Deficiency of Klc2 Induces Low-Frequency Sensorineural Hearing Loss in C57BL/6 J Mice and Human

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Abstract
The transport system in cochlear hair cells (HCs) is important for their function, and the kinesin family of proteins transports numerous cellular cargos via the microtubule network in the cytoplasm. Here, we found that Klc2 (kinesin light chain 2), the light chain of kinesin-1 that mediates cargo binding and regulates kinesin-1 motility, is essential for cochlear function. We generated mice lacking Klc2, and they suffered from low-frequency hearing loss as early as 1 month of age. We demonstrated that deficiency of Klc2 resulted in abnormal transport of mitochondria and the down-regulation of the GABAA receptor family. In addition, whole-genome sequencing (WGS) of patient showed that KLC2 was related to low-frequency hearing in human. Hence, to explore therapeutic approaches, we developed adeno-associated virus containing the Klc2 wide-type cDNA sequence, and Klc2-null mice delivered virus showed apparent recovery, including decreased ABR threshold and reduced out hair cell (OHC) loss. In summary, we show that the kinesin transport system plays an indispensable and special role in cochlear HC function in mice and human and that mitochondrial localization is essential for HC survival.

Keywords Hair cell · Mouse model · AAV · Low-frequency hearing loss

Introduction
According to the WHO in 2020, over 5% of the world’s population – about 466 million people – have disabling hearing loss, and this number is expected to increase to over 900 million by 2050. Hearing loss affects every aspect of a person’s life. Sensorineural hearing loss can be congenital or acquired and results from damage to the organ of Corti caused by genetic factors, drugs, age, noise exposure, etc. [1–8]. As the auditory sensory cells, HCs in the cochlea play a role in converting mechanical signals into electrical signals, and proper cargo transport is indispensable for these cells’ development and function [9–11]. Myosin motor proteins in cochlear HCs have been widely studied because they transport the components of the actin assembly machinery that are necessary for regulating...
Kinesin-1 is one kind of motor protein that mediates cargo transport toward the plus-ends of microtubules using the energy from the hydrolysis of ATP, and dysfunction of kinesin-1 is associated with numerous diseases including Alzheimer’s disease, cancers, and diabetes [25–27]. Kinesin-1 consists of two kinesin heavy chains (KHCs) that are widely believed to bind to microtubules and to hydrolyze ATP and two kinesin light chains (KLCs) that mediate cargo binding and regulate kinesin-1 motility. Klc2, one of the KLCs, is a widely expressed molecular motor [28] that mediates the transport of numerous cargos, including intracellular organelles, synaptic vesicles, and microtubule dimers. Batut et al. discovered that Klc2 interacts with Smad2 and transports it to the nucleus where it triggers downstream signaling pathways [29], and huntingtin is anterogradely transported in axons by means of the binding between HAP1 and KLCs [30]. Additionally, a study showed that GSK-3 phosphorylates KLC2 in order to regulate AMPAR trafficking by the kinesin system [31]. Thus, Klc2-mediated cargo transport plays an important role in various physiological activities and ensures the normal activities and function of cells.

To investigate whether the Klc2-mediated transport system plays an important role in the auditory system, Klc2-null mice were generated using CRISPR/Cas9 technology. We found that Klc2 is essential for the function of HCs, but not for SGN (spiral ganglion) or StV (stria vascularis) function. Klc2-null mice showed low-frequency sensory neural hearing loss at 1 month of age, and they were completely deaf at 8 months of age. In addition, Klc2-null mice showed normal gross morphology, indicating that the Klc2-mediated transport system is only essential in the OHCs of the cochlea. Strikingly, we found that mitochondrial distribution was altered in OHCs, indicating that mitochondrial transport mediated by Klc2 is vital for the function and survival of OHCs. Furthermore, a 21-year-old male with an indel variant c.1444-8_1444-6delTCC in KLC2 gene (NM_001134775.1) was screened out by WGS, and he showed low-frequency hearing impairment, which is corresponding to the phenotype of Klc2-null mice. It proved that Klc2 is necessary for both mice and human. Moreover, we designed and packaged adeno-associated virus (AAV) to cure and recover the hearing impairment in Klc2-null mice and achieved a curative effect. Altogether, our work shows for the first time that the transport of mitochondria mediated by kinesin-1 in auditory OHCs relies on Klc2, and deficiency of Klc2 results in OHC death and hearing loss.

