Preventing autosomal-dominant hearing loss in Bth mice with CRISPR/CasRx-based RNA editing

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CRISPR/RfxCas13d (CasRx) editing system can specifically and precisely cleave single-strand RNAs, which is a promising treatment for various disorders by downregulation of related gene expression. Here, we tested this RNA-editing approach on Beethoven (Bth) mice, an animal model for human DFNA36 due to a point mutation in Tmc1. We first screened 30 sgRNAs in cell cultures and found that CasRx with sgRNA3 reduced the Tmc1Bth transcript by 90.8%, and the Tmc1 wild type transcript (Tmc1 wt) by 44.3%. We then injected a newly developed AAV vector (AAV-PHP.eB) based CasRx into the inner ears of neonatal Bth mice, and we found that Tmc1Bth was reduced by 70.2% in 2 weeks with few off-target effects in the whole transcriptome. Consistently, we found improved hair cell survival, rescued hair bundle degeneration, and reduced mechanoelectrical transduction current. Importantly, the hearing performance, measured in both ABR and DPOAE thresholds, was improved significantly in all ages over 8 weeks. We, therefore, have validated the CRISPR/CasRx-based RNA editing strategy in treating autosomal-dominant hearing loss, paving way for its further application in many other hereditary diseases in hearing and beyond.

INTRODUCTION

According to the World Health Organization (WHO), hearing loss is one of the most common sensory defects, with approximately 5% of the world’s population suffers from disabling hearing loss, and 34 million of these are children.1-4 In children, hearing loss affects cognitive, language, and psychosocial development.1,2 Almost half of all cases of deafness cases are caused by genetic factors,3 and among the different types of hereditary hearing loss, 20–25% of nonsyndromic hearing loss (NSHL) cases are autosomal dominant.5-9 To date, over 100 genes have been confirmed to be relevant to NSHL (https://hereditaryhearingloss.org/), and the prevalence of autosomal dominant inheritance increases while that of autosomal recessive inheritance decreases in aging populations.5 For example, TMC1 is the sixth most commonly inherited deafness gene, and mutations in TMC1 result in both dominant and recessive NSHL.7 Its protein product TMC1 is believed to have ten transmembrane domains, and together with TMC2, it forms the pore of a channel complex that is required for mechano-electrical transduction of sound in both auditory and vestibular hair cells.5-7 The TMC1 point mutation (c.1235T > A; p. M418K) in humans, which is identical to the Tmc1 mutation (c.1235T > A; p.M412K) in Bth mice, a transversion T > A locates in exon 13 of Tmc1 sequence. This mutation causes DFNA36 hearing loss,10,11 so Bth mice would be an appropriate model for NSHL research.

Currently, few treatments are available to slow or reverse genetic deafness in clinic.11 With an increasing understanding of heredity in relation to hearing loss, interest in gene therapies for hearing loss has grown.13 Gene replacement was first used to successfully restore hearing in mice with a null mutation in the gene coding for vesicular glutamate transporter-3 (VGLUT3).14 Subsequent studies also confirmed the usefulness of gene replacement in treating hereditary hearing loss,15-19 but gene replacement cannot precisely regulate gene expression according to the needs of the cells and it would be less effective when the mutant transcript is dominant-negative. Gene editing technology as a novel method of gene therapy has been applied to the treatment of genetic hearing loss, and delivering the CRISPR/Cas system into the inner ear successfully ameliorates hearing loss in Bth model mice.20-22 In addition, packaging cytosine base editors into dual AAVs restored gene function in Baringo mice that carry a recessive loss-of-function point mutation in the Tmc1 gene, and this demonstrated that in vivo base editing could partially and transiently rescue auditory function.23,24 However, genome editing...
might induce off-target mutations in DNA sequences that are similar to the targeting sequence, which limits the usefulness of such technology, especially for therapeutic and clinical applications.\textsuperscript{25,26} Genetic therapies on the RNA level, however, only modify the expression of target RNA without affecting the DNA. In recent years, RNA regulation has been used to treat hearing loss in mice. For example, antisense oligonucleotides were applied in a mouse model to rescue the inner ear mutation of \textit{Ush1c} (c.216G > A), the splice variant causes disruption of wild-type spliceing, which results in a frameshift and a truncated protein;\textsuperscript{27} RNA interference and artificial microRNA reduced RNA expression and protected against hearing loss.\textsuperscript{28–30} However, widespread off-target transcript silencing by these traditional RNA regulating tools has been a consistent concern.\textsuperscript{11,12}

CRISPR/Cas13, as a new RNA interference tool, is a class 2 type VI CRISPR/Cas RNA endonuclease initially used to mitigate viral infection in bacteria\textsuperscript{31} and it has higher specificity than traditional RNA interference tools.\textsuperscript{34} Four members of the Cas13 protein family have been identified, including Cas13a (previously known as C2c2),\textsuperscript{35} Cas13b,\textsuperscript{36} Cas13c,\textsuperscript{37} and Cas13d.\textsuperscript{38} It has been reported that PspCas13b and CasRx have higher activity and specificity than other Cas13s.\textsuperscript{36,39} As the most compact Cas13 enzymes at present, CasRx can be easily packaged into AAVs,\textsuperscript{12} and this makes it convenient to deliver the CRISPR/CasRx system in vivo. CasRx has been applied as a therapeutic tool in mouse models of liver and eye ailments,\textsuperscript{39–41} and compared to other gene editing systems the RNA editing system can provide a much safer approach for gene silencing without permanently altering the genome.\textsuperscript{42} In addition, the protospacer flanking sequence (PFS) is necessary for most Cas13s, limiting the selection of sgRNA sequences, especially for specific pathogenic point mutations. In contrast, there is no PFS restriction in the CasRx system, and thus a broader array of gRNAs can be designed and screened.

