In some embodiments, the PAM sequence may be 1-10 nucleotides upstream or downstream of the sequence to which the guide RNA binds. For example, Cas9 recognizes the 5′-NGG-3′ PAM (SpCas9). Liu et al. Comput Struct Biotechnol J. 18:35-44, 2020, which is incorporated here by reference in its entirety, is a review providing guide RNA design considerations, parameters, and tools.

The following U.S. patents and patent publications are incorporated herein by reference in their entireties for their disclosure regarding gene editing, including but not limited to disrupting vectors, replacement vectors, and methods thereof: Patent No. 8,697,359, 20140170753, 20140179006, 20140179770, 20140186843, 20140186958, 20140189896, 20140227787, 20140242664, 20140248702, 20140256046, 20140273230, 20140273233, 20140273234, 20140295556, 20140295557, 20140310830, 20140356956, 20140356959, 20140357530, 20150020223, 20150031132, 20150031133, 20150031134, 20150044191, 20150044192,

20150045546, 20150050699, 20150056705, 20150071898, 20150071899, 20150071903, 20150079681, 20150159172, 20150165054, 20150166980, and 20150184139.

Polynucleotide Delivery

The dual vector system provides for the safe and efficient delivery of exogenous gene constructs to relevant cell targets. In one embodiment, cells in the organ of Corti in the cochlea are targeted. The organ of Corti includes two classes of sensory hair cells: inner hair cells, which convert mechanical information carried by sound into electrical signals transmitted to neuronal structures, and outer hair cells which serve to amplify and tune the cochlear response, a process required for complex hearing function.

In some embodiments, the dual vector system comprises viral vectors. The viral vectors generally contain the minimum required viral sequences for packaging and subsequent integration into a subject. Adeno-associated virus (AAV) vectors used in gene therapy may only contain inverted terminal repeat (ITR) sequences from the AAV genome. These are necessary for packaging and integration into a host genome. Suitable methods for the delivering or administering nucleic acids to cells are available and well known to those skilled in the art, and although more than one route can be used for administering a particular composition, one route may provide a more effective or immediate result than another route.

Methods of delivering viruses (which also can be referred to as viral particles) containing a transgene to inner ear cells are known in the art. As described herein, about 108 to about 1,012 viral particles can be administered to a subject, and the virus can be suspended within a suitable volume (e.g., 10 μL, 50 μL, 100 μL, 500 μL, or 1000 μL) of, for example, artificial perilymph solution.

In some embodiments, a vector described herein (e.g., disrupting vector, replacing vector) comprises a promoter (e.g., an Espin promoter, a protocadherin 15 (PCDH15) promoter, a protein tyrosine phosphatase receptor type Q (PTPRQ) promoter, a myosin VI (Myo6) promoter, a Potassium Voltage-Gated Channel Subfamily Q Member 4 (KCNQ4) promoter, a myosin VIIA (Myo7a) promoter, a synapsin promoter, a glial fibrillary acidic protein (GFAP) promoter, a cytomegalovirus (CMV) promoter, a CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site (CAG) promoter, a chicken β-actin (CBA) promoter, a CBH promoter, a U6, type III RNA polymerase promoter, and a tetraspan membrane protein of hair cell stereocilia (TMHS) or lipoma HMGIC fusion partner-like 5 (LHFPL5) promoter) that drives expression of a downstream polynucleotide. One or both vectors may comprise at least one promoter selected from but not limited to CMV and U6.

In some embodiments, a therapeutically effective amount of the dual vector system of the invention is injected through the round window or the oval window, or the utricle, typically in a

relatively simple (e.g., outpatient) procedure. In some embodiments, viruses are delivered to the appropriate position within the ear during surgery (e.g., a cochleostomy or a canalostomy).

In some embodiments, delivery vehicles (e.g., polymers) are used to facilitate the transfer of agents across the tympanic membrane and/or through the round window or utricle, and any such delivery vehicles can be used to deliver the viruses described herein. See, for example, Arnold et al., 2005, Audiol. Neurootol., 10:53-63. Other viral vectors that can be used include, for example, a vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus. Retroviral vectors are particularly well developed and have been used in clinical settings.

In some embodiments, the compositions and methods described herein facilitate the delivery to, and expression of, exogenous polynucleotides in at least 65% (e.g., at least 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99%) of inner and/or outer hair cells or delivery to, and expression in, at least 80% (e.g., at least 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99) of outer hair cells.

In one embodiment, a vector of the invention is an adeno-associated virus (AAV). In another embodiment, a vector of the invention is an Anc80 vector, which is used to transduce greater than about60% (e.g., 70%, 80%, 90%, 95%, or even 100%) of inner or outer hair cells. In one embodiment, the Anc80 is Anc80-0065 (SEQ ID NO:2), which is described in International Application No. PCT/US2018/017104, which is incorporated herein by reference in its entirety. However, WO 2015/054653, which is also incorporated herein by reference in its entirety, describes a number of additional ancestral capsid proteins that fall within the class of Anc80 ancestral capsid proteins.

In particular embodiments, the adeno-associated virus (AAV) contains an ancestral AAV capsid protein that has a natural or engineered tropism for hair cells. In some embodiments, the virus is an Inner Ear Hair Cell Targeting AAV, which delivers a transgene to the inner ear in a subject. In some embodiments, the virus is an AAV that comprises purified capsid polypeptides. In some embodiments, the virus is artificial.

In some embodiments, one or both vectors of the dual vector system may comprise a heterologous promoter (e.g., CMV promoter, Espin promoter, a PCDH15 promoter, a PTPRQ promoter and a TMHS (LHFPL5) promoter) that drives expression of a downstream polynucleotide. As used herein, a "heterologous promoter" refers to a promoter that does not naturally direct expression of that sequence (i.e., is not found with that sequence in nature).

Methods for packaging a transgene into a virus are known in the art and utilize conventional molecular biology and recombinant nucleic acid techniques. For example, Aponte-Ubillus, et al. (*Appl Microbiol Biotechnol.* 102:1045-1054, 2018) provides a review of molecular design of AAV vectors for gene therapy, the contents of which are incorporated by reference in its entirety.

In some embodiments, an AAV9-PHP.B vector is used to efficiently target inner ear cells. AAV9-PHP.B is described in International Application No. PCT/US2019/020794, the contents of which are incorporated herein by reference in their entirety. AAV-PHP.B encodes the 7-mer sequence TLAVPFK and efficiently delivers transgenes to the cochlea, where it showed remarkably specific and robust expression in the inner and outer hair cells. An AAV-PHP.B vector can comprise, but is not limited to, any of the promoters described herein.

Gene transfer can also be achieved using non-viral means involving transfection *in vitro*. Such methods include the use of calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell.

cDNA expression for use in polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Compositions and Methods of Treatment

The present invention provides methods of treating disease and/or disorders or symptoms thereof which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a dual vector system of the invention, wherein one vector disrupts a target gene comprising a mutation and the other replaces the target gene comprising the mutation with a wild-type version of the gene. Thus, one embodiment is a method of treating a subject suffering from or susceptible to a disease or disorder or symptom thereof associated with a mutation. The method includes administering to the mammal a therapeutic amount of the dual vector system described herein in an amount sufficient to treat a disease or disorder or symptom. The method of treating a subject in need thereof with the dual vector system described here may result in the amelioration, reduction, or repair of the genetic disease, or symptoms thereof, suffered by the subject in need.

The therapeutic methods of the invention (which include prophylactic treatment), in general, comprise administration of a therapeutically effective amount of a dual vector system described herein to a subject in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a disease, disorder, or symptom thereof. Determination of those subjects “at risk” can be

made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, Marker (as defined herein), family history, and the like).

Compositions are contemplated herein for the treatment of diseases or conditions associated with a mutation associated with a disorder. For therapeutic purposes, the dual vector systems described herein are used to treat a disease or condition (e.g., dominant progressive hearing loss) as described herein. The dual vector system may be administered directly to a region of the body (e.g., cochlea). In some embodiments, the region of the body to which the vectors are administered is affected by a disease or condition associated with a genetic mutation (e.g., dominant, recessive). In some embodiments, the compositions are formulated in a pharmaceutically-acceptable buffer such as physiological saline. Non-limiting methods of administration include injecting into the cochlear duct or the perilymph-filled spaces surrounding the cochlear duct (e.g., scala tympani and scala vestibuli). Injecting into the cochlear duct, which is filled with high potassium endolymph fluid, could provide direct access to hair cells. However, alterations to this delicate fluid environment may disrupt the endocochlear potential, heightening the risk for injection-related toxicity. The perilymph-filled spaces surrounding the cochlear duct, scala tympani and scala vestibuli, can be accessed from the middle ear, either through the oval or round window membrane. The round window membrane, which is the only non-bony opening into the inner ear, is relatively easily accessible in many animal models and administration of viral vector using this route is well tolerated. In humans, cochlear implant placement routinely relies on surgical electrode insertion through the round window membrane.

Treatment of human patients or non-human animals are carried out using a therapeutically effective amount of a dual vector system in a physiologically-acceptable carrier. The phrase "pharmaceutically acceptable" refers to those compounds of the invention, compositions containing such compounds, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The amount of the dual vector system or composition comprising the dual vector system described here may be in an amount effective for treating a subject suffering from a genetic disease (including disorder or condition), such that the symptoms or genetic disease itself is reduced, ameliorated, or eliminated with treatment.

The phrase "pharmaceutically-acceptable excipient" includes pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, carrier, solvent or encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the

patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, about 5 per cent to about 70 per cent, or about 10 per cent to about 30 per cent.

Additional suitable carriers and their formulations are described, for example, in the most recent edition of Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the therapeutic agent to be administered varies depending upon the manner and mode of administration, the age and disease status (e.g., the extent of hearing loss present prior to treatment).

Compositions are administered at a dosage that controls the clinical or physiological symptoms of the disease or condition, as may in some cases be determined by a diagnostic method known to one skilled in the art.

Therapeutic compounds and therapeutic combinations are administered in an effective amount. For example, about 108 to about 1012 viral particles can be administered to a subject, and the virus can be suspended within a suitable volume (e.g., 10 μL, 50 μL, 100 μL, 500 μL, or 1000 μL) of, for example, artificial perilymph solution.