Material and Methods

Animal Models

Klc2-null mice were generated by CRISPR/Cas9 technology in the C57BL/6 J background. Two different sgRNAs (sgRNA1: GCC CAT GAC TAA CGG ACA CC, sgRNA2: CAG TTT GCC CCA CTC ACT GCT GG, designed by benchling.com) were cloned into the plasmid pX330 (Addgene ID: #42,230) digested with BbsI (NEB: #R0539V). Plasmids were purified using EndoFree Plasmid Mini Kit (OMEGA, D6950) following the manufacturer’s protocol. Numerous of WT female mice were mated with males to obtain zygotes. These two plasmid mixtures (10 ng/µl for each plasmid) were co-injected into the zygote’s nucleus from a microinjection system and followed by incubating them for 2 h at 37 °C and 5% CO2. The zygotes were then transferred to the oviducts of pseudopregnant CD1 female mice, which mated with male mice after ligated. The newborns’ genomic DNA was extracted from the tails and amplified by PCR using three sets of primers (F1: 5′-AAT ACA GGG TCC TAG ACT CCA AC-3′; R1: 5′-TTA TCC TCC TCC ACC ATG AAA G-3′; R2: 5′-CCT AGC CAT CAC TTT GCC TAT A-3′).

Auditory Brainstem Responses (ABRs)

ABRs were measured as described previously [32]. Mice were anesthetized by pentobarbital sodium (50 mg/kg body weight) before the experiments and kept on a heating pad to maintain the body temperature at 37 °C. All recordings were completed in a custom-made soundproof acoustic chamber using a Tucker-Davis Technologies System (TDT, USA) workstation running the SigGen32 software (TDT, USA). Subcutaneous platinum needle electrodes were placed at the vertex of the skull, behind the ear, and underneath the back. Auditory thresholds (dB SPL) were defined as the lowest sound intensity sufficient to clearly elicit the first wave response.

Scanning Electron Microscopy

Mice were sacrificed, and their cochleae were quickly removed and fixed in 2.5% glutaraldehyde. After decalciﬁcation, the basilar membranes were isolated from the cochlea and divided into the apical, middle, and basal turns. The samples were subsequently treated with 1%
osmium tetroxide and 1% tannic acid, dehydrated through an ethanol gradient, dried with a critical point drier, and finally observed under a JEOL 7000 field emission gun scanning electron microscope after being sputter-coated with a layer of gold.

Transmission Electron Microscopy

Cochleae were dissected and fixed in 2.5% glutaraldehyde followed by decalcification in 10% EDTA. After dissection and segmentation, the basilar membranes were fixed with 1% osmium tetroxide and then dehydrated through an ethanol gradient and embedded in Epon-812. Ultrathin sections were cut with a diamond knife on an ultramicrotome (RMC, Powertome-XL) and were observed under a transmission electron microscope (Thermo, FEI Tecnai G2 F20) after uranyl acetate and lead citrate staining.

Protein Extraction and Western Blot

Mice were sacrificed, and their cochleae were dissected and homogenized in ice-cold cell lysis buffer (10 mM Tris, pH = 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 0.2 mM PMSF) and then lysed for 30 min on ice and centrifuged at 10,000 x g at 4 °C for 30 min. The supernatant was transferred to a clean centrifuge tube, and the protein concentration was quantified with a BCA kit. Samples were mixed with 5 x loading buffer and heated to 100 °C for 5 min. Equal amounts of each sample were separated by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with 5% milk in TBST for 1 h at room temperature and then incubated with primary antibodies in BSA solution at 4 °C overnight. After washing with TBST, the PVDF membranes were incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. After washing with TBST, the bands were detected with an ECL western blot kit. The following antibodies were used: anti-Klc2 (Proteintech, 17,668–1-AP), anti-Myh14 (CST, 8189P), and anti-Gapdh (Boster, BM1623).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (q-PCR)

Total RNA was extracted using Trizol reagent (Ambion, 262,304) following the manufacturer’s protocol. The cDNAs were synthesized, and q-PCR was performed with a 10 µl mixture containing 5 µl SYBR Green qPCR Mix (SparkJade AH0104), 0.2 µl primer F, 0.2 µl primer R, 1 µl cDNA template (after dilution), and 3.6 µl ddH2O. All procedures were performed in triplicate, and the relative expression was calculated using the 2–ΔΔCT method. The following primers were used: Klc1 (F: 5'-AGC GGG AGT TTG GAT CTG TG-3'; R: 5'-AGC CAC TCT CTG TTA ACG TGA-3'), Klc2 (F: 5'-AGA AGC TGA GTT AGG AGC AGA-3'; R: 5'-GAT GGC TTG ATA ATG CCA GGA T-3'), Klc3 (F: 5'-CCC AAG AGA ACA CAT GGC TTC-3'; R: 5'-GCC GTG CAT ACT GCC TCA G-3'), Klc4 (F: 5'-GTA CCG CAA ACG CGG TAA ATA-3'; R: 5'-GCC CTG GTT TTG ACA TAG GAG G-3').

