A synthetic AAV vector enables safe and efficient gene transfer to the mammalian inner ear

Lukas D Landegger1–3,12, Bifeng Pan2,4,12, Charles Askew2,4,11,12, Sarah J Wassmer5,6, Sarah D Gluck2,4,7, Alice Galvin4, Ruth Taylor6, Andrew Forge6, Konstantina M Stankovic1,2,7,13, Jeffrey R Holt2,4,9,13 & Luk H Vandenberghe5,6,10,13

Efforts to develop gene therapies for hearing loss have been hampered by the lack of safe, efficient, and clinically relevant delivery modalities1,2. Here we demonstrate the safety and efficiency of Anc80L65, a rationally designed synthetic vector3, for transgene delivery to the mouse cochlea. Ex vivo transduction of mouse organotypic explants identified Anc80L65 from a set of other adeno-associated virus (AAV) vectors as a potent vector for the cochlear cell targets. Round window membrane injection resulted in highly efficient transduction of inner and outer hair cells in mice, a substantial improvement over conventional AAV vectors. Anc80L65 round window injection was well tolerated, as indicated by sensory cell function, hearing and vestibular function, and immunologic parameters. The ability of Anc80L65 to target outer hair cells at high rates, a requirement for restoration of complex auditory function, may enable future gene therapies for hearing and balance disorders.

Hearing loss is the most common sensory disorder worldwide, with half of prelingual deafness due to genetic causes4. More than 300 genetic loci linked to hereditary hearing loss and >100 causative genes have been identified4,5. Age-related hearing impairment affects the quality of life of over a quarter of individuals >65 years old. While medication history and noise exposure are known contributing factors to presbycusis, several genetic factors have been identified6. Sensory cells of the adult mammalian cochlea lack the capacity for self-renewal7,8. Current therapies for hearing loss employ various strategies depending on the level and exact position of impairment, including sound amplification (hearing aids), enhanced sound transduction (middle ear prostheses/active implants), and direct neuronal stimulation (cochlear implants) to compensate for permanent damage to primary sensory hair cells or to spiral ganglion neurons, which form the auditory nerve and relay acoustic information to the brain2.

These opportunities, while potentially transformative, remain far from optimal in restoring complex hearing function and may have deficiencies in frequency sensitivity, natural sound perception, and speech discrimination in noisy environments.

Therapeutic gene transfer to the cochlea could improve on the current standard of care for both genetic1,2,9–13 and age-related or environmentally induced hearing loss1,2,12,13. This approach would require the development of methods for safe, efficient delivery of transgene constructs to the relevant cell types in the organ of Corti in the cochlea. The organ of Corti includes two classes of sensory hair cells: inner hair cells (IHCs), which convert mechanical information carried by sound into electrical signals transmitted to neuronal structures, and outer hair cells (OHCs), which amplify and tune the cochlear response, a process required for complex hearing function14. Other potential targets in the inner ear include spiral ganglion neurons, columnar cells of the spiral limbus, which are important for the maintenance of the adjacent tectorial membrane15–17, and supporting cells, which have protective functions and can be triggered to transdifferentiate into hair cells up until an early neonatal stage18–21.

Direct access to hair cells for gene therapy may be achievable through vector injection into the cochlear duct. However, interventions that alter the delicate high-potassium endolymph fluid in the duct could disrupt the endocochlear potential, leading to damage of the sensory cells and irreversible hearing loss. The perilymph-filled spaces surrounding the cochlear duct, scala tympani, and scala vestibuli can be accessed from the middle ear, either through the oval window membrane or the round window membrane (RWM). The RWM, which is the only non-bony opening into the inner ear, is relatively easy to access in many animal models, and administration of viral vector using this route has been well tolerated10,11,22.

In humans, cochlear implant placement routinely relies on surgical electrode insertion through the RWM23.