Methods of Treating Diseases Associated with Mutations

The invention provides dual vector systems and methods for using such systems to treat a disease or disorder associated with a mutation. In one embodiment, the dual vector system is used to treat dominant progressive hearing loss (e.g., Deafness, Autosomal Dominant 36, or dominant

progressive deafness 36, (DFNA36)). DFNA36 presents as sensorineural hearing loss, i.e., high frequency loss followed by low frequency loss leading to profound loss of all frequencies, as well as tinnitus. Typically, onset occurs between 5 to 28 years of age. DFNA36 is associated with dominant mutations (acquired or inherited) in the *TMC1* gene of affected individuals. Autosomal recessive deafness, e.g., DFNB7 or DFNB11, is also caused by mutation in the same *TMC1* gene. To disrupt *TMC1* in a subject having a dominant mutation in *TMC1*, a first nucleic acid vector ("the disrupting vector") encoding a Cas9 polypeptide, e.g., an SaCas9-KKH or SpCas9-KKH protein, is used. A second vector ("the replacing vector") encoding a wild-type TMC1 protein and a guide RNA (gRNA) that targets TMC1 is used (**FIGs. 1-2**). The Cas9-KKH protein disrupts the TMC1 target gene inducing frame shifts and premature stop codons. Expression of wild-type TMC1 from the second vector replaces the disrupted gene, thereby restoring hearing loss associated with a dominant mutation in TMC1.

In addition to the TMC1 p.M418K mutation (DFNA36), 15 dominant mutations in genes that are targetable with SaCas9-KKH were identified (**FIG. 3** and **Table 1**). All known dominant human mutations for specific PAM targeting using SaCas9 and SaCas9-KKH were analyzed. SaCas9 has a unique PAM requirement of 'GRRT', while SaCas9-KKH has a PAM requirement only of 'RRT'. Of 17,783 dominant entries in the ClinVar database, the SaCas9 GRRT PAM site was evident in 1,328 variants (7.5%), while the SaCas9-KKH PAM site is able to distinguish mutant from wild-type for 3,759 dominant alleles (21.1%) (**FIG. 3**).

Table 1: Dominant deafness variants potentially targetable with Cas9-KKH

Deafness locus	OMIM Disease	SNP ID	WT	Variant	Protein	Gene	Link
DFNA11	.0015 Deafness, Autosomal Dominant 11	RS=121965084	CAATG	CATTG	ASN458ILE	MYO7A	www.omim.org/entry/276903#0015
DFNA12	.0001 Deafness, Autosomal Dominant 12	RS=281865415	AGCTC	AGTTC	GLY1824ASP	TECTA	www.omim.org/entry/602574#0001
DFNA13	.0006 Deafness, Autosomal Dominant 13	RS=121912947	GCGCC	GCACC	ARG549CYS	COL11A2	www.omim.org/entry/120290#0005
DFNA17	.0008 Deafness, Autosomal Dominant 17	RS=80338828	GGCGG	GGTGG	ARG705HIS	MYH9	www.omim.org/entry/160775#0008
DFNA20	.0002 Deafness,	RS=104894544	TCTTC	TCATC	LYS118MET	ACTG1	www.omim.org/entry/102560#

	Autosomal Dominant 20						0002
DFNA22	.0001 Deafness, Autosomal Dominant 22	RS=121912557	GTGTT	GTATT	CYS442TYR	MYO6	www.omim.org/entry/600970#0001
DFNA22	.0006 Deafness, Autosomal Dominant 22	RS=121912561	AACGA	AATGA	ARG849TER	MYO6	www.omim.org/entry/600970#0006
DFNA25	.0001 Deafness, Autosomal Dominant 25	RS=121918339	GGCAC	GGTAC	ALA211VAL	SLC17A8	www.omim.org/entry/607557#0001
DFNA36	.0007 Deafness, Autosomal Dominant 36	RS=786201027	GATGT	GAAGT	MET418LYS	TMC1	www.omim.org/entry/606706#0007
DFNA39	.0004 Deafness, Autosomal Dominant Non syndromic Sensorineural 39, With Dentino genesis Imperfecta 1	RS=121912987	AGGTT	AGTTT	VAL18PHE	DSPP	www.omim.org/entry/125485#0004
DFNA3b	.0001 Deafness, Autosomal Dominant 3b	RS=104894414	GACGC	GATGC	THR5MET	GJB6	www.omim.org/entry/604418#0001
DFNA41	.0001 Deafness, Autosomal Dominant 41	RS=587777692	ACGTA	ACTTA	VAL60LEU	P2RX2	www.omim.org/entry/600844#0001
DFNA48	.0004 Reclassified - Variant Of Unknown Significance	RS=61753849	GACAT	GAAAT	GLU385ASP	MYO1A	www.omim.org/entry/601478#0004
DFNA66	.0001 Deafness, Autosomal Dominant 66	RS=876661402	TCGTT	TCATT	ARG192TER	CD164	www.omim.org/entry/603356#0001
DFNA68	.0001 Deafness, Autosomal	RS=864309524	GCCGT	GCGGT	ARG185PRO	HOMER2	www.omim.org/entry/604799#0001

	Dominant 68						
DFNA9	.0001 Deafness, Autosomal Dominant 9	RS=12190 8927	AGTAT	AGGAT	VAL66GLY	COCH	www.omim.org /entry/603196# 0001
DFNA9	.0005 Deafness, Autosomal Dominant 9	RS=12190 8930	CATCC	CAACC	ILE109ASN	COCH	www.omim.org /entry/603196# 0005
DFNA9	.0006 Deafness, Autosomal Dominant 9	RS=12190 8931	CTGCT	CTACT	ALA119THR	COCH	www.omim.org /entry/603196# 0006

EXAMPLES

Example 1: Dual Vector System

The invention provides a dual vector gene therapy system, which includes a first vector that disrupts a target gene, and a second vector that provides for the replacement of the disrupted target gene. The first step of the gene editing strategy utilized a vector encoding a highly efficient and selective Cas9 enzyme (e.g., SaCas9-KKH, SpCas9-KKH) having a PAM site that recognized a sequence in a carefully chosen site at the 5' end of the coding region of the target gene. A guide RNA (gRNA) selective for a site adjacent to the PAM was designed to recognize both mutant and wild-type alleles. The guide RNA and PAM are specific for the target gene. When the Cas9-KKH (e.g., SaCas9-KKH, SpCas9-KKH) and the guide RNA were introduced together into the same cell, the Cas9-KKH generated insertions/deletions (indels) that resulted in frame shifts and premature stop codons, thereby disrupting the target gene, resulting in a functional null allele. In the case of dominant mutations, both mutant and WT alleles were disrupted. In the case of recessive mutations, both recessive alleles were targeted for disruption.

The second step involved the "replacement" of the target gene. In this case, a conventional gene replacement approach was used, where a vector that deliverd the wild-type coding sequence for the target gene was utilized. In one embodiment, the vector included a cell-type specific promoter driving expression of the wild-type coding sequence. The coding sequence used an alternate codon sequence that was degenerate, i.e. the wild-type amino acid sequence was preserved, but the DNA sequence used alternate codons in the region of the PAM and a guide RNA that "replaced" sequence that was not targeted for disruption by Cas9.

Dual vector transduction was required for the "disrupt and replace" strategy to be effective. One vector carried the Cas9 coding sequence for the disrupt portion of the gene editing strategy and

the second vector carried the wild-type (WT) coding sequence for the replace portion of the gene editing. For highly efficient vectors, dual transduction is possible in most cells.

Nevertheless, it is possible that some cells will receive only a single viral transduction event. For those cells transduced by just the "replacement" vector, the wild-type sequence will provide recovery of function, at least for recessive mutations. If cells are transduced by just the "disrupt" vector, there is a possibility that the WT allele may be disrupted (for heterozygous dominant genotypes) in cells that do not also receive the "replacement" vector, which could lead to an immediate loss-of-function of both dominant and WT alleles. To ensure that the WT gene target was not disrupted without being replaced, i.e., in the subset of cells transduced with just the "disrupt" vector, the guide RNA was provided in the "replace" vector. In this way, only cells that received both the "disrupt" and "replace" vectors will undergo the full "disrupt and replace" events.

Example 2: Analysis of Dual-vector system in Tmc Knockout Mice *in vivo*

Animals

All animals were bred and housed in facilities. All studies involving animals were approved by the HMS Standing Committee on Animals (Protocol No. 03524) and the Boston Children's Hospital Institutional Animal Care and Use Committee (Protocol Nos. 2878 and 3396). All experiments were conducted in accordance with the animal protocols.

Null allele ("knockout") mice that were TMC1 deficient (TMC1-/-) were generated and served as a mouse model for human hearing loss (e.g., Deafness, Autosomal Dominant 36, or dominant progressive deafness 36, (DFNA36) phenotype caused by dominant (aquired or inherited) *TMC1* gene mutations, Met to Lys at position 412 (M412K) and Thymine to Adenine at position 1253 (T1253A) of the *Tmc1* gene. The 'Beethoven' (Bth) deaf mutant mouse is a model for autosomal dominant DFNA36. The Bth mouse model was found to accurately recapitulate human hearing loss of the DFNA36 phenotype caused by TMC1 mutations that result in the hair cell degeneration and progressive hearing loss in mice.

Inner ear injections

Inner ears of $TMC1^{-/-}$ or $TMC1^{WT/WT}$ mouse pups were injected at postnatal day 1 (P1) with 1 µl of AAV9-PHP.B virus at a rate of 60 nl/min. Vector 1 carried a CMV promoter and the coding sequence for SaCas9-KKH. Vector 2 carried a CMV promoter driving the WT *TMC1* sequence with a mutation that did not affect the amino acid sequence and was not recognized by the SaCas9-KKH coding sequence. Vector 2 also contained the U6 promoter and guide RNA sequence that recognized WT and mutant *TMC1* alleles. Both vectors were packaged into AAV9-PHP.B. Use of a single vector to replace mutant TMC1 in mouse inner ears occurred in other embodiments. WT *TMC1*

encoded in either Anc80 or AAV9-PHP.B replaced mutant TMC1 in mouse inner ears in order to recover auditory function. Pups were anesthetized using hypothermia exposure in ice water for 2-3 minutes. Upon anesthesia, a post-auricular incision was made to expose the otic bulla and visualize the cochlea. Injections were made manually with a glass micropipette. After injection, a suture was used to close the skin cut. Then, the injected mice were placed on a 42°C heating pad for recovery. Pups were returned to the mother after they recovered fully within ~10 minutes. Standard post-operative care was applied after surgery. Sample sizes for *in vivo* studies were determined on a continuing basis to optimize the sample size and decrease the variance. At P5 to P7, organs of Corti were excised from injected ears. Organ of Corti tissues were incubated at 37°C, 5% CO_2 for 8-10 days, and the tectorial membrane was removed immediately before electrophysiology recording.