RNA-seq

Total RNA were extracted as describe above; the WT and Klc2-KO groups contained 3 different samples, respectively. RNA-seq was performed by BGI (Shenzhen, China) using the BGISEQ-500 platform. The sequencing data was filtered with SOAPnuke (v1. 5. 2) [33] by (1) removing reads containing sequencing adapter; (2) removing reads whose low-quality base ratio (base quality less than or equal to 5) is more than 20%; and (3) removing reads whose unknown base (‘N’ base) ratio is more than 5%; afterwards, clean reads were obtained and stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2. 0. 4) [34]. Bowtie2 (v2. 2. 5) [35] was applied to align the clean reads to the reference coding gene set; then, expression level of gene was calculated by RSEM (v1. 2. 12) [36]. Differential expression analysis was performed using the DESeq2 (v1. 4. 5) [37] with Q value ≤ 0. 05. GO enrichment analysis of annotated different expressed gene was performed by phyper based on hypergeometric test.

Immunohistochemistry

Cochleae were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, and the basilar membranes were isolated and divided into three sections after decalcification. Samples were then permeabilized in 0.02% Triton X-100 and washed three times (10 min each time). After blocking in 10% goat serum in PBS, the basilar membranes were incubated with the primary antibody at 4 °C overnight. The basilar membranes were then washed three times with PBS and incubated with fluorescence-conjugated secondary antibody diluted in PBS for 1 h. F-actin filaments were detected with FITC or TRITC-conjugated phalloidin, and nuclei were stained with DAPI. Samples were imaged using Zeiss LSM780 and Leica confocal microscopes. The following antibodies were used: anti-Myosin7a (Proteus Biosciences, 25–6790), anti-parvalbumin (Sigma, P3088), anti-spectrin (Sigma, mab1622), anti-caspase3 (CST, 9661S), anti-Lc3b(CST,2775S), anti-prestin (sc-22692), anti-3NT (Abcam, ab110282), anti-4HNE (Abcam, ab46545), anti-Kcnma1 (Alomone, APC-021), and anti-HAtag (CST, 3724S).
AAV Preparation and Round Window Injection

The AAV was created using triple transfection of HEK293T cells and extracted by iodixanol gradient purification. Briefly, pAAV-CAG-Klc2-HA, pHelper, and pANC80L65 were obtained from a kit (OMEGA, D6950-01) and co-transfected into HEK293T cells. Culture medium and cells were collected after 72 h and chloroform was added. After centrifugation, the supernatant was precipitated first with 1.0 M NaCl and then with 10% PEG 8000, and the precipitate was resuspended with PBS and treated with benzonase.

Klc2 binds to cuticular plate-related proteins

Klc2-mediated cargo transport has always been a research hotspot, but this has mainly focused on neurons [38–40], and little research on such transport in the auditory system has been performed. The light chains of kinesins play an important role in the transport processes as they can be connected to different kinds of cargo [29–31]. Here, we explored whether Klc2 plays a role in the auditory system. First, we use western blot to determine the Klc2 expression pattern in wild-type (WT) mice (Fig. 1A), and we find that Klc2 is widely expressed in various organs. The q-PCR results show that the cochlea had their highest expression of Klc2 at P14 (Fig. 1B), which is when the development of the organ of Corti is nearly complete. This suggested that Klc2 is related to the maintenance of hearing function. To investigate the specific function of Klc2 in the cochlea, we used CRISPR/Cas9 technology to generate Klc2-null mice. Two specific sgRNAs were designed to target Klc2 between intron 2 and intron 11, which abolishes the whole coding sequence of Klc2. Genomic DNA from mouse pups is extracted for PCR genotyping using the primers shown in Fig. 1C. Potential off-target sites were predicted by an online database (https://cm.jefferson.edu/Off-Spotter/), and every potential off-target site was amplified by PCR followed by sequencing. No off-target effects were found. In the Klc2-null mice, Klc2 could not be detected in the cochlea by q-PCR (Fig. 1D) or by western blot (Fig. 1E), indicating that Klc2 was successfully knocked out.

