Improved TMC1 gene therapy restores hearing and balance in mice with genetic inner ear disorders

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Fifty percent of inner ear disorders are caused by genetic mutations. To develop treatments for genetic inner ear disorders, we designed gene replacement therapies using synthetic adeno-associated viral vectors to deliver the coding sequence for Transmembrane Channel-Like (Tmc) 1 or 2 into sensory hair cells of mice with hearing and balance deficits due to mutations in Tmc1 and closely related Tmc2. Here we report restoration of function in inner and outer hair cells, enhanced hair cell survival, restoration of cochlear and vestibular function, restoration of neural responses in auditory cortex and recovery of behavioral responses to auditory and vestibular stimulation. Secondarily, we find that inner ear Tmc gene therapy restores breeding efficiency, litter survival and normal growth rates in mouse models of genetic inner ear dysfunction. Although challenges remain, the data suggest that Tmc gene therapy may be well suited for further development and perhaps translation to clinical application.

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Hearing loss is the most common neurological disorder and affects an estimated 466 million people worldwide, 35 million of which are children (World Health Organization). In addition to direct loss of auditory function, a number of secondary consequences can lead to diminished quality of life, including impaired cognitive development in children, social isolation, depression, and dementia. Despite the devastating consequences of hearing loss, there are no biological treatments for auditory dysfunction. The current standards of care provide only partial restoration for a limited patient population and include hearing aids, which boost sound levels and require some residual function, and cochlear implants, which bypass the sensory organ and directly stimulate the eighth cranial nerve.

In addition to the auditory organ, the inner ear includes five vestibular organs which mediate sensitivity to linear head moment and gravity, as well as rotational head movements around three orthogonal axes. Together, the vestibular end organs help maintain stable posture and gait and contribute to the sense of balance. Loss of vestibular function can lead to vertigo (i.e., the illusion of motion), imbalance, blurred vision and orthostatic intolerance. Vestibular dysfunction is a risk factor for falling, which is one of the most common reasons elderly patients seek medical attention. Unfortunately, there are no biological treatments for genetic vestibular dysfunction.

In a continuing effort to develop biological treatments for inner ear dysfunction, here we focus on development of improved gene therapy strategies to restore function in mouse models of genetic inner ear disorders. In particular, we focus on transmembrane channel-like 1 (TMC1) which encodes a protein required for the proper function and survival of inner ear hair cells. Recent evidence demonstrates that TMC1, and probably TMC2, are pore-forming components of mechanosensory transduction channels in auditory and vestibular hair cells. Mutations in Tmc1/TMC1 cause both dominant and recessive forms of deafness in mice and humans. Estimates suggest three to eight percent of genetic hearing loss may be due to TMC1 mutations. Some recessive TMC1 mutations cause moderate to severe hearing loss with postlingual onset. These patients may retain viable hair cells amenable to gene therapy intervention.

Prior gene therapy strategies that targeted TMC1 focused on gene replacement using conventional adeno-associated viral (AAV) vector serotypes to restore function in mouse models of recessive TMC1 deafness, DFNB7/11. Gene silencing using either AAV delivery of microRNA13 or ribonucleotide protein complexes to deliver CRISPR/Cas914 to target dominant TMC1 mutations. While these therapeutic strategies revealed no adverse effects, they yielded only partial recovery of auditory function. The recovery was limited due to inefficient delivery of the therapeutic DNA constructs to the targeted cell types, inner and outer hair cells of the cochlea. Although these approaches demonstrated successful targeting of IHCs, efficient targeting of OHCs has been problematic for these and other studies. Outer hair cells (OHCs) amplify and tune auditory information, which is transduced and transmitted by inner hair cells (IHCs). Both cell types are required for the fully functional cochlea, which enables exquisite sensitivity to faint sounds and fine pitch perception.

To address the limited recovery of auditory function with conventional AAV vectors, we previously screened a number of viral capsids and identified a synthetic AAV (sAAV), known as Anc80L65, which transduced IHCs and OHCs with high efficiency17. In prior work, the Anc80L65 capsid was shown to efficiently transduce a variety of cell types, including, retina, liver, and muscle. In the ear, we used the Anc80L65 capsid in a gene therapy study focused on Usher Syndrome, type IC, and found an unprecedented level of auditory recovery in deaf mice exposed to sAAVs encoding the wild-type Usher 1C gene.

For the current study we used a mouse model of DFNB7/11 recessive deafness to investigate whether sAAVs encoding Tmc1 can enhance auditory recovery, beyond that previously reported for Tmc1 packaged in conventional AAV1 capsids15,16. In addition, we ask whether restoration of function in the auditory periphery can drive neuronal function at the level of auditory cortex, which would be required for perception of complex sounds and speech. We also investigate the ability of exogenous Tmc1 and Tmc2 to restore vestibular and balance function in mouse models of genetic vestibular dysfunction. Since both normal hearing and balance contribute to quality of life in humans, we investigate whether sAAVs encoding TMC proteins improve secondary measures of recovery in mouse models of inner ear dysfunction. Although challenges remain, we conclude that efficient delivery of wild-type Tmc1 restores hearing, balance and several secondary outcomes, suggesting inner ear gene therapy for TMC1 mutations may be well-suited for further development and perhaps translation to the clinic.

Results

Tmc1 gene therapy restores sensory transduction. In prior work, we assessed Tmc1 gene therapy using an AAV2/1 serotype with a chicken beta actin (Chal) promoter driving expression of wild-type Tmc1 in a mouse model of DFNB7/11. We reported recovery of sensory transduction in inner hair cells (IHCs), but little or no recovery in outer hair cells (OHCs), most likely due to lack of expression or targeting in OHCs of AAV2/1-injected cochlea12. As a result, recovery of auditory function was modest. More recently, we demonstrated that synthetic AAV2/Anc80L65, together with a cytomegalovirus (Cmv) promoter, efficiently expressed eGFP in both IHCs and OHCs when injected via the round window membrane (RWM) at early postnatal stages. For the present study, we combined the two strategies and generated gene therapy vectors that encoded a Cmv promoter, an identical therapeutic gene, Tmc1ex1, followed by a WPRE sequence, packaged into synthetic AAV2/Anc80L65 capsids: AAV2/Anc80L65-Cmv-Tmc1ex1-WPRE (for brevity: sAAV-Tmc1). We injected 1 μL of sAAV-Tmc1 (1.4 × 1014 gc/mL) between postnatal day (P) 0 and P2 via RWM of Tmc1Δ/Δ;Tmc2Δ/Δ mice.