Hearing tests

To determine whether the dual-vector system of the disclosure using AAV vectors (see e.g., **FIGs. 1, 2, 5A)** were capable of and the extent of recovering hearing loss, Auditory Brainstem Responses (ABRs) and Distortion Product Otoacoustic Emissions (DPOAEs) were measured in the TMC1 knockout ($TMC1^{-/-}$) mice. **FIG. 4** demonstrates that the single vector with WT TMC1 encoded in either Anc80 or AAV9-PHP.B that was used to replace mutant Tmc1 in the inner ears of mice resulted in recovery of auditory function. ABR and DPOAE measurements were recorded using the EPL Acoustic system (Massachusetts Eye and Ear, Boston). Acoustic stimuli were generated with 24-bit digital Input/Output cards (National Instruments PXI-4461) in a PXI-1042Q chassis, amplified by a SA-1 speaker driver (Tucker–Davis Technologies, Inc.), and delivered from two electrostatic drivers (CUI CDMG15008-03A) in a custom acoustic system. An electret microphone (Knowles FG-23329-P07) at the end of a small probe tube was used to monitor ear-canal sound pressure. ABRs and DPOAEs were recorded from mice during the same session. ABR signals were collected using subcutaneous needle electrodes inserted at the pinna (active electrode), vertex (reference electrode), and rump (ground electrode). ABR potentials were amplified (10,000×), pass-filtered (0.3–10 kHz), and digitized using custom data acquisition software (LabVIEW) from the Eaton-Peabody Laboratories Cochlear Function Test Suite. Sound stimuli and electrode voltage were sampled at 40-μs intervals using a digital I-O board (National Instruments) and stored for offline analysis. Threshold was defined visually as the lowest decibel level at which peak 1 could be detected and reproduced with increasing sound intensities. ABR thresholds were averaged within each experimental group and used for statistical analysis. ABR and DPOAE measurements were performed by investigators blinded to the genotype.

Mice were anesthetized with intraperitoneal (i.p.) injection of xylazine (5–10 mg/kg) and ketamine (60-100 mg/kg), and the base of the pinna was trimmed away to expose the ear canal. Three

subcutaneous needle electrodes were inserted into the skin, including a) dorsally between the two ears (reference electrode); b) behind the left pinna (recording electrode); and c) dorsally at the rump of the animal (ground electrode). Additional aliquots of ketamine (60-100 mg/kg i.p.) were given throughout the session to maintain anesthesia if needed. Prior to ABR testing, the sound pressure at the entrance of the ear canal was calibrated for each individual test subject at all stimulus frequencies. ABR and DPOAE data were collected under the same conditions and during the same recording sessions.

DPOAEs were recorded first. Primary tones were produced at a frequency ratio of 1.2 (the frequency ratio of f1 and f2 primary tones (f2/f1 = 1.2)) for generating DPOAEs at 2f1-f2, where the f2 level was 10 dB sound pressure level below f1 level for each f2/f1 pair. The tones were presented with f2 varied between 5.6 and 32.0 kHz in half-octave steps and L1–L2 = 10 decibel sound pressure level (dB SPL). At each f2, L2 was varied between 10 and 80 dB in 10 dB increments. DPOAE threshold was defined from the average spectra as the L2-level eliciting a DPOAE of magnitude 5 dB above the noise floor. The mean noise floor level was under 0 dB across all frequencies. At each level, waveform and spectral averaging were used in order to increase the signal-to-noise (s/n) ratio of the recorded ear-canal sound pressure. DPOAE at 2f1-f2 had an amplitude that was extracted from the averaged spectra, as well as the noise floor at neighboring points in the spectrum. Interpolation from plots of DPOAE amplitude versus sound level resulted in iso-response curves. Threshold was defined as the f2 level required to produce DPOAEs above 0 dB.

ABR experiments were then performed at 32° C in a sound-proof chamber. To test hearing function, mice were presented with stimuli of broadband "click" tones as well as the pure tones between 5.6 and 32.0 kHz in half-octave steps, all presented as 5-ms tone pips. The responses were amplified (10,000 times), filtered (0.1–3 kHz), and averaged with an analog-to-digital board in a PC-based data-acquisition system (EPL, Cochlear function test suite, MEE, Boston). Across various trials, the sound level was raised in 5 to 10 dB steps from 0 to 110 dB SPL. At each level, 512 responses were collected and averaged for each sound pressure level (with stimulus polarity alternated) after "artifact rejection." Threshold was determined by visual inspection of the appearance of Peak 1 relative to background noise. Data were analyzed and plotted using Origin-2015 (OriginLab Corporation, MA). Thresholds averages ± standard deviations are presented unless otherwise stated. The majority of these experiments were not performed under blind conditions.

Example 3: Screening of gRNAs for selective disruption of the Bth allele mediated by SpCas9 nuclease.

FIG. 5A illustrates the constructs of a dual AAV vector system that express SpCas9 nuclease and guide RNA (gRNA). **FIG. 5B** presents various guide RNA (gRNA) sequences that were

designed to target the Beethoven (Bth) mutation found in the mouse and human TMC1 genes as compared to the wild type (WT) sequence (in grey boxes). The designed gRNA sequences included PAM sequences that are underlined, the nucleotide corresponding to the Bth mutation depicted by an asterisk (*) over the aligned nucleotides, and for the human sequences, nucleotides corresponding to the wild type sequence shown by # over the particular nucleotide of gRNA 16 and gRNA 22.

Sequencing chromatograms of the *TMC1* gene in mouse embryonic fibroblast (MEF) cells (**FIG. 5C**) and near-haploid human (HAP) cells (**FIG. 5E**) were also performed. The position corresponding to the T-to-A Bth point mutation was identified (*). Note the predominant T in this position in Bth/+ cells transfected with gRNA 15, which indicates selective SpCas9-mediated cleavage of the Bth allele. The arrowhead shows the SpCas9 cleavage site in **FIG. 5C**. Indels (arrowhead) were shown to be present only in the SpCas9-expressing HAP $TMC1^{Bth}$ cells transfected with gRNA 16 of **FIG. 5E**.

Sequence traces were analyzed by deconvolution (TIDE, Tracking Indels by Decomposition, Desktop genetics, UK). Aberrant sequences were quantified downstream of the CRISPR cut site. Analysis was performed on forward vs. reverse traces and efficiency was averaged. **FIGs. 5D amd 5F** show TIDE quantification of gRNA-induced cleavage efficiency based on the relative presence of the wild type (+) or Bth sequence in Bth/WT (+) cells transfected with the indicated gRNA-expressing constructs. Significant selectivity for the Bth allele of gRNA 12, 15, and 16.

Example 4: *In vivo* Dual vector system for SpCas9/gRNA targeting of the Bth allele.

To confirm that the dual vector system works, AAV9-PHP.B-GFP and AAV9-PHPB-RFP were injected and images of the apical-mid-cochlear sections were shown in green (GFP), red (RFP), and Myo7a (blue) (**FIG. 6**). Auditory function was then shown to recover when $Tmc1^{Bth/+}$ mice were transduced with AAV9-PHP.B-spCas9 and AA9-PHP.B-gRNA15. **FIG. 7A** presents ABR waveform families recorded from mice (24 weeks) using 11.3 kHz tone bursts at increasing sound pressure levels (dB; y-axis). Thresholds were determined for wild type $Tmc1^{+/+}$ and $Tmc1^{Bth/+}$ mice with AAV9-PHP.B-spCas9 and AAV9-PHP.B-gRNA15.

FIG. 7B shows the ABR and DPOAE thresholds as a function of stimulus frequency for $TMC1^{Bth/+}$ un-injected controls (bold line beneath "Red") to $TMC1^{Bth/+}$ mice dual injected with AAV9-PHP.B-spCas9 and AAV9-PHP.B-gRNA15 (bold line above "Blue") at varying ages. These data were plotted as a function of mice age (4, 6, 12, and 24 weeks) for WT $TMC1^{+/+}$, $TMC1^{Bth/+}$, and $TMC1^{Bth/+}$ with the dual infection of spCas9 and gRNA15. The dual vector injected mice at all age groups had a lower DPOAE threshold (dB) as compared to $TMC1^{Bth/+}$ and was similar to TMC1+/+ threshold levels or above, yet below that of $TMC1^{Bth/+}$ demonstrating recovery of auditory function.

Example 5: AAV9-PHP.B-SpCas9/gRNA dual vector transduction preserves hair cell survival.

FIG. 8A compares individual hairs in sections from the apex, middle, and basal cochlear turns of $TMC1^{+/+}$ wild-type and uninjected $TMC1^{Bth/+}$ mice. Clearly the hair cells in the $TMC1^{Bth/+}$ mice are in a disarray as opposed to the uniform hair cells of the wild-type, $TMC1^{+/+}$, and the uniformity is restored in the $TMC1^{Bth/+}$ mice dual injected with AAV9-PHP.B-spCas9 and AAV9-PHP.B-gRNA15. The number of inner (left panel) and outer (right panel) hair cells per 100 μm is lost in $TMC1^{Bth/+}$ mice, while $TMC1^{Bth/+}$ mice dual injected with AAV9-PHP.B-spCas9 and AAV9-PHP.B-gRNA15 increased the numberof both inner and outer hair cells at the apex, middle, and base (**FIG. 8B**). In **FIG. 8C**, the ABR thresholds were found to be linear as a function of percentage hair cell survival (left panel) and of percentage of green fluorescent protein (GFP) (right panel) in dual infected mice.

Example 6: Sequencing analysis of *in vivo* SpCas9/gRNA dual injection.

The genomic DNA sequences of AAV9-SpCas9 and gRNA 15 of the $TMC1^{Bth}$ from the Apex and Base halves are presented with the number of reads for each (see, e.g., **FIG. 9A**). The indel profile s for AAV9-PHP.B-SpCas9 with AAV9-PHP.B-gRNA 15 for $TMC1^{Bth}$ (left panel) or $TMC1^{WT}$ (right panel) were presented (**FIG. 9B**) where the indel size (nucleotides, nt) (x-axis) are compared to the percent of modified reads (y-axis) based on the information presented in **FIG. 9A**.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

CLAIMS

What is claimed is:

1. A dual vector system comprising a first vector comprising a polynucleotide encoding a Cas9-KKH polypeptide and a second vector comprising a polynucleotide encoding a guide RNA (gRNA) that binds a target gene comprising a mutation and a polynucleotide encoding a wild-type version of the target gene.