There is still a lack of studies using the CRISPR/Cas13 RNA editing system for hereditary deafness therapy. To explore the potential therapeutic effects of CRISPR/Cas13, we screened 30 sgRNAs that match the single point mutation at all possible positions to target the pathogenic allele of \textit{Tmc1}, and we compared the editing specificity and efficiency between PspCas13b and the CasRx system to select an optimal sgRNA. We used a newly developed AAV vector, AAV-PHP.eB,\textsuperscript{43} with high transduction efficacy for inner ear hair cells to deliver CasRx and sgRNA to the natal mouse cochlea and successfully downregulated the expression of the \textit{Tmc1} transcript. The change of mRNA expression ratio of \textit{Tmc1} in CasRx compared to the control. The point mutation A was presented in the 5' end of the sequence (Fig. 1a). We co-transfected the reporters, the PspCas13b or CasRx expression vectors, and the sgRNA expression vectors into 293T cells, and after 48 h of transfection the RNA editing system disrupted the expression of the fused RNA and the fluorescence intensity of the cells was measured as an indication of the RNA knockdown efficiency. Next, the ratio of the averaged fluorescence intensities was calculated between targeted and non-targeted sgRNA cell wells (Fig. 1b, c), for mCherry-\textit{Tmc1} \textsuperscript{fl} or mCherry-\textit{Tmc1} \textsuperscript{NT} cell wells. Lower ratio indicates higher targeting specificity. As expected, CasRx and PspCas13b resulted in a dramatic decrease in mCherry expression in 293T cells (Supplementary Fig. S2). The lowest ratios of fluorescence intensities of the cells were 9.2 ± 0.13% and 17.44 ± 0.48% when using the CasRx and PspCas13b systems, respectively, to target mCherry-\textit{Tmc1} \textsuperscript{fl} (Fig. 1b, c). Further, in order to compare the specificity of the two systems, we measured the mean fluorescence intensities by targeting mCherry-\textit{Tmc1} \textsuperscript{fl} and we analyzed the ratio of the mean fluorescence intensities between mCherry-\textit{Tmc1} \textsuperscript{fl} and mCherry-\textit{Tmc1} \textsuperscript{mRNA} interference (Fig. 1d). The sgRNA3 in the CasRx system exhibited the lowest ratio at 0.089113, which decreased about 90.8% mCherry-\textit{Tmc1} \textsuperscript{fl} and 44.3% mCherry-\textit{Tmc1} \textsuperscript{mRNA} respectively (Fig. 1d). The additional experiment results showed in Fig. S3, the knockdown efficiency of sgRNA3-mediated knockdown of \textit{Tmc1} \textsuperscript{fl} and \textit{Tmc1} \textsuperscript{mRNA} were 91.9% and 43.4%, respectively (Supplementary Fig. S3a), and the ratio was 0.07854677 (Supplementary Fig. S3b). The results showed that mCherry integrated density significantly decreased by 83.7% which was mediated by sgRNA3 in CasRx system (Fig. 1e), indicating the efficient knock-down of the \textit{Tmc1} \textsuperscript{fl} transcript. Taken together, these results demonstrated that sgRNA3 in CasRx was the ideal sgRNA for efficient knockdown of the \textit{Tmc1} \textsuperscript{fl} transcript with significantly less knockdown of \textit{Tmc1} \textsuperscript{mRNA} transcript.

Off-target analysis of CasRx-mediated RNA knockdown in 293T cells

Four experiment groups were conducted, including CasRx + sgRNA3 + mCherry-\textit{Tmc1} \textsuperscript{fl}, CasRx + sgRNA3 + mCherry-\textit{Tmc1} \textsuperscript{mRNA}, sgRNA3 and EGFP, 3 replicates per group. 5 × 10\textsuperscript{4} EGFP\textsuperscript{+} positive cells per sample were collected by FACS. Then we extracted the total RNA of these cells for RNA-Sequencing. We first examined the RNA-Sequencing data at the whole genome level, and we found the gene expression profiles of both CasRx + sgRNA3 + mCherry-\textit{Tmc1} \textsuperscript{fl} and CasRx + sgRNA3 + mCherry-\textit{Tmc1} \textsuperscript{mRNA} are comparable to that of EGFP (Supplementary Fig. S4). To further quantify possible off-target effects, we screened the top 10 most likely off-target genes according to the 30 bp sgRNA sequence by aligning on human whole genome (Fig. 2a). Among these 10 genes, two were not detectable in expression for all groups. For the other 8 genes, we found no significant expression difference in CasRx + sgRNA3 + mCherry-\textit{Tmc1} \textsuperscript{fl}, CasRx + sgRNA3 + mCherry-\textit{Tmc1} \textsuperscript{mRNA} or sgRNA3, when compared to EGFP (Fig. 2b and Supplementary Table S4). These results suggested that CasRx-mediated RNA knockdown had no off-targets in 293T cells.

Specific targeting mediated by CasRx in vivo

Previous results demonstrated that sgRNA3 in the CasRx system had the highest efficiency in targeting \textit{Tmc1} \textsuperscript{fl} transcript in 293T cells, to determine whether AAV-CasRx + sgRNA3 targets \textit{Tmc1} \textsuperscript{fl} in vivo, an AAV vector encoding sgRNA3 and CasRx was used to downregulate the \textit{Tmc1} \textsuperscript{fl} transcript in the inner ears of \textit{Bth} mice, and non-target sgRNA was packaged in the same vector as a control (Fig. 3a). We used the engineered AAV-PHP.eB, which is the more efficient and further evolved AAV variant of the PHP serotype, as the delivery vector.\textsuperscript{44} Toordered the validate ability of AAV-PHP.eB to deliver genes into inner hair cells (IHCs) and outer hair cells (OHCs), we injected AAV-PHP.eB encoding EGFP into the right inner ear of postnatal day (P1)–P2 mice through the round window membrane. The cochleae were collected 2 weeks after injection, and the organs of Corti were dissected for immunohistochemistry (Fig. 3b). We observed a nearly 100% viral transduction efficiency in IHCs and OHCs showed over 95% viral transduction efficiency
that decreased from the apical to basal turns (Supplementary Fig. S5a), which was consistent with our previous research.43