To determine the efficacy of viral targeting, expression of exogenous Tmc1, and restoration of sensory transduction in auditory hair cells, we visualized uptake of FM1-43, a styryl dye which permeates hair cell sensory transduction channels. Since both Tmc1 and Tmc2 are expressed at early postnatal stages (<P10), we injected inner ears of Tmc1Δ/Δ;Tmc2Δ/Δ mice which lack expression of endogenous TMC1 and TMC2 proteins, and thus do not take up FM1-4322. Likewise, age-matched, sham injected Tmc1Δ/Δ;Tmc2Δ/Δ mice also lacked FM1-43 uptake. However, in Tmc1Δ/Δ;Tmc2Δ/Δ mice seven days post-injection with sAAV-Tmc1, we found robust FM1-43 uptake in most IHCs (91 ± 8%, n = 4 cochleae) and half of the OHCs (50 ± 17%), suggesting expression of exogenous TMC1 and the presence of functional sensory transduction (Fig. 1a).

To assess sensory transduction currents we recorded from IHCs and OHCs of Tmc1Δ/Δ;Tmc2Δ/Δ mice injected on P1 with sAAV-Tmc1. Cochleas were harvested on P5-P7 and maintained in organotypic culture. On the equivalent of P7, Tmc1Δ/Δ;Tmc2Δ/Δ hair cells which did not take up FM1-43, lacked sensory transduction (Fig. 1b, left). FM1-43-positive OHCs within the same culture displayed robust sensory transduction (Fig. 1b, center left). Additionally, IHCs displayed sensory transduction at the equivalent of P8–9 (Fig. 1b, center right), and these responses were maintained to the equivalent of P30 (Fig. 1b, right), the latest time point tested. Hair cells from all three experimental groups displayed sensitivity, as indicated from stimulus-response
Tmc1 gene therapy restores cochlear function. To investigate the extent of recovery from sAAV-Tmc1 at the level of the whole cochlea, we measured auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) beginning at P28. Since TMC2 is not expressed in cochlear hair cells at mature stages,22,23, we used Tmc1ΔΔ mice, which in the absence of gene therapy are profoundly deaf. Tmc1ΔΔ mice were injected at P1-P2, and cochlear function was assessed at P28 to P30. Figure 2a shows representative families of ABR waveforms recorded in response to 11.3-kHz tone bursts of varying sound intensity. The families were selected to illustrate responses of an uninjected Tmc1ΔΔ mouse (left), two Tmc1ΔΔ mice injected with sAAV-Tmc1 with ABRs that displayed the lowest and median thresholds, a Tmc1ΔΔ mouse injected with 1 μL of AAV2/1-Cmv-Tmc1 (8.1 × 1014 gc/mL), and an aged-matched Tmc1ΔΔ/wild-type control (right). Of 11 mice injected with sAAV-Tmc1, all showed improvement in auditory function relative to uninjected Tmc1ΔΔ mice and relative to the mean response of six Tmc1ΔΔ mice injected with AAV2/1-Tmc1 (Fig. 2b). Variability in the extent of the recovery may have been due to injection variability, local variability of viral titer within the cochlea or variability of transduction efficiency. The variability is consistent with prior reports of variable gene therapy recovery.15,16. The source and methods for reducing variability in mouse gene therapy studies will require additional study.

ABR thresholds for the 11 Tmc1ΔΔ mice injected with sAAV-Tmc1 ranged from 35 to 90 dB and corresponded to best frequencies of 8–16 kHz (Fig. 2b). ABR thresholds of sAAV-Tmc1-injected mice were significantly lower than the six Tmc1ΔΔ mice injected with AAV2/1-Tmc1 at all frequencies tested between 5 and 16 kHz (p ≤ 0.00024, paired t-test). ABR peak I amplitudes were generally monotonic increasing functions of sound level (Fig. 2c), while peak I latencies were monotonic decreasing functions of sound intensity (Fig. 2d), both consistent with curves of normal hearing mice. We also injected sAAV-Tmc1 into the inner ears of four WT C57BL/6 mice and found no change in auditory function relative to uninjected WT controls, which suggested the injection technique, the viral capsid and overexpression of Tmc1 had no detrimental effect on inner ear function (Supplementary Fig. 1).

To assess OHC function at the level of the whole cochlea, we measured DPOAEs, a proxy for cochlear amplification and a requirement for sensitivity to soft sounds and fine frequency selectivity. In prior work, we found no recovery of DPOAEs in Tmc1ΔΔ mice injected with AAV2/1-Cha-Tmc1.12 For the current study, we found that AAV2/1-Cmv-Tmc1 injected mice also lacked recovery of DPOAEs, likely the result of limited viral transduction in OHCs with the AAV2/1 serotype. However, following sAAV-Tmc1 injection, all 11 Tmc1ΔΔ mice had improved DPOAE responses (Fig. 2e), relative to uninjected Tmc1ΔΔ mice, consistent with more efficient OHC transduction and the enhanced ABR thresholds. Both the recovery of ABR and DPOAE thresholds were durable over time with no significant (p > 0.05) differences at four, six, and twelve weeks of age (Fig. 2f, g).

Since the cochlear aqueduct, which provides a fluid connection between the inner ear and cerebral spinal fluid, is patent in neonatal mice, we examined cochlear function in contralateral ears of Tmc1ΔΔ mice injected with sAAV-Tmc1 on the ipsilateral side. Contralateral ears displayed recovery of ABR thresholds, albeit to a lesser extent than injected ears (Supplementary Fig. 2). The performance of the contralateral ear was not correlated with the level of performance in the injected ear, likely due to lower viral distribution to the contralateral ear. However, in contralateral ears with recovery, ABR and DPOAE thresholds were durable from 6 to 12 weeks, similar to the durable recovery observed in injected ears.