2. The dual vector system of claim 1, wherein one or both vectors comprises at least one promoter.

3. The dual vector system of claim 2, wherein the at least one promoter is selected from: Espin promoter, a protocadherin 15 (PCDH15) promoter, a protein tyrosine phosphatase receptor type Q (PTPRQ) promoter, a myosin VI (Myo6) promoter, a Potassium Voltage-Gated Channel Subfamily Q Member 4 (KCNQ4) promoter, a myosin VIIA (Myo7a) promoter, a synapsin promoter, a glial fibrillary acidic protein (GFAP) promoter, a cytomegalovirus (CMV) promoter, a CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site (CAG) promoter, a chicken β-actin (CBA) promoter, a CBH promoter, a U6, type III RNA polymerase promoter, and a tetraspan membrane protein of hair cell stereocilia (TMHS) or lipoma HMGIC fusion partner-like 5 (LHFPL5) promoter.

4. The dual vector system of claim 1, wherein the target gene comprises a mutation associated with a disease or condition.

5. The dual vector system of claim 1, wherein the target gene is *TMC1*.

6. The dual vector system of claim 1, wherein the mutation is associated with hearing loss.

7. The dual vector system of claim 1, wherein the mutation is DFNA36.

8. The dual vector system of claim 1, wherein the Cas9-KKH is SaCas9-KKH or SpCas9-KKH.

9. The dual vector system of claim 1, wherein the guide RNA is selected from: gRNA 12, gRNA 15, and gRNA 16.

10. A dual vector system comprising:

a) a first AAV9-PHP.B vector comprising a nucleotide sequence encoding Cas9-KKH; and

b) a second AAV9-PHP.B vector comprising a nucleotide sequence encoding a guide RNA that binds a *TMC1* gene comprising a DFNA36 mutation and a polynucleotide encoding a wild-type *TMC1* gene.

11. The dual vector system of claim 10, wherein the guide RNA is any one selected from: gRNA 12, gRNA 15, and gRNA 16.

12. A composition comprising the dual vector system of any one of claims 1-11.

13. A method of modifying the genome of a cell, the method comprising contacting the cell with the dual vector system of any one of claims 1-11.

14. A method of genome editing, the method comprising contacting a cell with the dual vector system of any one of claims 1-11.

15. A method of treating a subject suffering from a genetic disease, the method comprising administering to the subject in need thereof, the dual vector system of any one of claims 1-11.

16. The method of claim 15, wherein the genetic disease is an autosomal dominant disease.

17. The method of claim 15, wherein the genetic disease is DFNA36 hearing loss.

18. The method of claim 15, wherein the target gene is *TMC1*.

19. The method of claim 15, wherein the administering step comprises contacting inner ear cells with the dual vector system.

20. The method of claim 19, wherein administering occurs by injecting.

moter

FIG. 1

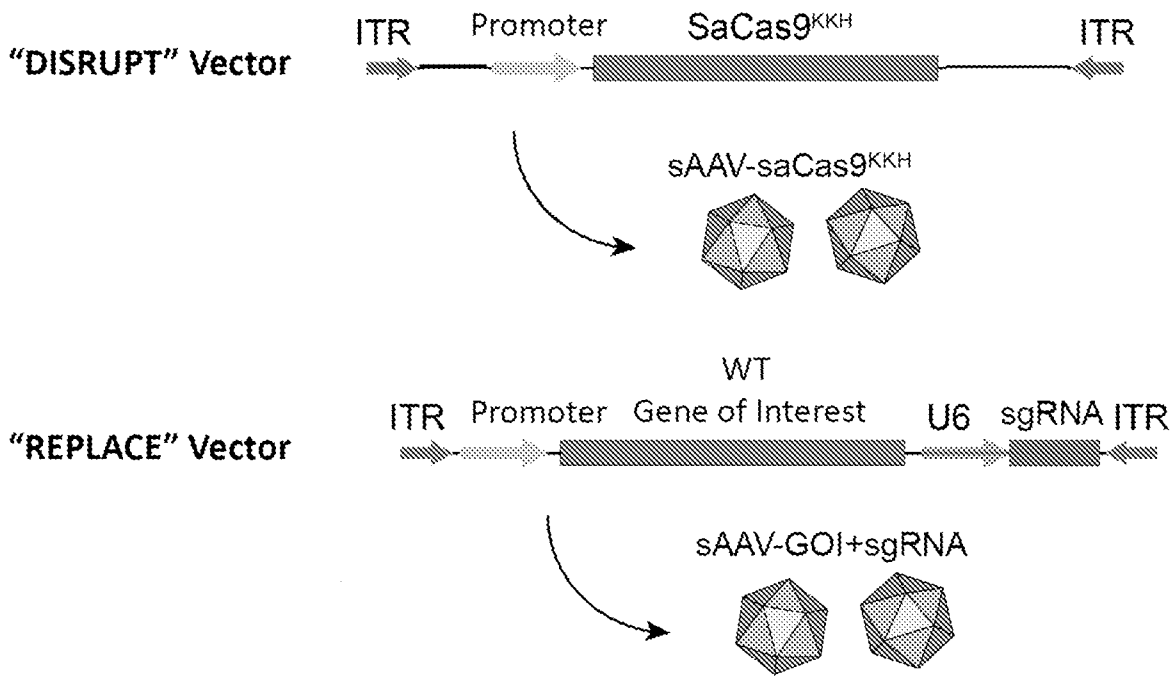
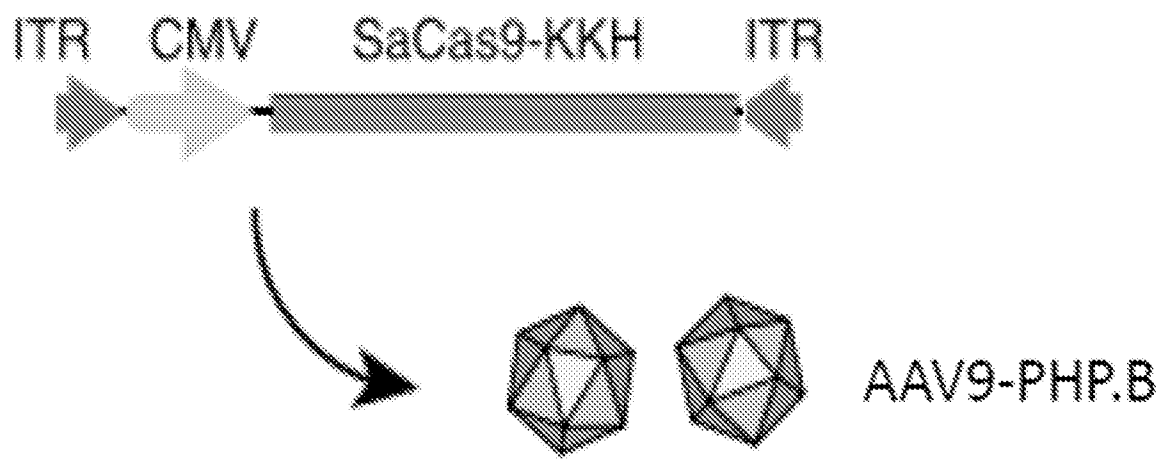

"DISRUPT" Vector
ITR
Promoter
SaCas9KKH
ITR
sAAV-saCas9KKH
WT
"REPLACE" Vector
ITR
Promoter
Gene of Interest
U6
sgRNA
ITR
sAAV-GOI+sgRNA

FIG. 2

VECTOR 1: SaCas9-KKH



VECTOR 2: MmTmc1PAMmut + gRNA Tmc1-WT (18 + 1 nt)