1Eaton Peabody Laboratories, Department of Otolaryngology, Massachusetts Eye and Ear, Boston, Massachusetts, USA. 2Department of Otolaryngology, Harvard Medical School, Boston, Massachusetts, USA. 3Department of Otolaryngology, Vienna General Hospital, Medical University of Vienna, Vienna, Austria. 4Department of Otolaryngology, F.M. Kirby Neurobiology Center, Boston Children’s Hospital, Boston, Massachusetts, USA. 5Grousbeck Gene Therapy Center, Schepps Eye Research Institute and Massachusetts Eye and Ear, Boston, Massachusetts, USA. 6Ocular Genomics Institute, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, USA. 7Harvard Program in Speech and Hearing Bioscience and Technology, Boston, Massachusetts, USA. 8Center for Auditory Research, UCL Ear Institute, London, UK. 9Department of Neurology, Boston Children’s Hospital and Harvard Medical School, Boston, Massachusetts, USA. 10Harvard Stem Cell Institute, Harvard University, Cambridge, Massachusetts, USA. 11Present address: Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. 12These authors contributed equally to this work. 13These authors jointly directed this work. Correspondence should be addressed to L.H.V. (luk_vandenberghe@meei.harvard.edu), J.R.H. (jeffrey.holt@childrens.harvard.edu), or K.M.S. (konstantina_stankovic@meei.harvard.edu).

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Several non-viral\textsuperscript{24,25} and viral (e.g., adenovirus, AAV, lentivirus, herpes simplex virus I, vaccinia virus) gene transfer vectors have been tested in the cochlea, often with only transient or suboptimal gene transfer as a result\textsuperscript{1}. Only adenovirus has progressed to a clinical program (NCT02132130)\textsuperscript{2,26}. For other target organs, such as the liver and the retina, AAV has shown clinical efficacy and safety in hemophilia B\textsuperscript{27}, two types of inherited blindness\textsuperscript{28,29}, and familial lipoprotein lipase deficiency\textsuperscript{30}. Previous studies of AAV serotypes for in vivo inner ear injection via different routes of administration highlighted the difficulties in targeting OHCs, particularly via RWM injection\textsuperscript{31}, and resulted in only partial correction of hearing in mouse models of inherited deafness\textsuperscript{9–11}. To identify vectors that target both IHCs and OHCs with high efficiency, we evaluated natural AAVs alongside a recently developed synthetic AAV called Anc80L65. This IHCs and OHCs with high efficiency, we evaluated natural AAVs an

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\caption{Transduction of organotypic explants of murine cochlea with natural AAV serotypes and Anc80L65. Representative confocal projections of an in vitro comparison of several AAV serotypes for eGFP transgene expression in cochlear explants of C57BL/6 mice. (a–g) Expression at the cochlear base for all serotypes, and apex and base for Anc80L65 after incubation with $10^{15}$ genome-containing (GC) particles for 48 h. Scale bar, 100 μm. Top: Myo7A+TuJ1, bottom: eGFP only, middle: overlay. S. cells, supporting cells; OHC, outer hair cell; IHC, inner hair cell. (h–k) Percentage of eGFP-positive hair cells per 100 μm after 48 h or 48 h + 5 d of incubation. Mean indicated by horizontal bar. Each condition had at least $N = 3$ for 48 h, and $N = 2$ for 48 h + 5 d, unless otherwise noted.}
\end{figure}

In a first selection, we incubated iodixanol-purified high-titer preparations of single-stranded AAV1, 2, 6, 8, 9, and Anc80L65 encoding eGFP driven from the cytomegalovirus immediate-early (CMV) promoter, at equal doses of $10^{15}$ genome-containing (GC) particles, with organotypic cochlear explants from C57BL/6 or CBA/CaJ mice harvested at postnatal day (P) P4. We performed histology of the inoculated cochleas after 2 d of vector exposure. eGFP expression was qualitatively brighter in cochlear cultures exposed to Anc80L65, with expression apparent in many cochlear cell types (Fig. 1 and Supplementary Fig. 1). Morphometric quantitative analysis was performed to determine transduction rates for IHCs, OHCs, supporting cells, limbus cells, and spiral ganglion neurons. For IHCs and OHCs, vector transduction efficiency was quantified as the percentage of eGFP-positive cells in representative 100-μm sections taken from the basal and apical regions of the cochlea. Anc80L65 targeted IHCs and OHCs at efficiencies of 60–100% on average in apical and basal regions of both mouse strains tested (Fig. 1h and Supplementary Fig. 1g, h). Anc80L65 showed consistently and qualitatively brighter IHC and OHC eGFP expression as compared to AAV2 (Fig. 1 and Supplementary Fig. 1).