To investigate ability of sAAV-Tmc1 to restore function at later time points, we injected Tmc1ΔΔ mice at P1, P4, P7, and P14. ABR and DPOAE thresholds were recorded at P28. We found elevated ABR thresholds and little or no recovery of DPOAEs following RWM injection at later stages (Supplementary Fig. 3). To investigate whether the lack of recovery was due to decreased expression of exogenous transgene we used the same RWM...
**Fig. 2** sAAV-Tmc1 restores ABRs and DPOAEs in Tmc1Δ/Δ mice.  

a) Families of ABR waveforms recorded at P28-P30 from uninjected Tmc1Δ/Δ mouse (left) and from Tmc1Δ/Δ mice injected with sAAV-Tmc1 or AAV2/1-Tmc1 and one WT control, as indicated above. ABRs were recorded using 11.3-kHz tone bursts at sound pressure levels increasing by 5-dB until peak amplitudes reached 0.55 μV. Thresholds determined by the presence of Peak 1 and is indicated by colored traces. Scale bar applies to all families.

b) ABR thresholds plotted as a function of stimulus frequency for eleven Tmc1Δ/Δ mice injected with sAAV-Tmc1 tested at P28-P30 (gray traces). Best (green), and median (blue) recovery are indicated. Black lines show mean ± S.D. (n = 6 mice) for WT and Tmc1Δ/Δ uninjected mice as indicated. Red line shows mean ± S.D. (n = 6 mice) for Tmc1Δ/Δ injected with AAV2/1-Tmc1. Colors correspond to conditions indicated in a.

c) Peak 1 amplitudes measured from 11.3-kHz ABR waveforms, as shown in a, for eleven Tmc1Δ/Δ mice injected with sAAV-Tmc1. Colors correspond to conditions indicated in a.

d) Peak 1 latencies measured from 11.3-kHz ABR waveforms, as shown in a, for eleven Tmc1Δ/Δ mice injected with sAAV-Tmc1. Colors correspond to conditions indicated in a.

e) DPOAE thresholds plotted as a function of stimulus frequency for eleven Tmc1Δ/Δ mice injected with sAAV-Tmc1 tested at P28-P30. Colors correspond to conditions indicated in a.

f) Mean ± S.D. (n = number of mice) ABR thresholds plotted as a function of stimulus frequency for Tmc1Δ/Δ mice injected with sAAV-Tmc1 at P1 tested at 4, 6, and 12 weeks.

g) Mean ± S.D. DPOAE thresholds plotted as a function of stimulus frequency for the same Tmc1Δ/Δ mice shown in panel G injected with sAAV-Tmc1.
Fig. 3 sAAV-Tmc1 promotes survival of IHCs and OHCs in Tmc1\(^{-/-}\) mice. a Composite of five images from a whole cochlea harvested at 12 weeks old from a Tmc1\(^{+/+}\) mouse injected with sAAV-Tmc1 on P1 reconstructed from microdissected tissue. The tissue was stained for Myo7a (green). b Tonotopically mapped cochlear sections (image width = 100 μm) at 8, 16, and 32 kHz regions for WT (top), Tmc1\(^{+/+}\) (second), and Tmc1\(^{+/+}\) injected with sAAV-Tmc1 on P1 with the worst (third) and best (bottom) ABR performances as indicated in Fig. 2. c, d The number of surviving IHCs (c) and OHCs (d) in sections indicated in b from 3 WT mice, 3 uninjected Tmc1\(^{+/+}\), and eight Tmc1\(^{+/+}\) mice injected with sAAV-Tmc1 on P1 showing mean ± S.D. Number of surviving IHCs in the 8 and 16 kHz was significantly greater in injected ears than in uninjected Tmc1\(^{+/+}\) ears at (t-test: p = 0.015, 0.03, 0.19, respectively). Number of surviving OHCs in the 8 and 16 kHz was significantly greater in injected ears than in uninjected Tmc1\(^{+/+}\) ears at (t-test: p = 1E−7, 0.002, 0.16, respectively). e The percentage of surviving hair cells at 12 weeks of age was correlated with ABR thresholds measured between 5.6 and 11 kHz from sAAV-Tmc1-injected Tmc1\(^{+/+}\) mice. The data were fit with a linear regression with r = −0.67 and p = 6.2 × 10^{-4} (n = 7 mice; 21 measurements).
we found that the number of surviving hair cells correlated \(r = 0.67\) with lower ABR thresholds in the low frequency region of the cochlea, between 5.6 and 11 kHz (Fig. 3e).

**Tmc1 gene therapy restores responses in auditory cortex.** Since several genes that cause hearing loss when mutated are known to be expressed in the cochlea and in central auditory pathways, we asked whether recovery of peripheral auditory function in sAAV-Tmc1-injected mice is sufficient to drive neural responses in auditory cortex.

To investigate central auditory function after sAAV-Tmc1 gene therapy, we recorded multi-unit (MU) activity in the auditory cortex (ACtx) of awake, head-fixed Tmc1\(^\Delta/\Delta\) mice (Fig. 4a). Eleven Tmc1\(^\Delta/\Delta\) mice were divided into two groups, seven sAAV-Tmc1-injected mice with improvement in ABR thresholds (ABR+: thresholds < 70 dB at any frequency) and four sAAV-Tmc1-injected mice with minimal improvement in ABR thresholds (ABR−: thresholds > 75 dB). The variability in ABR thresholds was likely due to the success of the injection technique. We found that ACtx neurons of both ABR+ and ABR− mice had higher spontaneous activity than C57BL/6 wild-type control mice (Figs. 4a top, b, Kruskal-Wallis test \(\chi^2(2) = 32.22\); pairwise comparisons: control vs. ABR+, \(p = 2.1e^{-05}\); control vs. ABR−, \(p = 8.5e^{-06}\)). To test whether cochlear gene therapy supported sound processing in regions of the central auditory cortex, we presented broad band noise (BBN) bursts at different sound levels. Representative raster plots from ACtx neurons recorded from the paradigm in Fig. 4b. Startle response amplitudes normalized to mouse weight measured at P28-P30 and plotted as a function of sound intensity and as mean ± S.D. for 6 control C57BL/6 mice (black), ten individual Tmc1\(^\Delta/\Delta\) mice injected with sAAV-Tmc1 (light green, mean ± S.D. in bold green), and five uninjected Tmc1\(^\Delta/\Delta\) mice (red).