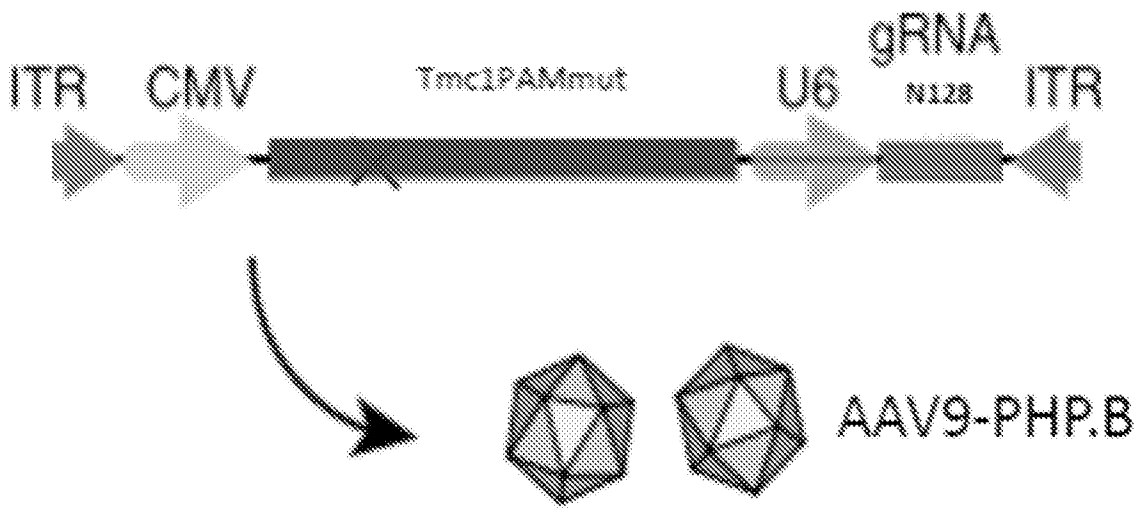


FIG. 3

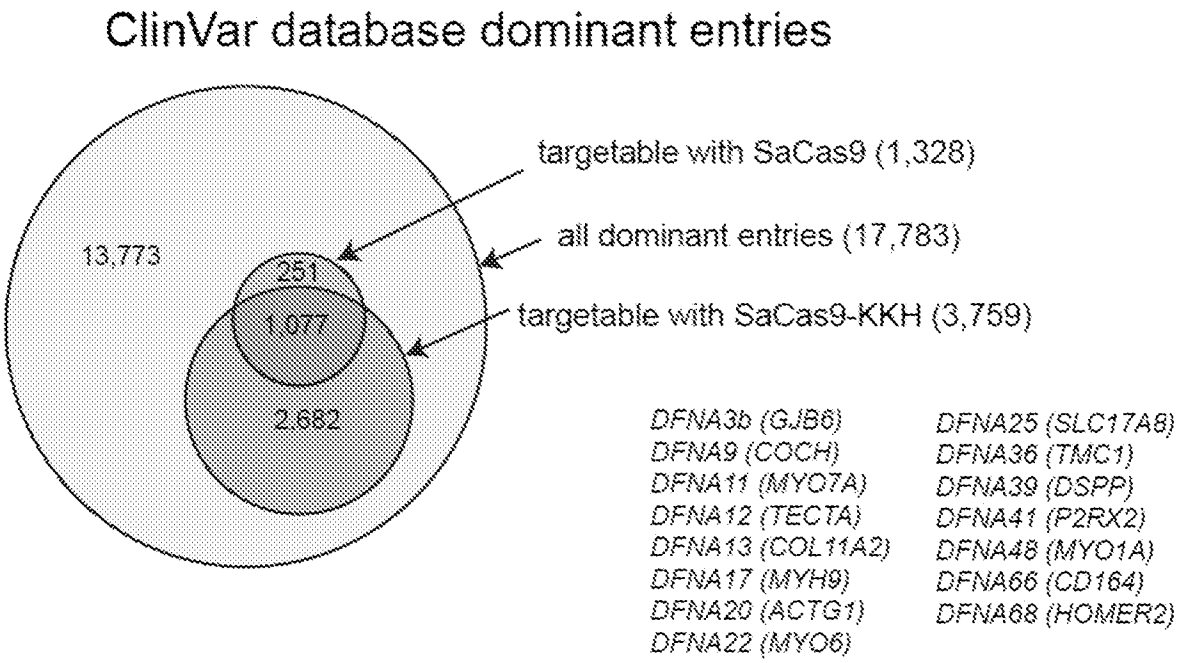

ClinVar database dominant entries
targetable with SaCas9 (1,328)
all dominant entries (17,783)
targetable with SaCas9-KKH (3,759)
13,773
251
1,077
2,682
DFNA3b (GJB6)
DFNA9 (COCH)
DFNA11 (MYO7A)
DFNA12 (TECTA)
DFNA13 (COL11A2)
DFNA17 (MYH9)
DFNA20 (ACTG1)
DFNA22 (MYO6)
DFNA25 (SLC17A8)
DFNA36 (TMC1)
DFNA39 (DSPP)
DFNA41 (P2RX2)
DFNA48 (MYO1A)
DFNA66 (CD164)
DFNA68 (HOMER2)

FIG. 4

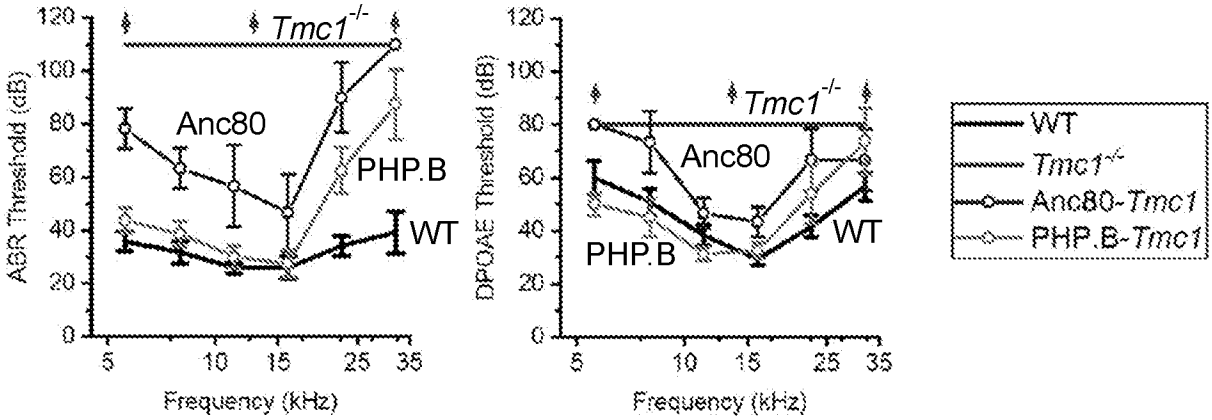
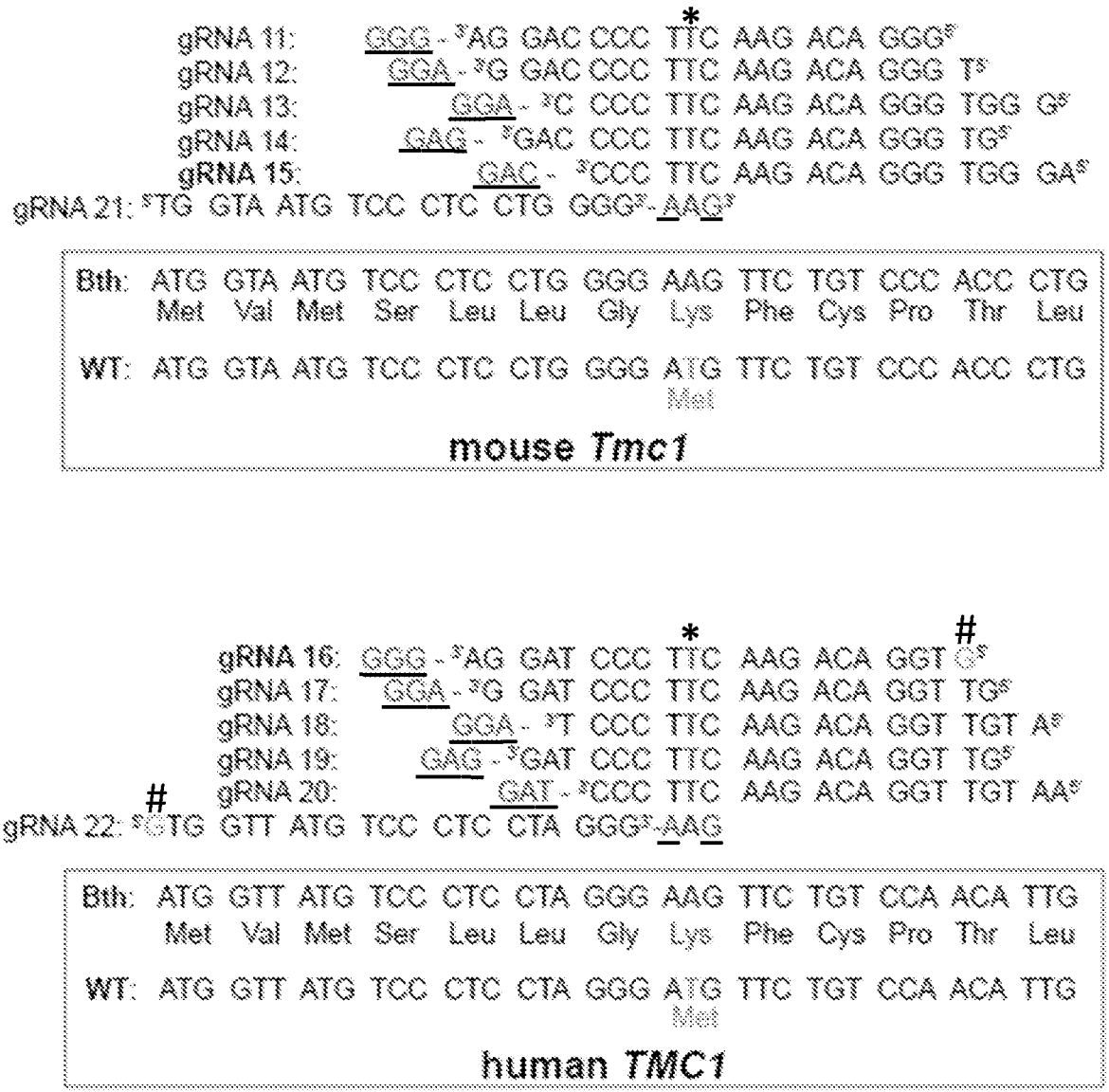

ABR Threshold (dB)
120
100
80
60
40
20
0
5
10
15
25
35
Frequency (kHz)
Tmc1-/-
Anc80
PHP.B
WT
DPOAE Threshold (dB)
Tmc1-/-
Anc80
PHP.B
WT
WT
Tmc1-/-
Anc80-Tmc1
PHP.B-Tmc1

FIG. 5A


ITR
ITR
ITR
ITR

FIG. 5B


gRNA 11: GGG - 5'AG GAC CCC TTC AAG ACA GGG 3'
gRNA 12: GGA - 5'G GAC CCC TTC AAG ACA GGG T 3'
gRNA 13: GGA - 5'C CCC TTC AAG ACA GGG TGG G 3'
gRNA 14: GAG - 5'GAC CCC TTC AAG ACA GGG TG 3'
gRNA 15: GAC - 5'CCC TTC AAG ACA GGG TGG GA 3'
gRNA 21: 5'TG GTA ATG TCC CTC CTG GGG 3' - AAG 3'
Bth: ATG GTA ATG TCC CTC CTG GGG AAG TTC TGT CCC ACC CTG
Met Val Met Ser Leu Leu Gly Lys Phe Cys Pro Thr Leu
WT: ATG GTA ATG TCC CTC CTG GGG ATG TTC TGT CCC ACC CTG
Met
mouse Tmc1

gRNA 16: GGG - 5'AG GAT CCC TTC AAG ACA GGT G 3'
gRNA 17: GGA - 5'G GAT CCC TTC AAG ACA GGT TG 3'
gRNA 18: GGA - 5'T CCC TTC AAG ACA GGT TGT A 3'
gRNA 19: GAG - 5'GAT CCC TTC AAG ACA GGT TG 3'
gRNA 20: GAT - 5'CCC TTC AAG ACA GGT TGT AA 3'
gRNA 22: 5'GTG GTT ATG TCC CTC CTA GGG 3' - AAG
Bth: ATG GTT ATG TCC CTC CTA GGG AAG TTC TGT CCA ACA TTG
Met Val Met Ser Leu Leu Gly Lys Phe Cys Pro Thr Leu
WT: ATG GTT ATG TCC CTC CTA GGG ATG TTC TGT CCA ACA TTG
Met
human TMC1