To control for possible differences between the AAV serotypes in the onset of transgene expression, which could lead to an underestimation of expression at the 2-d time point, we repeated the above experiment with a new set of cochleas and maintained the culture for an additional 5 d (referred to as 48 h + 5 d). A similar pattern of expression in IHCs and OHCs was observed in this longer-term study for AAV2 and Anc80L65 (Fig. 1j and Supplementary Fig. 1). Moderate increases in expression for AAV6, 8, and 9 in CBA/CaJ mice, particularly at the basal turn (Fig. 1k, and Supplementary Fig. 1), were noted. Other cell types were targeted by all serotypes, with limbus being more permissive than supporting cells, followed by spiral ganglion neurons (Supplementary Figs. 2–4). Consistently, Anc80L65 transduction yielded higher efficiencies and stronger expression, evidenced by brighter eGFP fluorescence (Fig. 1 and Supplementary Figs. 1–4).

Next, we evaluated the tropism and gene transfer efficiency of AAV1, 2, 6, 8, and Anc80L65 in vivo, using a protocol that is therapeutically relevant with respect to vector pharmacokinetics, anatomical and cellular barriers to transduction, and surgical approach. C57BL/6 animals were injected at P1 and cochleas were harvested, fixed and stained at P10 (Fig. 2). Consistent with prior reports\textsuperscript{9–11,32}, AAV1 transduced IHCs with moderate to high efficiency (Fig. 2a, b). Our results indicated that AAV2, 6, and 8 target low numbers of IHCs (Fig. 2a, b). Also, consistent with prior reports, transduction of OHCs was minimal (<5%) for all conventional AAV serotypes tested. In contrast, Anc80L65 transduced nearly 100% of IHCs and ~90% of OHCs (Fig. 2a–c) at a 20-fold (for AAV1) to threefold (for AAV2) lower dose. Transduction at equal doses of $1.36 \times 10^{12}$ GC for all serotypes resulted in substantial IHC and OHC transduction for Anc80L65, but minimal IHC targeting for AAV1, 2, and 8, and none
Figure 2  In vivo cochlear transduction of natural AAV serotypes and Anc80L65. (a) Confocal images of mouse organs of Corti, counterstained with Alexa546-phalloidin (red) and imaged for eGFP (green). A total of 5 (AAV1), 4 (AAV2), 2 (AAV8), 1 (AAV6), and 3 (Anc80L65) C57BL/6 mice were injected with 1 μL of AAV stock solution in one ear at the titer indicated above each panel. GC, genome-containing (particles). Scale bar, 50 μm. (b,c) Quantification of eGFP-positive IHCs (b) and OHCs (c) in the base and apex of a representative AAV-eGFP-injected cochlea for each vector. (d) Families of sensory transduction currents recorded at P7 (left) from eGFP-negative OHCs (black) and eGFP-positive OHCs (green). Hair bundles were deflected between −0.1 and 1 μm in 0.1-μm increments. Vertical scale bar, 200 pA; horizontal scale bar, 20 msec. Currents from eGFP-negative (black) and eGFP-positive (green) P35 IHCs are shown on the right. Vertical scale bar, 100 pA; horizontal scale bar, 20 msec. (e) Sensory transduction current amplitudes plotted for 102 IHCs and OHCs at the ages indicated at the bottom. Data from eGFP-negative (black) and eGFP-positive (green) are shown. The number of cells in each group are shown on the graph. All mice were injected at P1. (f) ABR thresholds plotted for four Anc80L65-injected ears (green) and four uninjected ears (black) together with data from one injected ear that had no eGFP fluorescence owing to injection-related damage (red). Error bars, mean ± s.d. (g) DPOAE thresholds are plotted for four Anc80L65-injected ears (green) and four uninjected ears (black) and one negative control ear with injection damage without eGFP fluorescence (red). Error bars, mean ± s.d. Injection titers for data points in b–g are as in a.
Since the perilymphatic solutions of the cochlea are continuous with those of the vestibular labyrinth, we wondered whether Anc80L65–eGFP injected via the cochlear RWM would transduce vestibular sensory organs. Indeed, whole-mounts of vestibular epithelia revealed robust eGFP expression in both type I and type II hair cells of the utricle, a vestibular organ sensitive to gravity and linear head movements, and in the semicircular canals, which are sensitive to rotational head movements (Fig. 4a). The sensory epithelium of a human utricle was exposed to 10^{10} GC Anc80L65-CMV.eGFP.WPRE for 24 h, cultured for 10 d, fixed, stained with Alexa546-phalloidin (red) and imaged for eGFP fluorescence (green). Scale bar, 100 μm. (b) The crista of the posterior semicircular canal from the same mouse described for panel a. Scale bar, 50 μm. (c) The sensory epithelium of a human utricle. The tissue was exposed to 10^{10} GC Anc80L65.CMV.eGFP.WPRE for 24 h, cultured for 10 d, fixed, stained with Alexa546-phalloidin (red) and imaged for eGFP fluorescence (green). Scale bar, 100 μm. (d) High-magnification view of a human epithelium in the utricle stained with Alexa546-phalloidin (red) and Myo7A (blue) and imaged for eGFP (green) transduced in identical conditions as in c. White arrows in the overlay panel indicate selected eGFP-positive/Myo7A-positive cells. Scale bar, 20 μm.