Behavioral recovery of auditory function was tested by measuring acoustic startle responses in Tmc1\(^\Delta/\Delta\) mice injected with sAAV-Tmc1. We found that all of 11 P1-P2-injected mice had significantly improved startle responses compared to uninjected Tmc1\(^\Delta/\Delta\) mice (Fig. 4h), suggesting that even though ABR and DPOAE thresholds remained elevated, the recovery was sufficient to drive auditory behavior.

**Gene therapy restores vestibular function in Tmc1−/− mice.** Cochlear perilymph is continuous with perilymph that bathes vestibular organs, hence, gene therapy reagents injected into the cochlea are distributed throughout the inner ear perilymphatic spaces and transduce both auditory and vestibular hair cells. Since both Tmc1 and Tmc2 are expressed in vestibular hair cells and Tmc1\(^\Delta/\Delta\);Tmc2\(^\Delta/\Delta\) mice suffer profound vestibular dystrophy, we intended whether Tmc gene therapy might improve vestibular function. We focused on Tmc2 because it is expressed in vestibular organs at neonatal and mature stages and thus generated AAV2/Anc80L65-Cmv-Tmc2-WPRE vectors (sAAV-Tmc2). Following RWM injection of 1 µL sAAV-Tmc2, we measured rotational vestibular ocular reflexes (RVOR) to sinusoidal head rotations at 8 weeks of age. Representative eye velocity responses to 1 Hz head rotation for a Tmc1\(^\Delta/\Delta\);Tmc2\(^\Delta/\Delta\) mouse, an sAAV-Tmc2-injected mouse and a wild-type mouse are shown in Fig. 5a. While the Tmc1\(^\Delta/\Delta\);Tmc2\(^\Delta/\Delta\) mouse exhibited virtually no eye movement responses to head rotation, the treated mouse exhibited compensatory RVORs that were comparable to the RVORs of the WT mouse. Mean RVOR gains (Fig. 5b) and
phases (Fig. 5c) for 14 mice are summarized for the three groups of mice. While the WT group (n = 5) exhibited high gain compensatory RVORs at all frequencies tested, the Tmc mutant mice (n = 4) exhibited virtually zero gain responses at the same frequencies and thus displayed little difference from responses of a dead mouse. However, the sAAV-Tmc2-treated group (n = 5) exhibited robust compensatory RVORs at these frequencies.

Fig. 5 Exogenous Tmc1 or Tmc2 restores vestibular function in Tmc2Δ/Δ; Tmc2Δ/Δ mice. a Rotational vestibular ocular reflexes (RVOR) to sinusoidal head rotations with representative eye velocity responses to 1 Hz head rotation for a wild-type mouse (black), a Tmc2Δ/Δ; Tmc2Δ/Δ mouse (red), and an sAAV-Tmc2-injected mouse (green). b Mean ± S.D. RVOR gains and c phases for WT group (n = 5, black), uninjected Tmc mutant mice (n = 4, red), and sAAV-Tmc2-treated group (n = 5, green). d Linear vestibular ocular reflexes (LVORs) to sinusoidal head translations with representative eye position measurements to 2 Hz head translations for a wild-type mouse (black), a Tmc2Δ/Δ; Tmc2Δ/Δ mouse (red), an sAAV-Tmc2-injected mouse (green). e Mean ± S.D. gains and f phases of the LVORs are shown for uninjected Tmc mutant group (n = 4, red), the treated group (n = 5, green) and the WT group (n = 5, black). g Eye velocity response curves due to a high acceleration and high frequency step rotation. h Mean gains ± S.D. for eye movements during the first 100 ms, as shown in g. For a–g, positive head movements is for rightward head rotation and positive eye movement is for eye movement toward the left ear. Eye movements in injected and wild-type groups were compensatory to head rotation. Stars indicate statistical significance (t-test). i Mean ± S.D. number of full body rotations observed during 5 min in a 42-cm-wide arena in 6-week-old control Tmc1Δ/Δ; Tmc1Δ/Δ mice, Tmc2Δ/Δ mice, and Tmc2Δ/Δ mice injected at either P1, P14 or P30 with sAAV-Tmc1 or sAAV-Tmc2. Significant recovery P < 0.001 was observed between the uninjected and injected mice. Number of mice test for each of eight conditions were: 6, 4, 6, 5, 7, 7, 7, respectively. Statistical analysis by one-way ANOVA. See Supplementary Movies 1-3. j Mean ± S.D. time spend on rotarod device recorded at 6 weeks of age in control Tmc1Δ/Δ; Tmc1Δ/Δ mice, Tmc2Δ/Δ mice, and Tmc2Δ/Δ mice injected at either P1, P14 or P30 with sAAV-Tmc1 or sAAV-Tmc2. Number of animals test for each of eight conditions were: 10, 5, 7, 10, 6, 7, 10, 10, respectively.

Their phases were not statistically different from those of the WT mouse. Although their gains were about 86% of that of the WT group, the differences between experimental mice and wild-type mice did not reach statistical significance at the tested frequencies (t-test: 0.2 Hz: p = 0.305; 0.5 Hz: p = 0.524; 1 Hz: p = 0.401; 2 Hz: p = 0.573; 4 Hz: p = 0.127).

To assess the effects of sAAV-Tmc2 gene therapy on the otolith system (utricle and saccule), we measured vertical vestibular ocular reflexes (LVORs) to sinusoidal head translations. Figure 5d shows representative eye position responses to 2 Hz head translations for a WT mouse (black), a Tmc2Δ/Δ; Tmc2Δ/Δ mouse (red), and an sAAV-Tmc2-treated mouse (green). While the Tmc mutant mouse exhibited virtually no eye movement responses to head translation, the treated mouse exhibited compensatory LVORs that were similar to the responses of the WT mouse. Mean gains (Fig. 5e) and phases (Fig. 5f) of the LVORs are shown for the three groups of mice. In contrast to the Tmc mutant group (n = 4), which exhibited virtually zero gain responses at these frequencies, the treated group (n = 5) exhibited compensatory responses. Phases of the injected mice were nearly the same as the WT group (n = 5). Gains were not significantly different from those of the WT group at all frequencies tested (t-test: 0.2 Hz, p = 0.3; 0.5 Hz, p = 0.14; 1 Hz, p = 0.053; 2 Hz: p = 0.762).