FIG. 5C

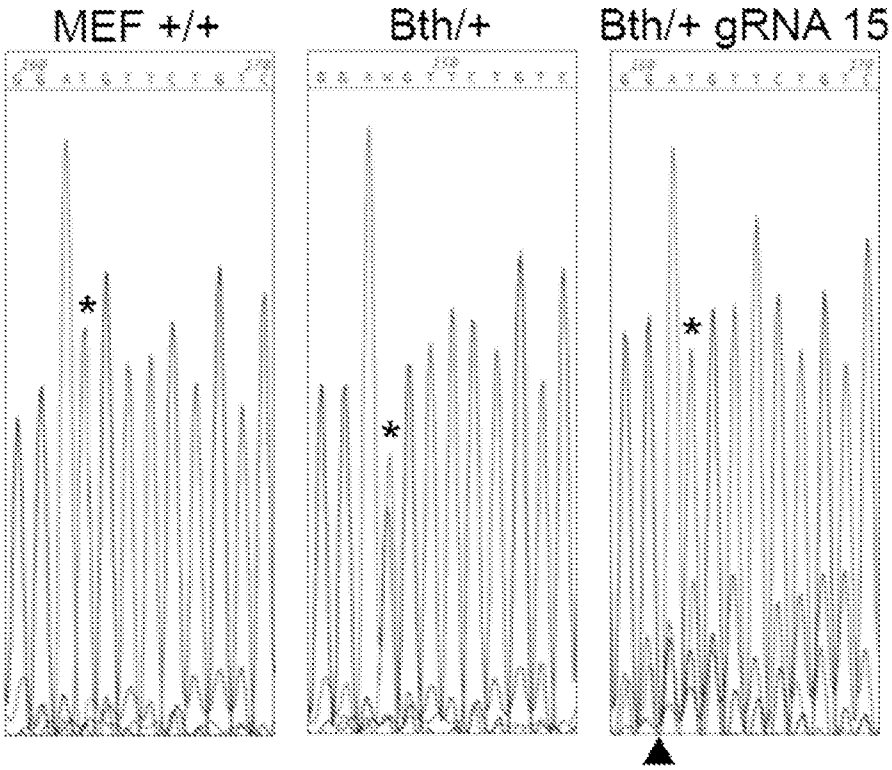

MEF +/+
Bth/+
Bth/+ gRNA 15
*
*
*

FIG. 5D

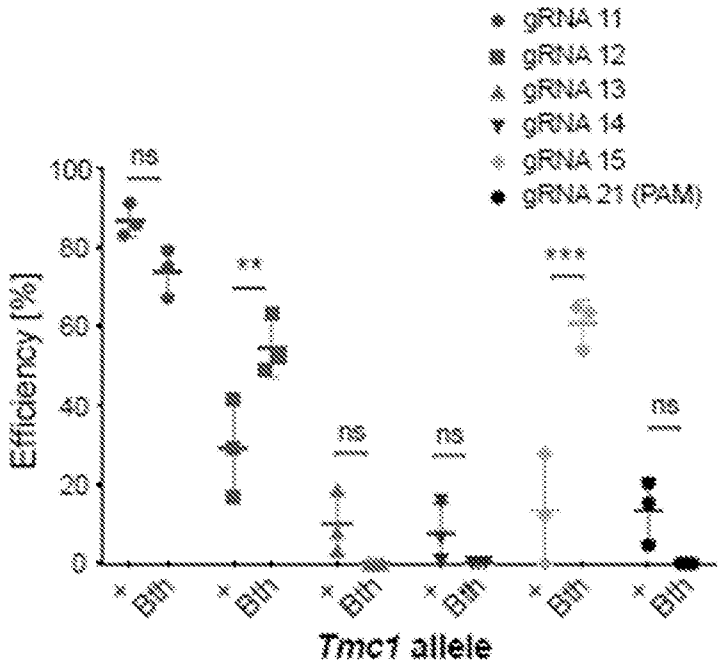

gRNA 11
gRNA 12
gRNA 13
gRNA 14
gRNA 15
gRNA 21 (PAM)
Efficiency [%]
100
80
60
40
20
0
ns
**
ns
ns
***
ns
+/+
Bth
Tmc1 allele

FIG. 5E

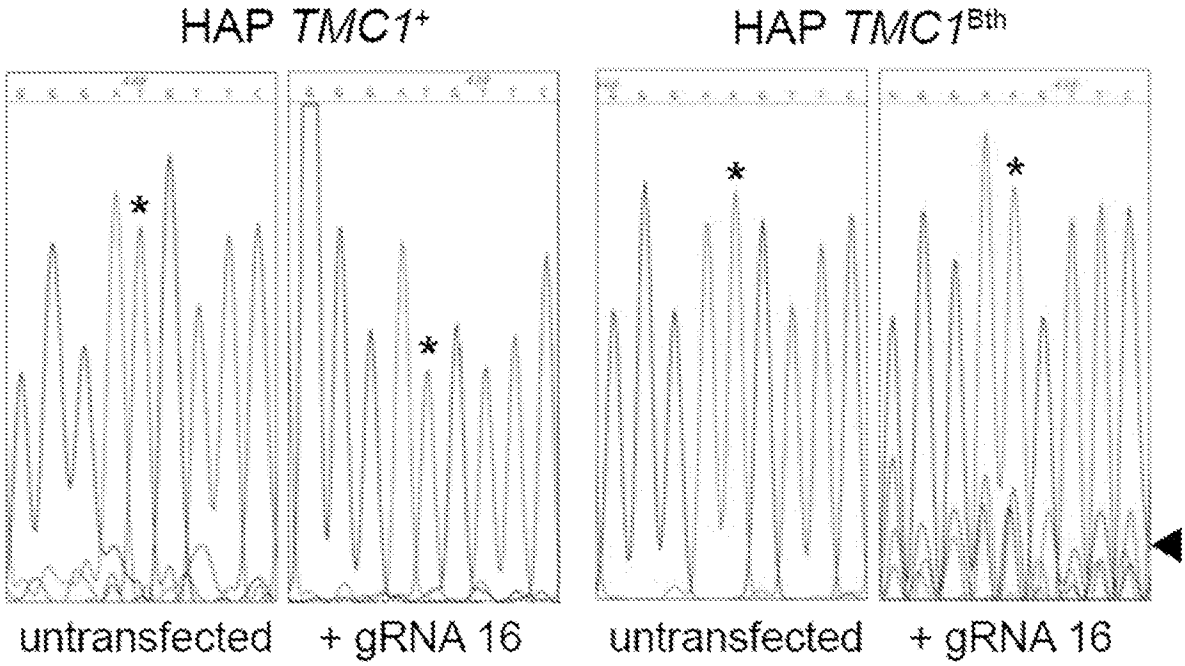

HAP TMC1+
HAP TMC1Bth
untransfected
+ gRNA 16
untransfected
+ gRNA 16

FIG. 5F

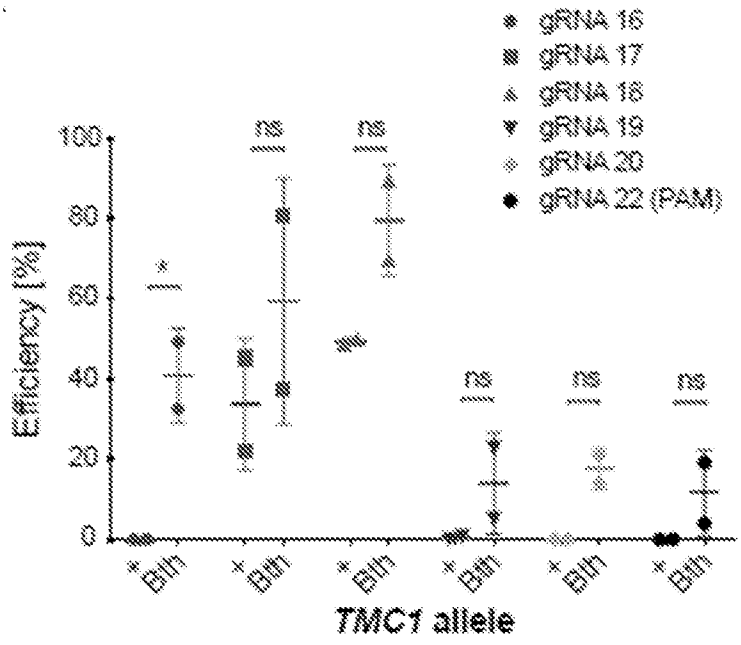

gRNA 16
gRNA 17
gRNA 18
gRNA 19
gRNA 20
gRNA 22 (PAM)
Efficiency [%]
100
80
60
40
20
0
ns
ns
ns
ns
ns
ns
+
Bth
TMC1 allele

FIG. 6

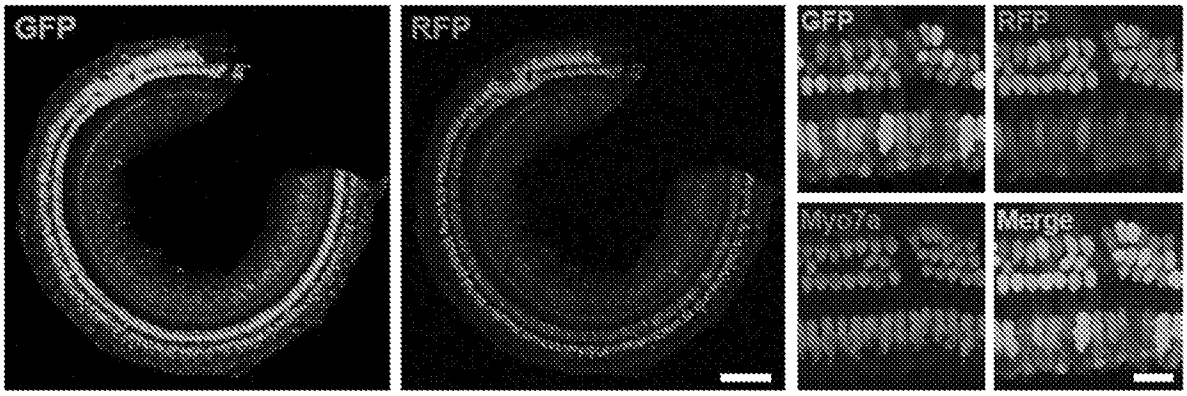

GFP
RFP
GFP
RFP
Myo7a
Merge

FIG. 7A

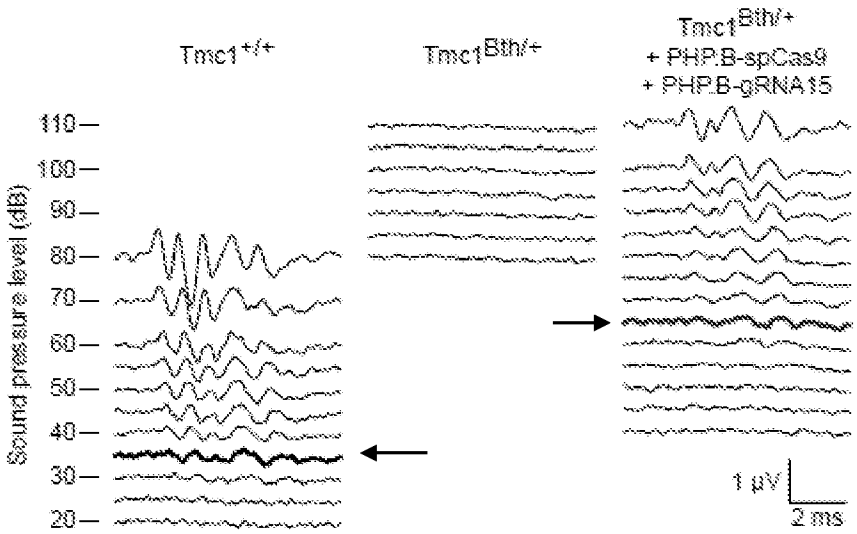

Tmc1+/+
Tmc1Bth/+
Tmc1Bth/+
+ PHP.B-spCas9
+ PHP.B-gRNA15
Sound pressure level (dB)
110
100
90
80
70
60
50
40
30
20
1 μV
2 ms