To determine the editing ability of CasRx in vivo, we performed targeted deep sequencing from whole cochlear tissues. The cochleae from Bth mice injected with AAV-CasRx + sgRNA3 showed that 14.88 ± 9.77% of the total Tmc1 transcripts were Tmc1Bth transcripts, which was significantly decreased compared to non-injected mice (52.83 ± 5.33%) and mice injected with AAV-casRx + NT (control AAV, non-targeting sgRNA) (53.39 ± 4.8%) (Fig. 3c and Supplementary Table S3). The actual ratios of the

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Fig. 1 Screening for efficient and specific sgRNAs for targeting the Tmc1Bth transcript. a Constructs used for the sgRNA screen mediated by the Cas RNA editing system. Five vectors were constructed, including the Cas expression vector, the mCherry-Tmc1Bth fluorescence reporter, the mCherry-Tmc1+ fluorescence reporter, the sgRNA expression vectors for targeting the Tmc1Bth transcript, and the non-targeting (NT) sgRNA expression vector. b Ratios of fluorescence intensity with sgRNAs compared to control sgRNA (NT) for targeting mCherry-Tmc1Bth mRNA and mCherry-Tmc1+ mRNA mediated by CasRx system. Data are shown as the mean ± SD (n = 3 biologically independent samples). c Ratios of fluorescence intensity with sgRNAs compared to control sgRNA (NT) for targeting mCherry-Tmc1Bth mRNA and mCherry-Tmc1+ mRNA mediated by PspCas13b system. Data are shown as the mean ± SD (n = 3 biologically independent samples). d Mean ratio of fluorescence intensities between mCherry-Tmc1Bth and mCherry-Tmc1+ mRNA. sgRNA3 has the lowest mean ratio of fluorescence intensity for all the 30 sgRNAs tested. e The integrated fluorescence intensity of cells with CasRx system. The integrated fluorescence density was significantly decreased with sgRNA3 targeting mCherry-Tmc1Bth mRNA compared to targeting mCherry-Tmc1+ mRNA, and integrated fluorescence density was decreased compared to targeting mCherry-Tmc1Bth and mCherry-Tmc1+ mRNA with non-targeting sgRNA. Data are shown as the mean ± SD (n = 5 biologically independent samples). ***p < 0.001, P-values were determined by one-way ANOVA with Sidak’s multiple comparisons test.

Fig. 2 Off-target analysis for RNA editing in 293T cells by RNA-Seq. a Off-target-1 to Off-target-10 are ten off-target sites detected by RNA-seq. Mismatches compared to the on-target site are shown and highlighted in color. The 30 bp sequence (On-target) targeted by the sgRNA3 is shown in the top row. b Off-target analysis for RNA editing in 293T cells by RNA-Seq. No significant difference was found in CasRx + sgRNA3 + mCherry-Tmc1Bth, CasRx + sgRNA3 + mCherry-Tmc1+ or sgRNA3, when compared to EGFP. Data are shown as the mean ± SD. ns no significance. Statistical analysis was performed by multiple unpaired t-test.
$Tmc_{1}^{Bth}$ and $Tmc_{1}^{+}$ transcripts were $1.1397 \pm 0.2584$, $1.1605 \pm 0.2183$, and $0.186 \pm 0.1457$ for non-injected, AAV-CasRx + NT, and AAV-CasRx + sgRNA3, respectively (Fig. 3d). This indicated a knockdown efficiency of 70.2% for the $Tmc_{1}^{Bth}$ transcript.

To expand our analysis of the expression of $Tmc_{1}$ in mouse, we used RT-qPCR to measure gene expression at the RNA level. We injected AAV-EGFP and AAV-CasRx + sgRNA3 in wild-type mice, and compared the expression in 2 weeks after injection. Both vectors showed highly expression in injected cochleae comparing to controls (Fig. 3e and Supplementary Fig. S5b). We next injected AAV-CasRx + sgRNA3 in $Bth$ mice, and observed decreased expression of total $Tmc_{1}$ compared to non-injected contralateral cochleae (Fig. 3e). RNA level of $Tmc_{1}^{Bth}$ was assessed with a pair of primers to specifically target $Tmc_{1}^{Bth}$ cDNA (Fig. 3f). $Tmc_{1}^{+}$ mRNA level was detected using specific primers by RT-qPCR. Results showed that mRNA levels of $Tmc_{1}^{+}$ and $Tmc_{1}^{Bth}$ were
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Fig. 3 CasRx selectively disrupts the Tmc1Bth transcript in Bth mice. a Schematic of the AAV vector encoding CasRx and sgRNA3 (upper), and a control NT vector (lower). b Outline of the in vivo experiments. Mice were injected with AAV (~5 × 10⁹ vg) at P1–P2, and the organs of Corti were dissected and cultured at P5, and hair cell physiology was measured at P15–P16. Injected mice were sequenced after 2 weeks following hearing tests (ABR and DPOAE) after 4, 8, and 12 weeks, immunohistochemistry, and scanning electron microscopy at 10 weeks after injection. c The percentage of deep sequencing reads of Tmc1Bth and Tmc1. Pie charts indicate the mean composition of Tmc1Bth and Tmc1 transcripts in these samples, showing the single-nucleotide difference between Tmc1Bth and Tmc1 transcripts (52.83 ± 5.33% for Tmc1Bth and 48.4% and 14.88 ± 9.77% Tmc1Bth transcript for non-injected, injected with AAV-CasRx + NT, and injected with AAV-CasRx + sgRNA3, respectively. n = 3 mice, data are shown as the mean ± SD). d Deep sequencing analysis of the ratios of transcripts between Tmc1Bth and Tmc1 for non-injected mice (n = 3 mice), mice injected with AAV-CasRx + NT (n = 3 mice), and mice injected with AAV-CasRx + sgRNA3 (n = 3 mice), respectively. Data are shown as the mean ± SD, **p < 0.01, P-values were determined by one-way ANOVA with Dunnett’s multiple comparisons test. e mRNA expressions in the cochlea at 2 weeks after injection as measured by RT-qPCR. The expression of CasRx mRNA (n = 11 mice), total Tmc1 mRNA (n = 5 mice), Tmc1Bth mRNA (n = 4 mice), and Tmc1Bth mRNA (n = 5 mice) between injected with AAV-CasRx + sgRNA3 and non-injected contralateral ears were showed in graphs. Relative mRNA expression levels were calculated with the ΔΔCt algorithm. Data are shown as the mean ± SD, **p < 0.05, ***p < 0.001, P-value was determined by unpaired two-tailed t-test. f Amplification of the Tmc1Bth sequence. The amplicon was detected by a pair of specific targeting primers with heterozygous templates, and the primers cannot amplify with the wild-type template. g Representative MET recordings and maximal MET current amplitudes of apical IHCs at the equivalent of P15–P16. h The MET current amplitude was 461.134 ± 74.978 pA, 442.458 ± 82.805 pA, and 344.409 ± 114.591 pA in Tmc1Bth/Bth (n = 3 mice) between injected with AAV-CasRx + sgRNA3 and non-injected contralateral ears were showed in graphs. Data are shown as the mean ± SD. The percentage of deep sequencing reads of Tmc1Bth transcripts in these samples, sequences show the single-nucleotide difference between Tmc1Bth and Tmc1 transcripts (52.83 ± 5.33%, 53.59 ± 4.8%, and 14.88 ± 9.77% Tmc1Bth transcript for non-injected, injected with AAV-CasRx + NT, and injected with AAV-CasRx + sgRNA3, respectively. n = 3 mice, data are shown as the mean ± SD). Deep sequencing analysis of the ratios of transcripts between Tmc1Bth and Tmc1 for non-injected mice (n = 3 mice), mice injected with AAV-CasRx + NT (n = 3 mice), and mice injected with AAV-CasRx + sgRNA3 (n = 3 mice), respectively. Data are shown as the mean ± SD, **p < 0.01, P-values were determined by one-way ANOVA with Dunnett’s multiple comparisons test.