Klc2 Is Required for the Normal Function of the Cochlea

Klc2-null mice had a normal gross morphology and did not show any apparent defects compared with their WT littermates. We analyze the auditory functions of Klc2-null mice as they aged, and at P40, the Klc2-null mice show significant low-frequency hearing loss (Fig. 2A), while high-frequency hearing was not affected. We perform H&E staining to explore the cochlear morphology of Klc2-null mice (Fig. 2C). The OHCs were lost, but the inner HCs (IHCs), the SGN, and the StV were normal compared to WT mice, which indicated that OHC death was the primary reason for the hearing loss seen in Klc2-null mice. Whole-mount staining of Myo7a and phalloidin, two HC-specific markers, shows that Klc2-null mice lost massive OHCs from the apical region at P40 (Fig. 2B and 2D). Immunofluorescence staining also performed on WT and Klc2-null mice basilar membrane in different time points before P40, and we confirmed that OHCs began to lose at P30 in Klc2-null mice. Thereafter massive cell loss occurs with further age, and the OHCs are nearly completely absent at 8 months of age, and hearing is almost completely lost (Fig. S1A–C). We further check the morphology of the stereocilia using SEM (Fig. 3), and no abnormalities are seen in the remaining OHCs, indicating that Klc2 functions mainly in the cell body of OHCs. In addition, we check the expression of other light chains of kinesin (Klc1, Klc3, and Klc4), and q-PCR of P30 WT and Klc2-null mice show no significant changes in these three genes (Fig. S1D), suggesting that the role of Klc2 in the cochlea is not related to these three genes. In conclusion, Klc2 appears to be essential for the survival of OHCs, and loss of OHCs is the reason behind the hearing loss in Klc2-null mice.

Klc2 Binds to Cuticular Plate-Related Proteins

Loss of Klc2 impairs cochlear HC function but does not affect the SGN and StV, so we sought to determine the role...
that Klc2 plays in HC survival. Klc2 is the light chain of kinesin-1, and it connects to the kinesin heavy chains and to the cargo molecules. We therefore carried out a co-immunoprecipitation from P30 mouse cochleae using Klc2-specific antibody, followed by protein identification by peptide mass spectrometry (CoIP-MS), to identify the possible cargos that are essential for auditory function in OHCs. Approximately 916 proteins are identified from the IgG samples and 663 proteins from the Klc2 antibody samples, and 128 proteins are left in the Klc2 antibody samples when the 535 common proteins were subtracted (Fig. S2A). As expected, some known Klc2-binding proteins like Kinh and Kif5c were included in these 128 proteins. Classification comparison was performed, and we identified a set of proteins that are reported to be localized to the cuticular plate (Tpm3, Myosin6, Sptb2, and Sptn1) [41–43]. Co-IP were performed to further validate the CoIP-MS result. We choose Myh14, a protein related to noise-induced hearing loss that we have reported on previously, and it shows strong binding affinity for Klc2 in the CoIP-MS data (p < 0.001). F Schematic diagram of kinesin-1, consisting of two heavy chains and two light chains, that transport intracellular cargos along microtubules.

Fig. 1 Klc2 knockout by CRISPR/Cas9 in C57BL/6 J mice. A Western blot analysis of Klc2 expression in WT mice. The expression of Klc2 in the cochlea was greater than that in the heart, liver, spleen, and lung but was less than that in the cerebellum and brain. B Q-PCR analysis showed that the expression of Klc2 was highest at P14 (p < 0.001 for P14, p < 0.01 for P50. C Schematic of the Klc2 knockout strategy. Two individual sgRNAs were specific to intron 2 and intron 11, and the whole coding sequence was abolished. D and E Q-PCR and western blot analysis of the cochlea verified the knockout of Klc2 in mutant mice (p < 0.001). F Schematic diagram of kinesin-1, consisting of two heavy chains and two light chains, that transport intracellular cargos along microtubules.
Knockout of Klc2 Disrupts the Distribution of Mitochondria