In order to reliably track eye movements, all VOR measurements were performed in ambient light conditions. To rule out the possibility that visual input drove the eye movement responses in Fig. 5a–f, we employed not only high frequency sinusoidal head rotations (e.g., 2 and 4 Hz), but also high acceleration and high frequency step rotations. Because the onset of visually-evoked eye movements requires at least 80 ms, the first 80 ms of the eye movement responses are primarily driven by vestibular inputs, i.e., open-loop responses without visual feedback. Figure 5g–h show that the sAAV-Tmc2-treated group
exhibited nearly identical response latencies to rapid head rotations as the WT group (p = 0.64, t-test). Thus, the sAAV-Tmc2 gene therapy in Tmc1Δ/Δ;Tmc2Δ/Δ mice promoted VOR responses at rates that were too rapid to be explained by visual compensation, supporting the conclusion for robust vestibular recovery.

These results not only confirmed a previous report of absence of RVOR in Tmc1Δ/Δ;Tmc2Δ/Δ mice, but also extend that finding to include loss of LVORs, suggesting that Tmc1/2 disruption affected both the semicircular canals and the otolith organs. Importantly, the data demonstrate that sAAV-Tmc2 gene therapy restores VOR responses to near wild-type levels.

To assess vestibular recovery at the behavioral level, we recorded open-field behavior over the course of five minutes in six-week-old control WT mice, Tmc1Δ/Δ; Tmc2Δ/Δ mice, and Tmc1Δ/Δ; Tmc2Δ/Δ mice injected at P1, P14, or P30 with sAAV-Tmc1 or sAAV-Tmc2. Figure 5i demonstrates that Tmc1Δ/Δ; Tmc2Δ/Δ mice, which have profound vestibular dysfunction, circle 10–12 times each minute (Supplementary Movies 1-3), significantly more than wild-type mice (p = 7.6E−4, ANOVA). Tmc1Δ/Δ; Tmc2Δ/Δ mice injected with sAAV-Tmc1 or sAAV-Tmc2 at all three time points displayed significantly less circling behavior (p = 4.4E−4, ANOVA).

To assess balance behavior on a more challenging task, we recorded rotarod performance at six weeks of age in the same cohort of mice. While wild-type mice maintain balance for 1.5 min on average, Tmc1Δ/Δ; Tmc2Δ/Δ mice typically fell off the device within 30–40 s (Fig. 5i). Injection of sAAV-Tmc1 or sAAV-Tmc2 at early postnatal stages (P1) yielded the best improvement in rotarod performance, with mice able to maintain balance for 1.5–3 min. Injection at later stages also yielded improved performance in balance behavior, though to a lesser extent, perhaps due to reduced viral transduction in vestibular hair cells when injected at later postnatal stages (Supplementary Fig. 6).

### Tmc1 gene therapy improves secondary outcomes. Tmc1Δ/Δ; Tmc2Δ/Δ mice suffer profound hearing and balance dysfunction, consequently, Tmc1Δ/Δ;Tmc2Δ/Δ mice do not breed well. When they do breed, survival of Tmc1Δ/Δ; Tmc2Δ/Δ offspring to Tmc1Δ/Δ; Tmc2Δ/Δ parents is reduced and those that do survive have stunted growth relative to wild-type C57BL/6 mice. In the course of this study we discovered that Tmc1Δ/Δ;Tmc2Δ/Δ mice injected with sAAV-Tmc1 were better breeders (Fig. 6a). During a 6–7 month mating period, four wild-type C57BL/6 breeding pairs produced 6–7 litters each, or ~1 litter/month. During a 6–12 month breeding period, five Tmc1Δ/Δ;Tmc2Δ/Δ breeding pairs produced an average of three litters/breeding pair or an average of 1 litter every four months. Tmc1Δ/Δ;Tmc2Δ/Δ breeding pairs in which both parents were injected with sAAV-Tmc1, produced 8–9 litters during the same 6–12 month time frame. In addition to the significant (p = 0.001, t-test) increase in the number of litters, we also found that there was an increase in survival rate among the litters born to sAAV-Tmc1-injected Tmc1Δ/Δ;Tmc2Δ/Δ breeding pairs. On average 92% of litters from WT parents survive to their typical wean date of P21, whereas only 22% of litters born to Tmc1Δ/Δ;Tmc2Δ/Δ parents survived to weaning age. In contrast, 81% of litters born to Tmc1Δ/Δ;Tmc2Δ/Δ mice injected with sAAV-Tmc1 survived until weaning (Fig. 6b).

We also tracked weight and growth of pups at four, eight and 12 weeks of age among four cohorts of mice: (1) WT C57BL/6, (2) Tmc1Δ/Δ;Tmc2Δ/Δ pups born to Tmc1Δ/Δ;Tmc2Δ/Δ parents, (3) Tmc1Δ/Δ;Tmc2Δ/Δ pups born to sAAV-Tmc1-injected parents, and (4) of sAAV-Tmc1-injected pups born to sAAV-Tmc1-injected parents. WT mice doubled their weight from ~12 to 24 g between 4 and 12 weeks of age (Fig. 6c). Tmc1Δ/Δ;Tmc2Δ/Δ pups born to Tmc1Δ/Δ;Tmc2Δ/Δ parents had similar weight to WT mice at 4 weeks of age, but failed to thrive, growing only ~50% during the following 8 weeks. Tmc1Δ/Δ;Tmc2Δ/Δ pups born to sAAV-Tmc1-injected parents did not fare any better and their growth remained stunted over the same time frame. However, sAAV-Tmc1-injected pups born to sAAV-Tmc1-injected parents showed a dramatic improvement in growth with weights that were indistinguishable from those of WT mice at all three time points tested. These data indicate that recovery of hearing and balance function has a significant impact on secondary measures including, breeding, survival and growth in mouse models of genetic inner ear disorder.