Prevention of progressive hearing loss by RNA knockdown in vivo

The convincing therapeutic effects were obtained because CasRx disrupted the Tmc1Bth transcript guided by sgRNA3 with less interfering in Tmc1 transcript. Hearing in Bth mice was protected when the Tmc1Bth transcript was disrupted and the level of the harmful protein decreased, while the control mice injected with AAV encoding a non-targeting (NT) RNA had progressive hearing loss (Supplementary Fig. S5e). To measure hearing function in the injected cochlea, we performed auditory brainstem response (ABR) tests every 4 weeks because the Tmc1 induction mechanisms progresses progressively into hearing loss. We measured tone-burst ABRs at frequencies of 4, 8, 16, 24, and 32 kHz, and ABR waveforms recorded at 8 kHz showed that injection of CasRx + sgRNA3 greatly improved hearing function compared to non-injected controls (Fig. 4a). At the fourth week after injection, Bth mice injected with AAV-CasRx + sgRNA3 had lower ABR thresholds at all frequencies (57 ± 9, 47 ± 11, 65 ± 8, 70 ± 8, and 75 ± 7 dB at 4, 8, 16, 24, and 32 kHz, respectively) compared to non-injected contralateral ears (77 ± 5, 67 ± 13, 78 ± 4, 78 ± 4, and 82 ± 5 dB at 4, 8, 16, 24, and 32 kHz, respectively) (Fig. 4b). ABR thresholds of Bth mice were not reduced at 4 weeks after injecting AAV-CasRx + NT (Supplementary Fig. S7a), and either AAV-CasRx + sgRNA3 nor AAV-Cas + NT had any impact on hearing in wild type mice (Supplementary Fig. S7b). At 8 weeks after injection, the ABR thresholds rose in both ears, but the treated ear still had lower thresholds at low frequency (72 ± 7, 65 ± 9, and 78 ± 7 dB for 4, 8, and 16 kHz, respectively) compared to non-injected ears (84 ± 7, 82 ± 6, and 87 ± 5 dB for 4, 8, and 16 kHz, respectively) (Fig. 4b).

We further measured click-evoked ABRs and found that the thresholds in the injected ears were significantly decreased at 4 and 8 weeks after injection, which was consistent with the pure tone ABR results showing that AAV-CasRx + sgRNA3 injection slowed the progressive hearing loss in Bth mice (Fig. 4c). Click-evoked ABR peak 1 (P1) amplitudes at 80 and 90 dB showed increase in AAV-CasRx + sgRNA3 injected Bth mice compared to non-injected mice at 4 weeks, latencies of P1 waves of injected Bth mice were also normalized by injection (Supplementary Fig. S7c, d).

We also measured the distortion product otoacoustic emissions (DPOAEs) to evaluate the function of OHCs (Fig. 4d). Ears injected with AAV-CasRx + sgRNA3 showed lower DPOAE thresholds at 8 kHz and 16 kHz at 4 and 8 weeks after injection (65 ± 10 and 71 ± 9 dB for 8 and 16 kHz at 4 weeks, and 76 ± 5 and 79 ± 3 dB for 8 and 16 kHz at 8 weeks), while the non-injected ears lacked DPOAE indicating a lack of OHCs function.

These results suggested that mRNA knockdown mediated by AAV-CasRx + sgRNA3 could improve the hearing function over a period 8 weeks.

Preservation of hair cells and stereocilia bundle morphology mediated by CasRx

To determine whether CasRx and sgRNA3 can preserve hair cells and hair bundle morphology, we sacrificed the mice at 10 weeks of age and performed confocal and scanning electron microscopy (SEM) analyses. We found that OHCs in the apical turn of the organ of Corti (8 kHz region) began to be lost, and OHC loss became more severe from the middle turn (16 kHz region) to the basal turn (32 kHz region), where the OHCs were almost completely absent (Fig. 5a). IHCs in the apical turn remained intact, while some IHCs were lost in the middle turn and IHCs were completely absent in the basal turn (Fig. 5a). These results were consistent with those of previous studies. In the cochleae injected with AAV-CasRx + sgRNA3, the survival of both IHCs and OHCs was improved, and the number of OHCs per 100 μm of the cochlea increased in the 8 and 16 kHz regions (37.0 ± 1.6, 39.8 ± 1.6 compared to 26.2 ± 10.1, 22.6 ± 9.1) while the IHC number increased in the 16 kHz region (11.4 ± 2.5 compared to 1.2 ± 1.3) (Fig. 5b).