FIG. 7B

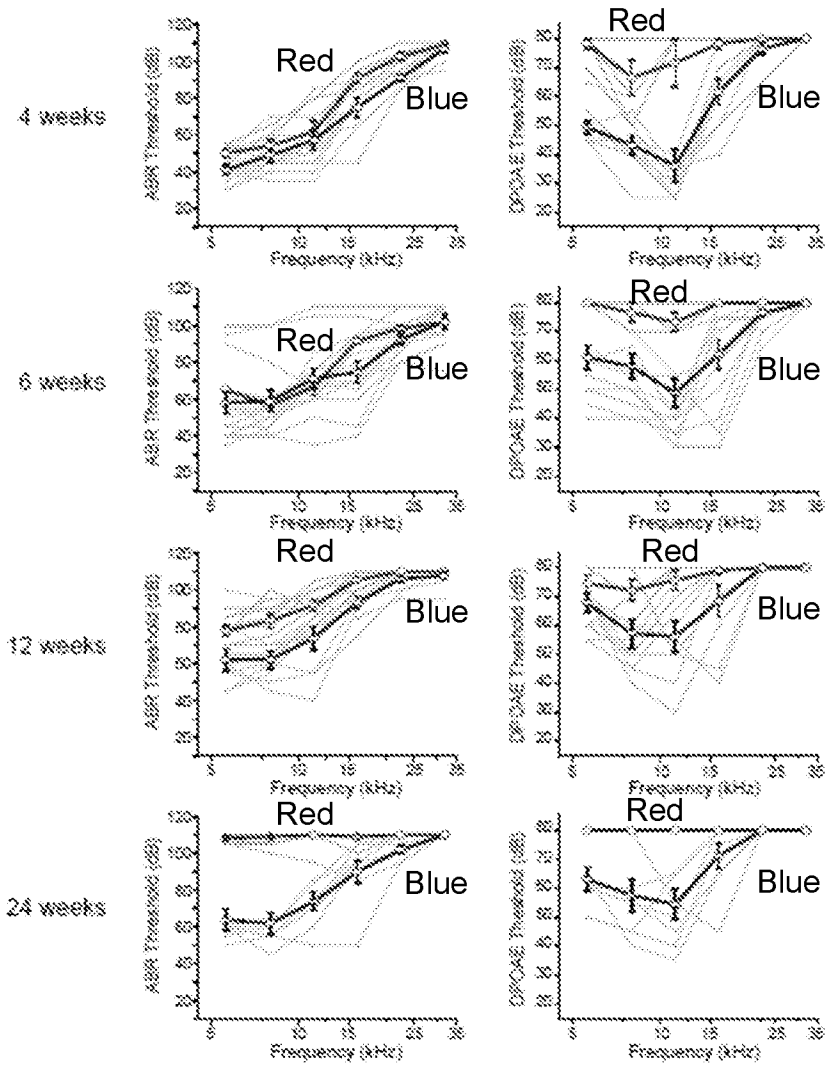


FIG. 7C

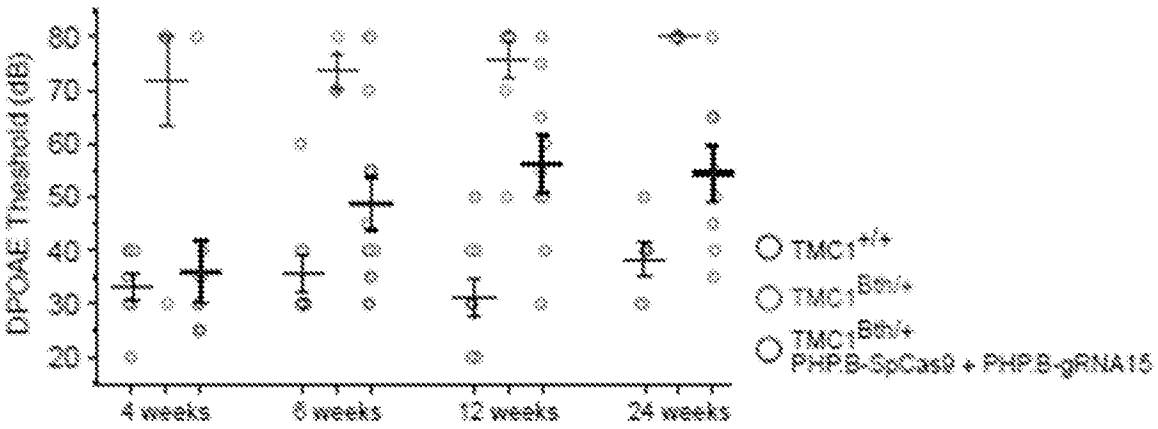

DPOAE Threshold (dB)
80
70
60
50
40
30
20
4 weeks
6 weeks
12 weeks
24 weeks
TMC1+/+
TMC1Bth/+
TMC1Bth/+
PHP.B-SpCas9 + PHP.B-gRNA15

FIG. 8A

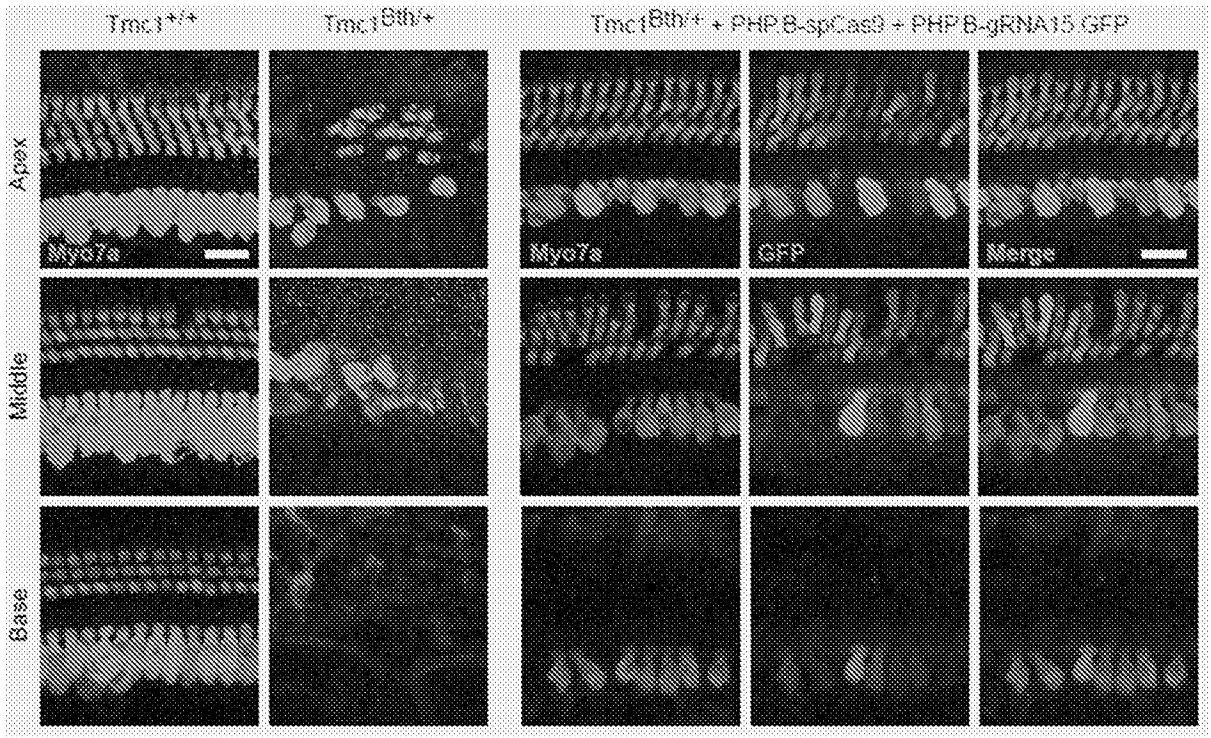

Tmc1+/+
Tmc1Bth/+
Tmc1Bth/+ + PHP.B-spCas9 + PHP.B-gRNA15 GFP
Apex
Middle
Base
Myo7a
Myo7a
GFP
Merge

FIG. 8B

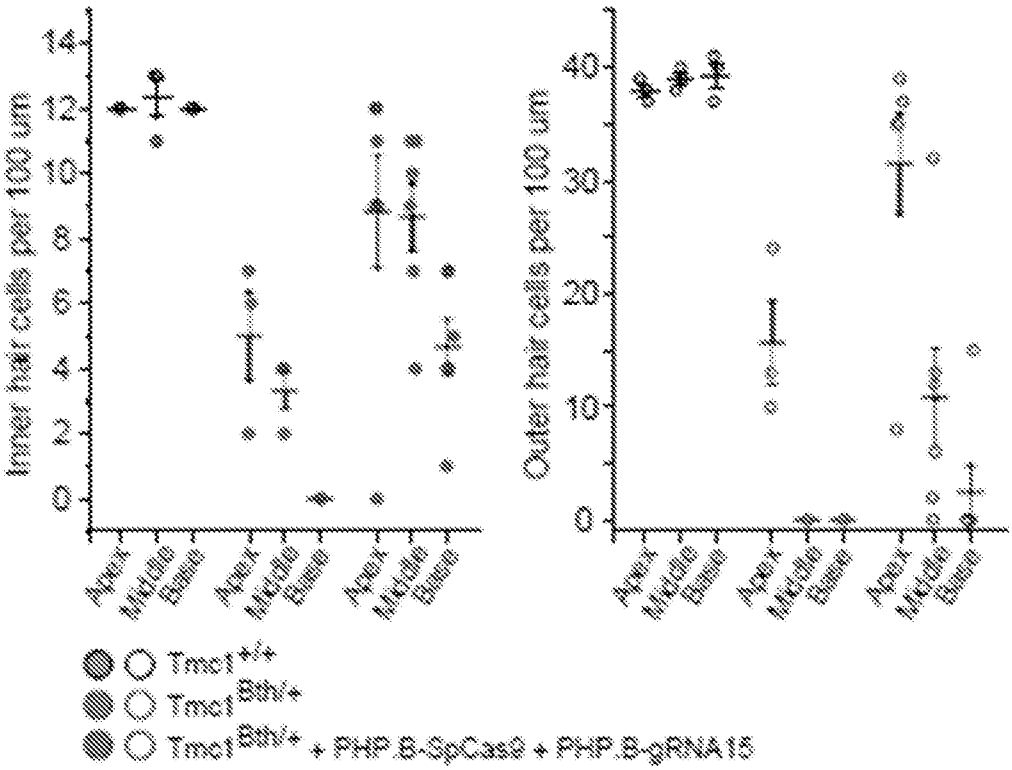

Inner hair cells per 100 um
Outer hair cells per 100 um
Apex
Mid-low
Base
Tmc1+/+
Tmc1Bth/+
Tmc1Bth/+ + PHP.B-SpCas9 + PHP.B-gRNA15