TEM was performed to check the subcellular structure of HCs in WT and Klc2-null mice at P30, which was when the hearing loss phenotype first appeared. We found that the structure of the cuticular plate was normal, further indicating that it was not the primary reason for HC death. However, we found that the distribution of mitochondria was affected in the HCs of the cochlea after careful examination. Normally, mitochondria are located along the plasma membrane, under the cuticular plate, and around the nucleus to provide the energy required for OHC activity [45], but the mitochondria are concentrated in the center of the cytoplasm of the OHCs from Klc2-null mice (Fig. 4A-C). It is noteworthy that the OHC nuclei of Klc2-null mice were normal at P30 (Fig. 4B), suggesting that there was still a period of time before these cells died, and the abnormal mitochondrial localization might be the primary factor that triggers a series of events that lead to HC death. We concluded that deficiency of Klc2 disrupts the mitochondrial transport system, which depends on kinesin and the microtubule network. The disordered mitochondrial transport system might subsequently affect normal mitochondrial function like energy production, apoptosis, and autophagy, thus leading to HC degeneration and hearing loss. Next, to explore the possible cause of OHC death, we co-stained parvalbumin with apoptosis marker caspase-3 and autophagy marker Lc3b, respectively. No positive signal for caspase-3 or Lc3b is observed (Fig. S3A). The expression pattern of prestin in Klc2-null mice is normal compared to WT mice (Fig. S3B), suggesting that the aberrant mitochondrial distribution did not affect the magnification ability of OHCs. It is reported that inaccurate distribution of mitochondria might cause problems with reactive oxygen species (ROS) production [46], but Klc2-null mice did not show this phenomenon (Fig. S3C). Interestingly, a decreased number of BK channels is seen in IHCs (Fig. 4E-F), which might be related to the special BK variant (called the DEC splice variant or MitoBK) that is specifically located in mitochondria [47–49]. In summary, we found that aberrant mitochondrial distribution resulted in HC degeneration, but this was not related to apoptosis, autophagy, or redox imbalance.

GABA Receptor Might be Associated with the Auditory Phenotype

To further explore the intracellular changes caused by defective mitochondrial distribution which leads to HC loss in Klc2-null mice, RNA-seq analysis was performed at P30. We found that 28 genes are up-regulated, and 366 genes are down-regulated (Fig. 5A). Moreover, 55 genes are classified as “Transporter activity” (Fig. 5B). From the gene ontology (GO) analysis, we found that expression of multiple GABA receptor family genes is decreased (GABA receptor subunits α1, β2, α3, δ, α2, α6, and γ1) in the Klc2-null mice (Fig. 5A and 5D). GABA, as one kind of inhibitory neurotransmitter, interacts with GABA receptors in order to regulate cellular activities. In the inner ear, the olivocochlear efferent system is both cholinergic and GABAergic, and several studies have shown that GABA receptor is essential for cochlear function [50–52]. Liberman et al. constructed a set of mouse models and showed that knockout of three mouse lines (α1, α2, and α6) did not affect cochlea function [51]. We therefore validate the down-regulated expression of the remaining genes in our RNA-seq results (genes show in Fig. 5D subtract subunits α1, α2, and α6) by q-PCR (Fig. 5E). In short, knockout of Klc2 led to the down-regulation of multiple GABA subunits, which might arise from abnormal mitochondrial localization, and this might be one reason for the disruption of the auditory system.

An Indel Variant in Human KLC2 Is Associated with Low-Frequency Hearing Impairment

A 21-year-old male (case 1,707,652) complaining bilateral hearing impairment was related to our research, with the onset age of 14 years old. Whole-genome sequencing found an indel variant c.1444-8_1444-6delTCC in KLC2 gene (NM_001134775.1) from the patient. This novel variation was not found in the 1000 Genomes Project, ExAC 65,000 exome allele frequency data, and 1751 ethnicity-matched controls. No family history is complained (Fig. 6A). Otoacoustic examination, distortion product otoacoustic emission (DPOAE), ABR, cochlear microphonics (CM), and speech recognition score (SRS) performed on him illustrated the typical auditory neuropathy. The audiogram of the proband with damaged SRS (20% and 48% for the left and right ear, respectively), DPOAE for the proband is normal (Fig. 6C), and no waves could be detected in ABR testing bilaterally (Fig. 6D). It is worth mentioning that our mouse model also showed low-frequency hearing loss which replicated phenotype in human exactly.
Therapeutic Approaches Using AAV