We next used SEM to evaluate the hair bundle morphology. The OHCs and IHCs of wild-type Tmc1Bth/Bth mice at 10 weeks of age showed neatly arranged hair bundles, while the non-injected mice showed severe bundle disorganization. Hair cells injected with…
AAV-CasRx + sgRNA3 had normal IHC and OHC morphology in the apical turn (Fig. 5c), and hair bundles were preserved in the middle region (Supplementary Fig. S8). These results were consistent with the ABR data showing the protection of hearing in the lower frequency region (4–8 kHz).

Off-target analysis of CasRx-mediated RNA knockdown in vivo
We performed RNA-seq in the cochleae collected 2 weeks after AAV injection. We screened the top 10 most likely off-target genes according to the 30 bp sgRNA sequence by aligning on mouse whole genome (Fig. 6a), and we analyzed the expression difference between the AAV-CasRx + sgRNA3-injected (n=3 mice) and non-injected (n=3 mice) groups. There was no difference in RNA expression for 9 of the 10 genes, one gene (Gm13492) was not detected (Fig. 6b and Supplementary Table S4). Nonetheless, the RNA expression differences were limited, suggesting that CasRx-mediated RNA knockdown had few off-target effects.
Fig. 5  Injection of AAV-CasRx + sgRNA3 (~5 × 10⁹ vg of AAV) protects hair cells and hair bundles. a Representative confocal images of 100 μm cochlear sections harvested 10 weeks after injection. Samples were stained with myosin7a (Red). The images are from Tmc1⁺/⁺, Tmc1Bth⁺/⁺ + AAV-CasRx + sgRNA3, and Tmc1Bth⁻/⁻ non-injected mice (n = 5 mice) at locations corresponding to 8 and 16 kHz. The IHCs and OHCs are indicated. Scale bar: 20 μm. b The number of OHCs (upper) and IHCs (lower) per 100 μm of the cochleae. Data are shown as the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Statistical analysis was performed by two-way Sidak’s multiple comparisons test. c SEM images of the apical cochlear sensory epithelium showing the morphology of the hair cell bundles. Tmc1⁺/⁺, Tmc1Bth⁺/⁺ + AAV-CasRx + sgRNA3, and Tmc1Bth⁻/⁻ non-injected samples were collected 10 weeks after injection. Scale bars: 20 μm (upper); 3 μm (lower).
DISCUSSION

**TMC1** mutations account for 4–8% of all cases of heritable hearing loss in the world. In this study, we used the CRISPR/CasRx system to downregulate the *Tmc1* mRNA transcript in the *Bth* mouse model of human genetic deafness with less knockdown of *Tmc1* transcript (there is only a single nucleobase difference between the two transcripts) both in vitro and in vivo. The new, highly efficient AAV-PHP.eB delivery system targeting for hair cells packaged CasRx and sgRNA3 was delivered into the cochlea of *Bth* mice, resulting in preservation of auditory function due to the preservation of hair cell survival and rescue of hair bundle degeneration. These results demonstrate that the CasRx system can successfully ameliorate dominant-negative hearing loss by specifically knocking down the mutant transcript.

The CasRx RNA editing system showed high knockdown efficiency in our study, and co-transfection of vectors encoding the exogenous *Tmc1* sequence, CasRx, and sgRNA into 293T cells resulted in more than 80% knockdown of the *Tmc1* mRNA transcripts. CasRx RNA knockdown also showed over 70% RNA knockdown in vivo, and two main factors contributed to the high efficiency. First, CasRx is the smallest protein among the Cas13 family of proteins, which makes it possible to package into a single AAV. Second, different AAV serotypes induce different transduction efficiencies, and several evolved AAVs have been confirmed to be safe and efficient for delivering genes into the inner ear. We previously demonstrated that the AAV-PHP.eB vector had extremely high transduction efficiency in cochlear IHCs and OHCs in vivo. The vector has potential to deliver CasRx and sgRNA to hair cells with a transduction efficiency of over 95% (Supplementary Fig. S5a). Previous study showed Cas9 and sgRNA induced target gene disruption at about 25% efficiency in hair cells following RNP delivery, indicating that AAV-PHP.eB is more effective compared to non-viral vector.