Anc80L65 may provide a valuable approach for gene delivery to human IHCs and OHCs, as well as to other inner ear cell types that are compromised by genetic hearing and balance disorders. Previous work has shown that Anc80L65 has an analogous safety profile in mouse and nonhuman primate after systemic injection, and is antigenically distinct from circulating AAVs, providing a potential benefit in terms of pre-existing immunity, which limits the efficacy of conventional AAV vectors.

Further validation of Anc80L65 as a gene transfer vector for use in human inner ear gene therapy will require targeting-efficiency studies in large-animal models; additional exploration of the window of opportunity for therapeutic intervention; and pharmacology and toxicology studies to investigate the safety of Anc80L65 upon cochlear administration. Given the promiscuity of expression of AAV, including Anc80L65, additional methods to maximize specificity and minimize biodistribution should be considered to limit expression outside of the therapeutic cochlear cell target. Considering that nonsyndromic auditory and vestibular dysfunction can be caused by dominant or recessive mutations in >100 genes, Anc80L65 may accelerate the development of novel gene therapy strategies for a wide range of inner ear disorders.

METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
L.D.L., R.P., C.A., S.W.D., and A.G. conducted, analyzed, and reported the mouse gene transfer experiments described, and R.T. and A.F. performed and led the human vestibular transduction experiments. L.H.V. provided vector materials. K.M.S., J.R.H., and L.H.V. conceived the study, led experimental design and oversaw analysis, J.R.H., L.H.V., and L.D.L. drafted the manuscript with topical input from all other authors.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Animal models and general methods. All experiments were approved by the respective Institutional Animal Care and Use Committees at Massachusetts Eye and Ear (protocol #15-003) and Boston Children’s Hospital (protocol #12-02-2146) as well as the Institutional Biosafety Committee (protocol #BC-000000447). Wild-type C57BL/6 and CBA/Ca mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and animals of either sex were used for experimentation in an estimated 50:50 ratio. Group sizes per experiment for the in vitro and in vivo transduction assays and subsequent endpoints were determined by access to specimen and technical feasibility. Reported observations on Anc80L65 transduction were qualitatively validated in subsequent experiments with various vector lots (except for the human vestibular tissue transduction due to the unique and limited nature of access to specimen). No statistical analysis between serotype transduction efficiencies was performed due to the limited access to specimen and qualitative nature of the reported findings.

Viral vectors. AAV2/1, 2/2, 2/6, 2/8, 2/9, and AAV2/Anc80L65 with a CMV-driven eGFP transgene and the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) cassette were prepared at Gene Transfer Vector Core (vector.mei.harvard.edu) at Massachusetts Eye and Ear as previously described by HEK293 transfection. We confirmed the identity of HEK293 cells (originally obtained from ATCC) morphologically, and we ensured that the cells remained mycoplasma free through regular testing (Bionique, Saranac Lake, NY, USA). AAV2/Anc80L65 plasmid reagents are available through http://www.addgene.com.