### Discussion
Here we demonstrate significant improvement in auditory and vestibular function using next generation gene therapy in a mouse model of the human recessive deafness DFNB7/11. The difference over prior gene therapy outcomes for recessive Tmc1 mutations.
studies have shown that eliminating ~95% of cochlear afferent sensitivity, we generated sAAV vectors with widths similar to those of wild-type mice. While the thresholds, we found stimulus-evoked responses in auditory pathways, we asked whether restoration of auditory function in the periphery was sufficient to drive neural responses in ascending auditory pathways. Thus, neuronal function in auditory cortex does not require Tmc1 expression locally within the cortex. Since some genes that cause deafness when mutated are expressed in both the cochlea and ascending auditory pathways, restoration of function in auditory cortex following peripheral injection of sAAV-Tmc1 was not assured. The neuronal responses in auditory cortex were correlated with recovery of ABR responses. This suggests that for Tmc1 gene therapy, the ABR, a less invasive measure, may be a good proxy for recovery of neural activity in higher auditory pathways, at least at early developmental stages. Whether normal function of auditory cortex can be recovered following gene therapy in the peripheral auditory system when introduced at mature stages remains to be determined. Further enhancement of cortical responses may also be achieved by injection of both ears and will require further investigation.

We also found significant recovery of vestibular function in Tmc1/Tmc2 double mutant mice injected with sAAV-Tmc2 vectors. Recovery of vestibular ocular reflexes in response to rotational or linear head movements suggested recovery of function in the semi-circular canal and otolith organs, respectively. Behavioral processing was documented by action potential recordings from the auditory cortex. This result has several implications: (1) Central auditory processing was documented by action potential recordings from auditory cortex, a more sensitive assay than the far-field electrical potentials measured by ABR. (2) Local delivery of Tmc1 gene therapy to the cochlea was sufficient to drive neural responses in ascending auditory pathways. (3) Thus, neuronal function in auditory cortex does not require Tmc1 expression locally within the cortex. Since some genes that cause deafness when mutated are expressed in both the cochlea and ascending auditory pathways, restoration of function in auditory cortex following peripheral injection of sAAV-Tmc1 was not assured. (4) The neuronal responses in auditory cortex were correlated with recovery of ABR responses. This suggests that for Tmc1 gene therapy, the ABR, a less invasive measure, may be a good proxy for recovery of neural activity in higher auditory pathways, at least at early developmental stages.
age-matched wild-type control pups. Thus, using measures of breeding success, survival rates and growth rates, the data suggest that, in addition to enhanced auditory and vestibular function, Tmcl gene therapy restores secondary measures in mouse models of genetic inner ear dysfunction. While normal auditory and balance function have been long recognized as contributing factors for quality of life in humans, here for the first time, we demonstrate that gene therapy restoration of hearing and balance enhance several secondary outcomes in a mouse model of human inner ear dysfunction.

Given the extent of the functional recovery quantified here using multiple physiological and behavioral assays, we suggest that Tmcl gene replacement therapy for recessive genetic inner ear dysfunction may be well suited for further development and translation to the clinic. Future studies that extend the recovery to high-frequency hearing or to the balance function have been long recognized as contributing factors for quality of life in humans, here for the first time, we demonstrate that gene therapy restoration of hearing and balance enhance several secondary outcomes in a mouse model of human inner ear dysfunction.

**Methods**

**Study design.** The aim of this study was to use the Anc80L65 capsid for delivery and expression of exogenous Tmc1 and Tmc2 in hair cells of the mouse cochlea and vestibular organs and to evaluate the ability of these vectors to restore auditory and vestibular function in mouse models of genetic inner ear disorders. Anc80L65 vectors containing the coding sequence for eGFP, Tmc1, or Tmc2 were injected in vivo through the round window membrane, and the outcomes were evaluated using FM1-43 uptake, single-cell recording, measurement of ABRs, DPOAEs, cochlear microphonics, and tonotopic mapping of whole cochleas, acoustic startle reflexes, multi-unit auditory cortex recordings, rotarod and open field analysis, vestibular ocular reflexes, and secondary indicators of functional recovery. Left ears were injected postnatally as indicated and later time points of injection were used to assess the effective therapeutic window. Each experiment was replicated as indicated by n values in the figure legends. All experiments with mice and viral vectors were approved by the Institutional Animal Care and Use Committee (protocols #18-01-3610R and #17-03-3396R) and the Institutional Biosafety Committee (protocol #IBC-RN00000447-1) at Boston Children’s Hospital.

**Mice.** Wild-type control mice were C57BL/6J (Jackson Laboratories) and mice that carried mutant alleles of Tmc1 and Tmc2 were on a C57BL/6J background. Three genotypes of Tmc mutant mice were used: Tmc1fi/ΔTmc1, Tmc1+/-, Tmc1fi/ΔTmc1, Tmc2fi/ΔTmc2, and Tmc1fi/ΔTmc2fi. Mice ages P0–P1, P4, P7, and P14 were used for in vivo delivery of viral vectors according to protocols approved by the Institutional Animal Care and Use Committee (protocols #18-01-3610R and #17-03-3396R) at Boston Children’s Hospital.

**Tissue preparation.** Temporal bones were harvested from mice at P7, P28, or 12 weeks after birth. For tissues harvested at P7, pups were euthanized by rapid decapitation and temporal bones were dissected in Minimum Essential Medium (Intralipid) supplemented with 10 mM HEPES, 0.05 mg/mL ampicillin, and 0.01 mg/mL ciprofloxacin at pH 7.4. The membranous labyrinth was isolated under a dissecting scope, Reissner’s membrane was peeled back, and the tectorial membrane and stria vasculosa were mechanically removed. Organ of Corti cultures were pinned flatly beneath a pair of thin glass fibers adhered at one end with Sylgard to an 18-mm round glass coverslip. The tissue was placed in culture and used acutely with GFP primers (GFP-F: AGAACGGCATCAAGGTGAAC; GFP-R: GAACTCTGCAAGCAGATGT), or ITR primers (LITR-F: GACCTTTGGTCGCCCGGCTTG; RITR-R: GCCGCGGTCAGAAAGATTGCGA). All four vectors were purified using an iodixanol step gradient followed by ion exchange chromatography. Virus aliquots were stored at −80 °C and thawed just prior to use for in vivo injections.