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**Fig. 6** Off-target analysis for RNA editing in vivo by RNA-Seq. **a** Off-target-1 to Off-target-10 were ten off-target sites detected by RNA-seq. Mismatches compared to the on-target site are shown and highlighted in color. The 30 bp sequence (On-target) targeted by the sgRNA3 is shown in the top row. **b** Comparison of FPKM values of the ten off-target sites at injected AAV-CasRx + sgRNA3 or non-injected ears. Data are shown as the mean ± SD, ns no significance. Statistical analysis was performed by multiple unpaired t-test.
CasRx-mediated RNA knockdown ameliorated the auditory function in Bth mice. In our results, we detected the hearing was improved about 10–20 dB at 4 and 8 weeks in Bth mouse, the hearing loss was still be prevented in 12 weeks at low frequencies (data not shown), our data were consistent and comparable with the amelioration effects by SaCas9 based DNA editing technology which showed an improved ABR function over 12 weeks after injection.\(^{21}\) The numbers of IHCs and OHCs in the treated ear were greater than in the untreated ears, which was generally consistent with the improved hearing. In the apical turn, the stereocilia bundles were disorganized in the Bth mouse model, and this prevented sound from being transmitted thus resulting in higher ABR thresholds at low frequency. This explains why the hearing thresholds at low frequencies were high even though the hair cells were present. We demonstrated that the hair bundle morphology of IHCs and OHCs in injected ears was much better than non-injected ears in apical turn and the hearing was preserved very well at low frequency. Furthermore, injection with control AAV (AAV-CasRx + NT) didn't rescue hearing on the heterogeneous mouse ears (Supplementary Fig. S7a), then we injected AAV-CasRx + sgRNA3 and control AAV on wild-type mouse and found that no ABR threshold shifts were observed comparing with non-injected ears (Supplementary Fig. S7b), indicating that the hearing loss was prevented by AAV-CasRx + sgRNA3 editing specifically, the injection of AAV-CasRx + sgRNA3 was safe and it did not affect normal hearing function. Despite our positive results, we found that auditory function was still not completely rescued by injecting AAV-CasRx + sgRNA3 compared to wild-type mice especially in the high-frequency region, explanations for the results include that degeneration of hair cells begins in the base and spreads apically, and the efficiency of RNA knockdown is relative lower to overcome the dominant-negative effects of mutant \(Tmc1\) protein in the basal turn compared to apical turn. Although the knockdown efficiency of mutant transcript is high, the interfering of wild-type transcript can not be ignored, and RNA base editing technology with improved precision and specificity remains to developed to directly convert the mutant base into normal, hopefully in the near future. Since the space of cochlea is limited, introducing more volume is impossible, a research team developed a surgical approach of injection route by round window membrane injection combining with semi-circular canal fenestration.\(^{58}\) We might need to improve the targeting efficiency for better recovery of auditory function by multiple-site injection in cochlea and selecting more effective AAV vector to improve the transduction efficiency in the basal turn. Another question needs to be explored more systematically is the window of injection time for effective rescuing of hearing function, as this type of dominant-negative hearing loss is postlingual and progressive, leaving a large window for therapy. The neonatal mouse inner ear is partly developed and is equivalent to human cochlea at 26-week gestational age,\(^{51}\) so that injection in mouse inner ear in a later postnatal time is necessary to investigate the protective effects in clinical practice. The previous report showed hearing function was improved by injection AAV-mediated RNAi in Bth mouse at ages before 8 weeks, but protective effects degraded when treatment-time was late.\(^{59}\) Whether injection of CasRx in juvenile and adult mice could prevent hearing loss is needed for future work.

RNA editing based on the CasRx system has some advantages in disease treatment. At the RNA expression level, CasRx-mediated knockdown has been shown to substantially reduce off-target effects compared to RNA interference knockdown,\(^{31,34,36,38}\) and we didn’t detect any off-target effects in vitro and in vivo. At the gene editing level, using the CRISPR system to target RNA can avoid the risks associated with permanent DNA alteration.\(^{42,52}\) In our study, we compared the CasRx and PspCas13b systems and found that CasRx had higher efficiency (Fig. 1b, c) and specificity (Fig. 1d). The potential toxic effects of CasRx system in vivo are difficult to be ruled out completely, but we did not detect anything significant. Consistently, a recent study showed that CasRx displayed no toxic effects by injection with up to 300 pg mRNA encoding CasRx protein into per zebrafish embryo, and in mouse embryos, injection with up to 25–50 pg CasRx together with sgRNA to downregulate target mRNA is tolerated without deleterious effects.\(^{53}\) The potential toxic effects of CasRx system delivered by AAV was also evaluated in vivo, study tested the knockdown effect via tail-vein injection of CasRx and sgRNA packaged in AAV8, the target mRNA level was significantly reduced after AAV injection in mouse liver, but the level of serum ALT and AST was similar in the AAV-injected mice and non-injected mice, indicating that CasRx system delivered by AAV had no toxic effects in vivo.\(^{57}\) These findings suggest that the CasRx system might be a safe and highly efficient RNA knockdown tool when applied in clinical therapeutics in the future.

In conclusion, 85% of all human autosomal-dominant non-syndromic hearing loss is caused by missense mutation.\(^{54}\) RNA knockdown strategy could be broadly applicable to this type of hearing loss. We applied CasRx RNA knockdown to prevent hearing loss in the Bth mouse model as a proof-of-concept study. It may also be worth determining whether the CasRx system is effective at treating deafness in adult mice. Anyway, we conclude that CasRx has great potential in treating human dominant-negative hearing loss in the future.

**MATERIALS AND METHODS**

**Plasmids**

The human codon-optimized CasRx gene was synthesized and cloned into a mammalian expression vector with two NLS (Nuclear localization sequence) under the control of the CAG promoter, and the human codon-optimized PspCas13b gene was synthesized and cloned into a mammalian expression vector with a NES (Nuclear export sequence) under control of the CAG promoter. We then constructed the CasRx sgRNA cloning backbone, which contains two direct repeats for cloning with BspQI enzyme, and constructed the PspCas13b sgRNA cloning backbone, which contains a 3′ direct repeat for cloning with BbsI enzyme. We synthesized sgRNAs as single-stranded DNA oligos. The sgRNA oligos were annealed and cloned under the U6 promoter using the BspQI enzyme in an sgRNA expression vector containing the U6 promoter element and two BspQI enzyme sites for the CasRx system, and they were cloned under the U6 promoter using the BbsI enzyme in an sgRNA expression vector containing the U6 promoter element and two BbsI enzyme sites for the PspCas13b system.

To construct the \(\text{mCherry-}Tmc1^{\text{1Bth}}\) reporter vector, 90 bp sequences of the \(Tmc1\) transcript that contained the c.1235T > A mutation were synthesized and cloned into the 3′ end of the \(\text{mCherry}\) gene in which the stop codon had been removed. To construct the \(\text{mCherry-}Tmc1^{+}\) reporter vector, 90 bp sequences of the \(Tmc1\) transcript that contained the c.1235T were synthesized and cloned into the 3′ end of the \(\text{mCherry}\) gene in which the stop codon had been removed (Supplementary Table 2).

**Cells culture and transfection**

We cultured 293T cells in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (v/v) (Gemi) at 37 °C with 5% CO\(_2\) under humidified conditions. Before transfection, cells were seeded on poly-D-lysine-coated 24-well plates and maintained at approximately 60–70% confluence. Cells were transfectioned using EZ Trans Reagent (Shanghai Life Lab) according to the manufacturer's protocols. For transfection, CasRx or PspCas13b-expressing plasmid (600 ng), sgRNA-expressing plasmid (300 ng), and \(\text{mCherry-}Tmc1^{\text{1Bth}}\) or \(\text{mCherry-}Tmc1^{+}\) reporter were mixed in each well. A total of 1 μg DNA and 3 μL EZ Trans Reagent were diluted in 40 μL DMEM separately. The diluted EZ
Trans Reagent was then added to the diluted DNA solution, mixed gently, and incubated for 15 min at room temperature to form DNA-EZ Trans Reagent complexes. The DNA-EZ Trans Reagent complexes were then directly added to each well and mixed gently by rocking the plate back and forth. At 6 h post-transfection, the complexes were removed and 0.5 mL complete growth medium was added to the cells.