In vitro explant cultures. A total of 156 cochlear explant cultures from mouse pups of both strains were prepared on postnatal day 4 (ref 34). In brief, murine temporal bones were harvested after decapitation and the cochlea was dissected to culture as organotypic explants connected to the spiral ganglion neuron region. Two specimens were obtained per cochlea, one (“apical”) consisting of the lower apical and one (“basal”) of the upper basal turn. For each serotype, a minimum of 3 (CBA/Ca, 48 h), 2 (CBA/Ca, 48 h + 5 d), 3 (C57BL/6, 48 h), 2 (C57BL/6, 48 h + 5 d) basal and apical specimens were inoculated (unless otherwise noted). Specimens were excluded if cochlear morphology was not retained during the culture. Sample numbers were chosen to inform on the variability of transduction and to provide a basis for selection for further in vivo evaluation. Explants were incubated with culture medium (98% Dulbecco’s Modified Eagle Medium (DMEM), 1% ampicillin, and 1% N2 supplement during the first 12 h, plus 1% FBS) and 10× GC AAV for 48 h in 50 µL. For the 48 h + 5 d condition, the medium with AAV was replaced with fresh media without AAV for an additional 5 d.

Human vestibular epithelia from utricles obtained from four consented, adult patients undergoing vestibular schwannoma tumor resection were cultured as previously described, exposed to 10× GC AAV for 24 h, and subsequently were standardized with additional control PBS before the start of the experiment. After obtaining the blood sample in a 1.1 mL Z-Gel micro tube (Sarstedt, Nümbrecht, Germany), it was spun down at 8,000 r.p.m. for 8 min and serum was stored together with the CSF sample (in PBS) at −80 °C until further use.

Hearing tests. Auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) data were collected as described previously. Stimuli tested in anesthetized mice varied between 10 and 90 dB sound pressure level at frequencies of 5.6, 8, 11.3, 16, 22.6, and 32 kHz. Four Anc80L65 injected ears, four uninjected ears, and one negative control ear with injection damage without eGFP fluorescence were analyzed at P28–P30.

Cerebrospinal fluid and blood sampling. Cerebrospinal fluid (CSF) sampling from the cisterna magna and intracardiac blood collection with thoracotomy were performed in a terminal procedure. Through the microcapillary tube, the maximum amount (up to 5 µL) of clear CSF per animal was collected in a volume of 60 µL PBS, leading to slightly different starting dilutions that subsequently were standardized with additional control PBS before the start of the experiment. After obtaining the blood sample in a 1.1 mL Z-Gel micro tube (Sarstedt, Nümbrecht, Germany), it was spun down at 8,000 r.p.m. for 8 min and serum was stored together with the CSF sample (in PBS) at −80 °C until further use.

Histological analysis. After a follow-up period of 5 to 29 d, animals were euthanized and cochlear whole-mounts were prepared as previously reported. Both cochlear whole-mounts and explants were stained with antibodies against myosin 7A (Myo7A, #25-6790 Proteus Biosciences, Ramona, CA, 1:400) and β-tubulin (Tuj1, #MMS-435P BioLegend, San Diego, CA, 1:200), together with corresponding secondary antibodies (Alexa Fluor 555 anti-mouse and Alexa Fluor 647 anti-rabbit, #A-21422 and #A-21245 Thermo Fisher Scientific, Waltham, MA, 1:1,000). Mounting of the specimens was followed by confocal microscopy. Every image of a given experimental series was obtained with the same settings, with laser intensity being chosen based on the specimen with the strongest eGFP signal to prevent fluorescence saturation. Z-stacks for overview images and zoomed-in pictures for the organ of Corti and spiral ganglion neuron areas were obtained. Three-dimensional reconstruction with Amira software was used to determine spiral ganglion neuron transduction more accurately. Staining for phalloidin was performed with Thermofisher Scientific A22283 antibody at a 1:200 dilution.

Quantification of eGFP-expression. For in vitro data, the percentage of eGFP-positive IHCs and OHCs was manually quantified along the cochlea, by dividing the number of eGFP-positive cells by the total number of outer or inner hair cells per one or two 100-µm sections per basal and apical sample for each specimen. All visible spiral ganglion neurons in a cochlear explant were evaluated regarding their eGFP expression. The areas of the spiral limbus and supporting cells were assessed with a qualitative approach (as explained above, adjusted for each experimental series) by means of a scale from 0 (no expression) to 3 (strongest signal). Control samples without AAV were used to exclude autofluorescence.