**Vector production.** Anc80L65 vectors carrying the coding sequence for eGFP, mouse Tmc1, or mouse Tmc2 driven by a cytomegalovirus (CMV) promoter were generated using a helper virus free system and a double transfection method. AAV2/1-CMV-Tmc1-WPRE, AAV2/Anc80-Cmv-eGFP-WPRE, AAV2/Anc80-Cmv-Tmc2-WPRE, and AAV2/Anc80-Cmv-Tmc2-WPRE were produced by the Viral Core at Boston Children’s Hospital at titers of 8.1E+14/g/mL, 1.4E+14/g/mL, 1.4E+14/g/mL, and 1.6E+14/g/mL, respectively. Titters were calculated by qPCR with GFP primers (GFP-F: AGAACGGCATCAAGGTGAAC; GFP-R: GAACTCTGCAAGCAGATGT), or ITR primers (LITR-F: GACCTTTGGTCGCCCGGCTTG; RITR-R: GCCGCGGTCAGAAAGATTGCGA). All four vectors were purified using an iodixanol step gradient followed by ion exchange chromatography. Virus aliquots were stored at −80 °C and thawed just prior to use for in vivo injections.

**In vivo injection of viral vectors.** Mouse pups were injected via the round window membrane (RWM) technique using beveled glass microinjection pipettes on postnatal day 0 (P0) between P0 and P30 as indicated. Pups were anesthetized by rapid induction of hypothermia for 2–4 min on ice water until loss of consciousness, and this state was maintained on a cooling platform for 10–15 min during the surgery. The surgical site was disinfected by scrubbing with Betadine and wiping with 70% Ethanol. A post-auricular incision was made to expose the transparent otic bulla, and the RWM was penetrated by the tip of the micropipette. Approximately 1 µL of virus was slowly introduced unilaterally into the left ear. The skin incision was closed using a 6-0 monofilament suture (Ethicon). EMLA cream (2.5% lidocaine and 2.5% prilocaine) was applied to the pinnae of mice under anaesthesia using sterile swabs to cover the surgical site (left mastoid prominence). Body temperature was maintained at a 37 °C warming pad for 30–60 min after surgery and before reintroduction into parental cage.

**Hair cell electrophysiology.** Organotypic cochlear cultures were bathed in standard artificial perilymph containing 137 mM NaCl, 0.7 mM NaH2PO4, 5.8 mM KCl, 1.3 mM CaCl2, 0.9 mM MgCl2, 10 mM HEPES, and 5.6 mM 0-glucose. AAV2/Anc80-Cmv-Tmc1-WPRE, or AAV2/Anc80-Cmv-Tmc2-WPRE, and AAV2/Anc80-Cmv-Tmc1-WPRE were added to the solution from concentra-
alternating polarity stimulus, 512 responses were collected and averaged for each sound pressure level. Wavesforms with amplitude larger than 15 µV (peakto-peak) were discarded as an artifact reject function. Prior to the onset of ABR testing, the flap of skin and cartilage that typically obscures the entrance of the external auditory meatus was trimmed away with dissecting scissors, and sound pressure at the entrance of the ear canal was calibrated for each individual test subject at all stimulus frequencies. Acoustic stimuli were delivered directly to the studied ear through a custom probe tube/speaker/microphone assembly (EPM Systems) consisting of two electrostatic earphones (CUI Miniature Dynamics) to generate primary tones and a Knowles miniature microphone (Electret Condenser) to record ear-canal sound pressure. Sound stimuli consisted of 5-ms tone bursts (0.5 ms rise/decay time of a rectangular hyperbolic cosine shape) delivered at 40 Hz. 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 Etonn-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.

DPOAE measurement. DPOAE data were collected under the same conditions, and during the same recording sessions, as ABR data. Primary tones were produced at a frequency ratio of 1.2 (2/1) for the generation of DPOAEs at 2f1−2f2. When the f1 level was 10 dB sound pressure level below f1 level for each f2/f2 pair. The f2 levels were swept in 10-dB steps from 20 to 80 dB. Waveform and spectral averages were used at each level to increase the signal-to-noise ratio of the recorded ear-canal sound. The probed amplitude of the DPOAE at 2f1−2f2 was extracted from the averaged spectra, along with the noise floor at nearby points in the spectrum. Isor response curves were interpolated from plots of DPOAE amplitude versus sound level. Threshold was defined as the f2 level required to produce DPOAEs above 0 dB.

Auditory cortex recordings. All procedures were approved by the Animal Care and Use Committee at the Massachusetts Eye and Ear Infrmary and followed guidelines established by the National Institutes of Health for the care and use of laboratory animals. Behavior and awake extracellular recordings were performed at 7–10 weeks old on C57BL/6j and Tmcd1−/− deficient mice injected at P1−2 with AAV2/Ance80L65-Cmv-Tmcd1x1.1.WPRE. A titanium head plate was affixed to the skull with dental cement (C&B Metabond) three days before the recording sessions began. The primary auditory cortex was first identified by anatomical markers (2.5 mm posterior to bregma lateral to the squamosal ridge) and through a coarse mapping of middle layer unit responses when possible. Before the first recording session, animals were briefly anesthetized with isoflurane (1.5% in oxygen) while a small craniotomy (0.5 × 1.0 mm) was made behind the external auditory meatus. At the midline of the brain, the dura was removed and a dural window was made. After cutting the dura mater, the brain was removed and a small craniotomy was made. The craniotomy was made by 3.5 SDs. For auditory responses latency was measured 10 dB above threshold. The offset of the spike collection window was the first bin after the response decreased to less than 5 SD above the spontaneous rate. Additional details on spike windowing and FRA boundary determination are described in Guo et al. (2012).

For both auditory and visual responses, latency to onset was defined as the time-point at which the post-stimulus firing rate exceeded spontaneous firing rate by 3.5 SDs. For auditory responses latency was measured 10 dB above threshold.

Acoustic startle reflexes. Mice were tested for startle reflexes in response to broadband auditory stimulation at varying intensities. The animals were tested in a sound shielded startle booth (Kinder Scientific) and the force generated by foot movement was sensed by piezoelectric motion sensor fixed beneath an elevated platform. Mice were placed in a smaller sub chamber anchored to the top of the platform. Mice were then released from the sub chamber and a sound was presented through the perforated door of the sound booth. Sound stimuli were calibrated with the door closed using a sound pressure level meter (Allied Electronics) with the microphone mounted in the position normally occupied by the animal holder. The generation of sound stimuli and recording of force amplitude signals was performed by startle monitor software (Kinder Scientific). Broadband white noise was presented at a background level of 60 dB throughout the experiment and auditory test stimuli consisted of 50-ms broadband white noise pulses in 10-dB steps from 60 to 120 dB. Different intensities of the test stimulus were presented in a pseudo-random order at randomized inter trial intervals (ITI) that varied between 8 and 22 s, with no ITI repetitions of the same intensity. More than three times the spontaneous rate of the intensities for one test subject and responses were normalized to the weight of the mouse tested. Startle response measurements were performed by investigators blinded to the genotype.