Fluorescence-activated cell sorting (FACS)
293T cells were harvested and subjected to flow cytometry 48 h after transfection. mCherry signals were immediately detected on a BD LSRFortessa flow cytometer (BD Biosciences) with FCS Express 5 software (De Novo Software). A total of 10,000 cell events were collected and analyzed with the FlowJo software for each sample.

Integrated fluorescence intensity
All fluorescence images were acquired with a Nikon Ti-E microscope (Tokyo, Japan), and the Image J software (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze the integrated fluorescence densities of mCherry. At 48 h post-transfection, we captured images of three randomly selected 1300 × 1300 μm regions in each cell well. We then used Image J to extract fluorescence intensity values from all cells in the region, which was averaged across three regions for each cell well. Use image J software to quantitatively analyze fluorescence intensity steps: Firstly, overlap all the 2D diagrams and select max intensity. Secondly, adjust threshold selects all the area of visual field of the cell image for calculation, and the selected area is highlighted in red. Subtract the background until the red highlighted plaque is the desired cell. Finally, calculate the mean integrated fluorescence intensity of each well cells and statistics the ratio between cell wells.

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Virus production
AAV (Phi.E8 serotype) viral vectors were produced by OBIO Technology Corp., Ltd. (Shanghai, China). AAV carrying a dual transgene cassette, a U6 promoter-driven sgRNA targeting Tmc1, and a CMV-driven RfxCas13 promoter were packaged as therapeutic vectors. The control AAV was constructed consistently with the therapeutic vector except that the sgRNA sequence was replaced by a NT sequence. The same serotype AAV encoding an EGFP tag was used for testing the transduction efficiency. Viral titers were 3.38 × 10<sup>12</sup> vg/mL for AAV-CasRx + sgRNA3 and 1.73 × 10<sup>12</sup> vg/mL for AAV-CasRx + NT. Virus aliquots were separated into small volumes and stored at –80 °C to avoid repeated freeze-thaw cycles.

Mice
All animals were bred and housed in our facilities with a 12 h light-dark cycle. Heterozygous Beethoven mice (Tmc1<sup>Bth+</sup>/+) were obtained as a gift from Dr. Andrew Griffith (Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University). Tmc1<sup>Bth+</sup> mice were inbred with Tmc1<sup>+/+</sup> or Tmc1<sup>Bth+</sup> C3HeB/FeJ (C3H) background mice (Jackson Laboratories) to propagate new pups. DNA was extracted from tail-clip biopsies using lysis solution mixed with proteinase-K at 55 °C for 8 h and then at 85 °C for 1 h, and genotyping was performed by PCR with primers in a 20 μL volume according to a previously published protocol.<ref>

Inner ear injection
Tmc1<sup>Bth+</sup> or wild-type mouse pups were injected between P1 and P2 with 1.5 μL virus (~5 × 10<sup>5</sup> vg) via the round window membrane. Pups were anesthetized on ice for 2–3 min until loss of consciousness. Upon anesthesia, a post-auricular incision was made to expose the otic bulla and visualize the round window membrane. Virus was slowly microinjected into the right cochlea using a glass micropipette. After injection, the skin incision was closed using a suture, and the pup was placed on a 42 °C heating pad for recovery. Pups were returned to the parental cage after they fully recovered.

Hair cell electrophysiology
Cochlea of Tmc1<sup>+/+</sup>, Tmc1<sup>Bth/Bth</sup>, or Tmc1<sup>Bth/Bth</sup> injected mice were harvested at P4-5 and cultured in DMEM (1X) medium with 1% FBS at 37 °C in a 5% CO<sub>2</sub> atmosphere. Whole-cell patch-clamp recording was performed in IHCs at P14–15, in a standard artificial perilymph solution containing (in mM) 137 NaCl, 5.8 KCl, 1.3 CaCl<sub>2</sub>, 0.9 MgCl<sub>2</sub>, 0.7 NaH<sub>2</sub>PO<sub>4</sub>, 10 HEPES, and 5.6 D-glucose, with pH adjusted to 7.40 and osmolarity to ~300 mmol/kg. Recording pipettes were pulled from borosilicate glass capillaries (18150F-4, World Precision Instruments Inc., Florida, USA) and filled with an internal solution containing (in mM) 140 CsCl, 0.1 EGTA, 1 MgCl<sub>2</sub>, 10 HEPES, 2 Mg-ATP and 0.3 Na-ATP (pH 7.20, ~295 mmol/kg). Mechano-electrical transduction (MET) currents were recorded under voltage-clamp with a holding potential of ~80 mV, through an EPC10/2 amplifier (HEKA, Lambrecht/Pfalz, Germany) driven by a PC running Patchmaster (HEKA). Current signals were filtered at 2 kHz and digitized at 200 kHz. Hair bundles were deflected with a fluid jet, delivered through a pipette with a tip of ~10 μm, positioned ~15 μm away. The fluid jet was driven by a piezoelectric disc (27 mm in diameter) with sinusoidal voltage commands applied (40 Hz, ±120 V).

Targeted deep-sequencing data analysis
To analyze the CasRx knockdown mutant sequence at the RNA level, total RNA from the cochlea and cDNA were obtained as described above. Target site sequences were amplified with primers TMC1-lib-F and TMC1-lib-R (Supplementary Table 2). PCR products were visualized on 2% agarose gels and purified using a purification kit (Qiagen). Pair-end reads (150 base pairs) were generated on an Illumina MiSeq platform. The reads from heterozygous samples were segregated based on the presence of the wild-type sequence (5′-ATG CCT CTT GAG GTT GTT CTC CAC C-3′ and its reverse complement 5′-GTT GGG ACA GAA CAT CCC CAG GGA CAT-3′) and the mutant sequence (5′-ATG CCT CTC CTT GGG AAG TTC TGT TGG CCC ACC-3′) and its reverse complement 5′-GTT GGG ACA CTT CCC CAG GGA CAT-3′). The knockdown efficiency was calculated as (theoretical proportion – actual proportion)/theoretical proportion) × 100%.