Immunological assays. Antibody titers against Anc80L65 in CSF and serum were determined through neutralization assays. Using a 96-well format, heat-inactivated CSF or serum samples (collected as described above) were serially diluted in serum-free medium (Life Technologies, Carlsbad, CA), and then treated with Anc80L65-luciferase (106 GC/well) for 1 h at 37 °C.
The sample/Anc80L65-luciferase mix was then transferred onto HEK293 cells, which were treated with adenovirus (MOI 20) the day before. After 1 h at 37 °C, diluted serum medium (1 part serum-free, 2 parts with serum) was added to each well. Two days later, the cells were treated with lysis buffer (Promega, Madison, WI) and frozen at ~80 °C for 30 min. The cells were then thawed at 37 °C for 15 min before being treated with substrate buffer (Tri-s-HCl, MgCl₂, ATP (Life Technologies, Carlsbad, CA), β-luciferin (Caliper Life Sciences, Hopkinton, MA)). Luminescence output was read using the Synergy BioTek Plate Reader (BioTek, Winooski, VT).

**Hair cell electrophysiology.** Cochleas were excised, mounted on glass coverslips and viewed on an Axio Examiner.A1 upright microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 63× water-immersion objective and differential interference contrast optics. Electrophysiological recordings were performed at room temperature (22 °C–24 °C) in standard solutions containing (in mM): 137 NaCl, 5.8 KCl, 10 HEPES, 0.7 NaH₂PO₄, 1.3 CaCl₂, 0.9 MgCl₂, and 5.6 d-glucose, vitamins (1:100), and amino acids (1:50) as in MEM (Life Technologies, Carlsbad, CA) (pH 7.4; ~310 mOsm/kg). Recording electrodes (3–4 MΩ) were pulled from R-6 glass (King Precision Glass, Claremont, CA) and filled with intracellular solution containing (in mM): 140 CsCl, 5 EGTA-KOH, 5 HEPES, 2.5 Na₂ATP, 3.5 MgCl₂, and 0.1 CaCl₂ (pH 7.4; ~280 mOsm/kg). The whole-cell, tight-seal technique was used to record mechanotransduction currents using an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Hair cells were held at −84 mV. Currents were filtered at 5 kHz with a low-pass Bessel filter, digitized at ≥20 kHz with a 12-bit acquisition board (Digidata 1440A, Molecular Devices, Sunnyvale, CA), and recorded using pCLAMP 10 software (Molecular Devices, Sunnyvale, CA). Hair bundles from IHCs and OHCs were deflected using stiff glass probes mounted on a PICMA chip piezo actuator (Physik Instrumente, Karlsruhe, Germany) driven by an LVPZT amplifier (E-500.00, Physik Instrumente, Karlsruhe, Germany) and filtered with an 8-pole Bessel filter (Model 3384 filter, Krohn-Hite Corporation, Brockton, MA) at 40 kHz to eliminate residual pipette resonance. Stiff glass probes were designed to fit into the concave aspect of the array of hair cell stereocilia for whole-bundle recordings (3- to 4-µm diameter for OHCs and 4- to 5-µm diameter for IHCs). For the whole-cell electrophysiology recording at >P10, cochlea tissues were dissected at P5–7 and incubated in MEM (1×) + GlutaMAX-I medium with 1% FBS at 37 °C, 5% CO₂ for up to 30 d.

**Statistical tests.** Descriptive statistics for in vitro and in vivo eGFP expression data are presented. Rotarod results were analyzed with a two-tailed t-test. Error bars, n values, and type of replicates for experiments are defined in the respective paragraphs and figure legends.

**Rotarod test.** Five C57BL/6 mice were tested for balance behavior on the rotarod device. Mice with impaired vestibular function are known to perform poorly on the rotarod device. Previous studies highlighted the ability of this rotarod test to detect balance dysfunction when only one ear is affected. Three mice injected at P1 and tested at P36 and two uninjected control mice at P79 were tested. All mice were tested using the following rotarod protocol. On day one, mice were trained to balance on a rod that was rotating at 4 r.p.m. for 5 min. On day two, the mice were tested in five trials with each trial separated by 5 min. For each trial, the rod accelerated 1 r.p.m. from a starting rate of 2 r.p.m. The time (in seconds) was recorded until the mice fell off the device.

**Data availability.** Anc80L65 sequences are available at GenBank accession number AKU89595 (ref. 3).