FIG. 8C

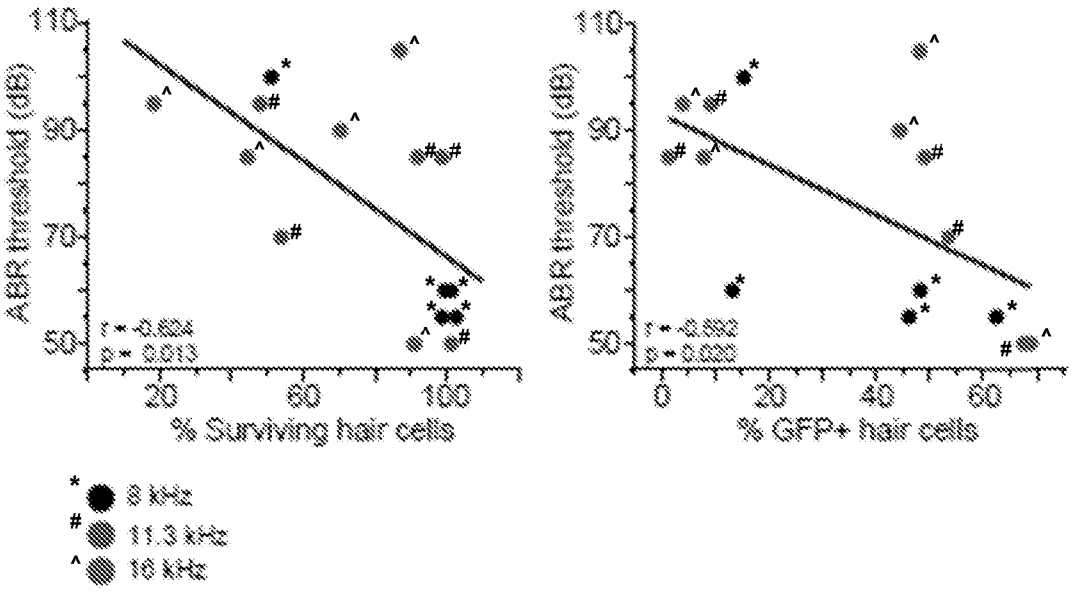

ABR threshold (dB)
% Surviving hair cells
% GFP+ hair cells
8 kHz
11.3 kHz
16 kHz

FIG. 9A

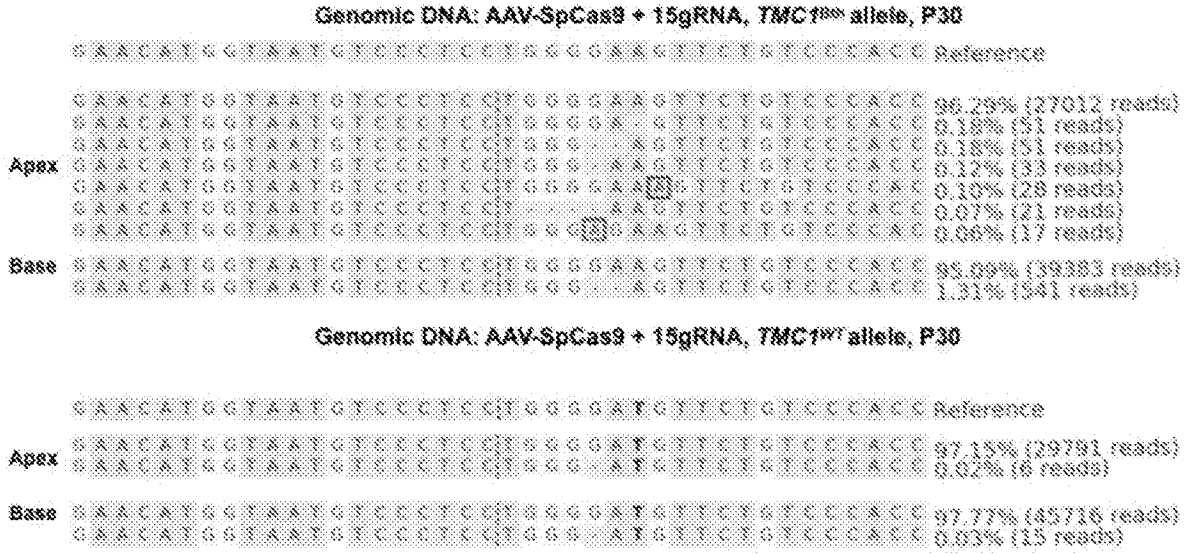
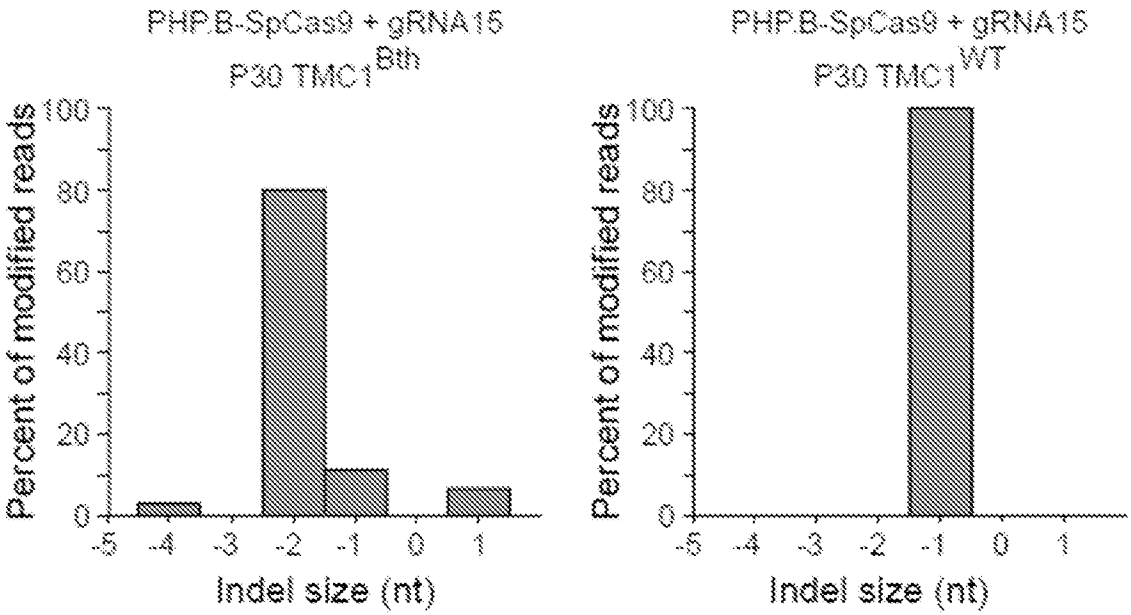

Genomic DNA: AAV-SpCas9 + 15gRNA, TMC1Bth allele, P30
Reference
Apex
96.29% (27012 reads)
0.18% (51 reads)
0.18% (51 reads)
0.12% (33 reads)
0.10% (28 reads)
0.07% (21 reads)
0.06% (17 reads)
Base
95.69% (39383 reads)
1.31% (541 reads)
Genomic DNA: AAV-SpCas9 + 15gRNA, TMC1WT allele, P30
Reference
Apex
97.15% (29791 reads)
0.02% (6 reads)
Base
97.77% (45716 reads)
0.03% (15 reads)

FIG. 9B


PHP.B-SpCas9 + gRNA15
P30 TMC1Bth
Percent of modified reads
Indel size (nt)
PHP.B-SpCas9 + gRNA15
P30 TMC1WT
Percent of modified reads
Indel size (nt)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/040608

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 9/16; C12N 9/22; C12N 9/78; C12N 9/99 (2020.01)
CPC - C07K 2319/71; C07K 2319/80; C12N 15/102; C12N 15/111; C12N 15/63 (2020.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

see Search History document

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

see Search History document

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

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/0073674 A1 (EDITAS MEDICINE, INC.) 16 March 2017 (16.03.2017) entire document	1-20
Y	US 2018/0282714 A1 (THE GENERAL HOSPITAL CORPORATION) 04 October 2018 (04.10.2018) entire document	1-20
Y	GAO et al. "Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents," Nature, 20 December 2017 (20.12.2017), Vol. 553, Iss. 7687, Pgs. 217-221. entire document	5, 10, 11, 16, 17
Y	US 9,758,781 B2 (SMITH et al) 12 September 2017 (12.09.2017) entire document	7, 10, 11, 18
Y	US 2017/0321214 A1 (THE BROAD INSTITUTE INC. et al) 09 November 2017 (09.11.2017) entire document	9, 11
Y	GYORGY et al. "Gene Transfer with AAV9-PHP.B Rescues Hearing in a Mouse Model of Usher Syndrome 3A and Transduces Hair Cells in a Non-human Primate," Molecular Therapy Methods & Clinical Development, 19 November 2018 (19.11.2018), Vol. 13, Pgs. 1-13. entire document	10, 11
P, X	GYORGY et al. "Allele-specific gene editing prevents deafness in a model of dominant progressive hearing loss," Nature Medicine, 03 July 2019 (03.07.2019), Vol. 25, Iss. 7, Pgs. 1123-1130. entire document	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2020	23 NOV 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver Telephone No. PCT Helpdesk: 571-272-4300