RT-qPCR of the cochlea
Cochlea from wild-type or heterozygous mice were dissected. Total mRNA was extracted from the organs of Corti by TRIzol (Invitrogen) and mRNA was reverse-transcribed using a cdNA synthesis supermix (YEASEN) according to the manufacturer’s protocol. One microliter of RT product was added to RT-qPCR SYBR (YEASEN) for subsequent RT-qPCR with the following steps: 95 °C for 5 min and 40 cycles of 95 °C for 10 s and 60 °C for 35 s. Primers were designed to detect the total Tmc1 expression level: q-TMC1-F2 and q-TMC1-R2. To detect the mutant transcript expression, the forward primer, q-TMC1-F4 was designed with a 3′ end ‘A’ that specifically bound to the mutant sequence; to detect the wild-type transcript expression, the forward primer, q-TMC1-F5 was designed with a 3′ end ‘T’ that specifically bound to the wild-type sequence, and q-TMC1-R2 was used as reverse primer.

Off-target analysis
For analyzing off-target RNA editing sites across the transcriptome, total RNA from different treatment samples was harvested using the RNeasy Plus Miniprep kit (Qiagen). A total of 1 μg RNA per sample was used as the input material for the RNA sample preparations. Sequencing libraries were generated using RNA Library Prep Kit for Illumina® (NEB, USA) following the
manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. There were at least 5 million reads per sample. Differential expression analysis of two groups was performed using the DESeq2 R package. DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting $P$-values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted $P$-value <0.05 found by DESeq2 were considered to be differentially expressed.

Auditory tests

ABRs and DPOAEs were recorded using the BioSigRZ system (Tucker-Davis Technologies, Alachua, FL, USA) in a soundproof chamber. Mice were anesthetized with intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). Stimuli were generated by digital input/output cards (PXI-4461 National Instruments) in a PXI-10420 chassis, amplified by a SA-1 speaker driver (Tucker–Davis Technologies, Inc.) and delivered to the studied ear by two electrostatic drivers (CUI Miniature Dynamics). An electret microphone (Electret Condenser) was used to record ear-canal sound pressure. ABR signals were collected using subcutaneous needle electrodes inserted at the pinna (active electrode), vertex (reference electrode), and rump (ground electrode).

Clicks were square pulses 100 ms in duration, and tone bursts were 3 ms in length at distinct 4, 8, 16, 24, and 32 kHz frequencies. The sound level was raised in 5 dB steps from 20 dB below threshold up to 90 dB, and electrical signals were averaged over 512 repeats. The ABR threshold was defined visually as the lowest sound pressure level (dB SPL) at which any wave could be detected. ABR thresholds were averaged within each experimental group and used for statistical analysis. Wave 1 amplitude was defined as the difference between the Wave 1 peak (P1) and the average of the 1 ms pre-stimulus baseline. DPOAE data were collected and recorded under the same conditions as ABR. Primary tones were produced at a frequency ratio of 1.2 (f2/f1) for the generation of DPOAEs at 2f1–f2, where the f2 level was 10 dB SPL below the f1 level for each f2/f1 pair. The f2 levels were swept in 5 dB steps from 20 to 80 dB. Waveform and spectral averaging were used at each level to increase the signal-to-noise ratio of the recorded ear-canal sound pressure. At each level, the amplitude of the DPOAE at 2f1–f2 was extracted from the averaged spectra, along with the noise floor. Iso-response curves were interpolated from plots of DPOAE amplitude versus sound level. The threshold was defined as the f2 level required to produce DPOAEs above 0 dB.

Immunohistochemistry of the cochlea

Injected and contralateral non-injected cochleae of 10-week-old adult mice were harvested after the animals were sacrificed by cervical dislocation. The temporal bones were perforated and perfused with 2.5% glutaraldehyde on the top of cochlea, fixed in 2.5% glutaraldehyde at 4 °C overnight, and decalciﬁed in 10% EDTA at 4 °C for 3–5 days. The decalciﬁed cochleae were dissected in pieces in 0.1 M PB for whole mounts. The dissected tissues were placed back into 2.5% glutaraldehyde, washed three times with 0.1 M PB, and ﬁxed in 1% osmium acid at 4 °C for 2 h. The tissues were dehydrated in an ethanol gradient and then dried in an HCP-2 critical point dryer for 2 h. The dried tissues were pasted on the sample tables and sprayed with an IB-3 ion sputtering instrument for 3 min. SEM images were acquired at 2.5 kV (low magniﬁcation) or 10.0 kV (high magniﬁcation) with a high vacuum ﬁeld emission SEM (Hitachi SU-8010).

Statistics

All data are shown as the mean ± SD. Statistical analysis of the results was performed using GraphPad Prism (GraphPad PRISM, Version 8.0). Student’s t-test was used to determine signiﬁcant differences between the means, and one-way analysis of variance (ANOVA) or two-way ANOVA was used for multiple comparisons. The level of signiﬁcance was set at $P < 0.05$.

DATA AVAILABILITY

The data supporting the ﬁnding of this study are available within the article and its supplementary Information files or available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Y.S., X.H. (Hu), G.L.L., X.H. (Huang), and H.L. contributed to project determination. Z.Z. and G.L. designed and performed most of the experiments, analyzed the data, and wrote the manuscript. G.L. and H.G. contributed to in vitro experiments and data analysis. Z.Z., C.C., F.W., X.W., and Z.X. contributed to in vivo experiments. Y.S. and G.L.L. designed and supervised the study. Y.C., H.T., D.W., M.H., and Z.Y.C. reviewed and edited the manuscript. All authors have read and approved the article.

ADDITIONAL INFORMATION

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