Vestibular ocular reflexes (VORs). Tests were performed on Tmcd1−2 double knockout and Tmcd1−/2 double knockout injected on P1 with sAAV-Tmcd2 from the inner ear, C57BL/6j, and wild-type C57BL/6j mice. All procedures were carried out in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. All surgical procedures were performed aseptically. Briefly, each mouse was implanted with a small head holder on the skull and was allowed seven days to recover before eye movement tests. Horizontal and vertical eye position signals were recorded using a video-based eye tracking system (ISCAN ETS-200, ISCAN, Burlington, MA). An infrared camera equipped with a zoom lens (Computar TV Zoom Lens, Computar Optics Group, Japan) was attached to the platform mounted on a servo-controlled rotator/sled (Neurokinetic, Pittsburgh, PA) and was focused on the left eye of the mouse through a multiple infrared LED illuminator. Vertical illumination and a reference corneal reflection (CR) for eye movement measurement. The eye tracker tracked the pupil center and the CR at a speed of 240 frames per second with a spatial resolution of 0.1°. Calibration was achieved by rotating the camera from the left 10° to the right 10° around the vertical axis of the eye. Following the calibration, a series of rotational and linear accelerations were delivered. To measure the steady state VOR responses, horizontal rotations were delivered at 0.2, 0.5, 1, 2, and 4 Hz (60°/s peak velocity) and horizontal translations were delivered at 0.2, 0.5, 1, and 2 Hz (0.1 g peak acceleration) along 45° right from naso-occipital direction (i.e., the translation direction is perpendicular to the visual axis of the left eye). Step responses were not used for the LVOR because preliminary tests in WT mice showed that the transient LVOR were too small to provide reliable estimates of otothyl function. To measure the transient linear VOR responses, rapid leftward or rightward head rotations were delivered, which steps reached a velocity of 100°/s within 100 ms with a peak acceleration of ~1500°/s/s. At least 30 cycles/30 trials per condition were recorded. Signals related to horizontal and vertical eye position and head rotation and translation were sampled at 1 kHz at 16 bits resolution by a CED Power 1401 system (Cambridge Electronics Devices, Cambridge, UK). Eye movement responses were analyzed using Spike2 (Cambridge Electronics Devices), Matlab (MatLab, Natick, MA) and SigmaPlot (Systat Software, San Jose, CA). Eye positions and head position signals were aligned using a band-pass of DC to 50 Hz to obtain eye velocity signals. As described in details in Stewart et al., gains and phases of the VORs were calculated by performing Fast Fourier Transform (FFT) on the de-saccaded eye velocity signal and head rotation velocity signal. Gains and phases of the LVORs were calculated by performing FFT on the de-saccaded eye position signal and head position signal. For the transient VORs, trials in the data stream were aligned at the onset of head rotation and averaged (~50 trials per condition) to obtain low-noise estimates of eye velocity as a function of time. Gains were calculated as the ratio of eye velocity and head velocity at 80 ms after head rotation onset. VOR measurements were performed by investigators blinded to the genotype.

Rotaror and open field assessment. Rotaror and Open Field balance assessments were performed on separate days in the Neurodevelopmental Behavioral Core at Boston Children’s Hospital. The rotaror performance test was performed over the course of two days. On day one, mice were placed in an enclosed housing on a rod that spins at a constant four r.p.m. for 5 min. If mice fell during this training session, it was repeated. On the second day, mice were placed on the spinning rod and the spinning rate accelerated at a rate of 0.1 r.p.m. s−1. The length of time the animals were able to remain on the device before the
dropping onto the instrumented floor of the housing was displayed on a timer and recorded after each test run. A 5-min resting period was imposed between trials and a total of five trials were performed for each mouse. The open field test was conducted using a 42-cm diameter circular frame inside a sound chamber with overhead LED lighting set to 30 lux. Mice were placed one at a time inside the circular open field and allowed to explore freely and undisturbed for 5 min. Behavior was recorded and tracked using Ethemvision XT, enabling measures of distance traveled, velocity, and number of crosses. A rotation was counted with the software if the mouse center-point had a cumulative turn of 270° over a minimum distance of 2.00 cm either counterclockwise or clockwise. Open field assessments were all conducted by investigators blinded to the genotype.

**Breeding, survival, and growth rates.** For analysis of birth rate, litter survival and weight, all experimental mice had genotypes of Tmc1Δ/Δ,Tmc2Δ/Δ. Control, wild-type mice were C57BL6J/ parents raising C57BL6J/ pups. Male and female mice were weighed with an electronic scale at 4, 8, and 12 weeks. Females and males were housed separately and tracked over 12 weeks and were excluded if they were pregnant or had ever been pregnant. Litter frequency and survival were calculated from breeding pairs that were either Tmc1Δ/Δ,Tmc2Δ/Δ uninject, Tmc1Δ/Δ,Tmc2Δ/Δ injected with SAAV-Tmc1, or C57BL6/ wild-type. PAres were paired with Tmc1Δ/Δ,Tmc2Δ/Δ male mice at six weeks and were housed together for 12 months. Births were manually recorded (litter size, date, and cage number). Females were retired after birthing and caring for 7 litters. To determine litter survival, litters across all breeding cages were classified as non-viable if none of the pups from the birthed litter survived to wean age (P21). Litters were classified as survival if any of the pups survived until wean age (P21). All data were recorded and evaluated using ANOVA and 2 sample independent t-tests for significance.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this Article.

**Data availability statement.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests: J.R.H. is a consultant to several companies focused on development of inner ear therapeutics. J.R.H. and G.S.G. hold patents on Tmc1 gene therapy which have not been licensed. The remaining authors declare no competing interests.

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