We next explored therapeutic strategies of hearing impairment caused by Klc2 deficiency. AAV has proved to be a safe and effective viral vector for genetic therapy in the inner ear [53–55]. Hence, we tested whether AAV containing the whole WT Klc2 cDNA sequence could rescue the deafness phenotype in Klc2-null mice. The synthetic vector Anc80L65 was used to viral packaging. At P2, Klc2-null mice were treated with an AAV solution by round window injection [53], and saline was injected into littermates as the vehicle control. In addition, we label the HA tag and observe high efficiency of infection at P14 (Fig. 7A), and western blot is performed to validate the expression of Klc2. Only the cochlear sample treated with AAV shows a clear band using both anti-HA and anti-Klc2 antibodies (Fig. 7B), indicating that Klc2 was re-expressed only in the cochlea and not in other tissue. We then wanted to know whether re-expressing Klc2 rescues Klc2-null mice from hearing loss. As expected, both the ABR threshold and OHC survival ratio show an apparent recovery in Klc2-null mice at P40 (Fig. 7C-D), and the mean hearing thresholds at 4 kHz, 8 kHz, and 12 kHz are reduced by about 27 dB (p < 0.001), 34 dB (p < 0.001), and 17 dB (p < 0.05), respectively. We concluded that Klc2 re-expression decreased OHC degeneration and prevented hearing loss, and AAV was an effective vector for Klc2 delivery.

Discussion

In this study, we show that Klc2, one of the light chains of kinesin-1, is vital for the low-frequency hearing in mice and is relevant for human. Klc2-null mice generated by CRISPR/Cas9 suffered from low-frequency hearing loss, and we filtered out a patient with defective KLC2 variant which also showed similar auditory symptom. Loss of Klc2 leads to the abnormal distribution of mitochondria in mice and thus leads to the death of HCs, and our study is the first to show that improper mitochondrial localization is related to OHC death and hearing loss.
Most hearing loss families and gene knockout models show that hearing loss occurs first at high frequencies, and many stressors generate high-frequency hearing damage. The unique physiological structures of the cochlea and the biological functions of HCs mean that HCs are more delicate in the high-frequency regions, but in our case, auditory dysfunction was seen at low frequencies. Hearing dysfunction at low frequencies is not without precedent. Wolfram syndrome spectrum disorder, caused by a WFS1 (DFNA6/14) mutation, leads to low-frequency sensorineural hearing impairment [56], while a protein-truncating mutation in DIAPH1 (DFNA1) results in autosomal-dominant low-frequency deafness [57, 58]. And we also screened out that a 21-year-old male with abnormal KLC2 gene (an indel variant c.1444-8_1444-6delTCC in NM_001134775.1) showed low-frequency hearing impairment. In addition, in some hereditary hearing loss families, including TECTA (DFNB21) [59–61], EYA4 (DFNA10) [62–64], COL11A2 (DFNA13) [65], and CCDC50 (DFNA44) [66], hearing impairment begins in the middle frequencies. These studies indicate that there are differences in the expression and/or specific functions of certain genes in the HCs in the different turns of the cochlea. Here we present a novel gene that has a
Fig. 5 RNA-seq showed the down-regulation of the GABAA receptor family in Klc2-null mice. A Histogram showing that 28 genes were up-regulated and 366 genes were down-regulated. B GO annotations of differentially expressed genes were classified and mapped, and “Transporter activity” (containing 55 genes) is marked separately. C GO mf enrichment analysis of 55 genes, with “GABAA receptor activity”, marked by a black circle. \( Q < 0.05 \). D Gene symbols and fold changes of seven genes included in “GABAA receptor activity”. E Q-PCR in Klc2-null mice to confirm the RNA-seq results. Negative numbers represent down-regulated expression, \( n = 3 \).
Fig. 6 Pedigree and audiological phenotype of case 1,707,652. A Pedigree of case 1,707,652. The affected subject is colored black; the proband is indicated by an arrow. B Audiogram of the proband. C Normal DPOAE result of the proband. D ABR waves were absent in the left ear and differentiated poorly in the right ear of the proband.
Fig. 7 AAV therapy resulted in some degree of phenotypic recovery. A Representative confocal images of the basilar membrane in Klc2-null mice at P14, with parvalbumin (red) labeling the HCs and HA-tag (green) labeling Klc2-HA. Klc2 was re-expressed in both the nuclei and cytoplasm of HCs, n = 3. Scale bar: 20 μm. B Protein was extracted from the cochlea and cerebellum at P14, and western blot using anti-Klc2 and anti-HA antibody was performed to verify the re-expression of Klc2, indicating that Klc2 was only re-expressed in the cochlea of the AAV group, n = 4. C The ABR threshold gap narrowed at P40 after AAV therapy, n = 6, ***p < 0.001 for 4 kHz and 8 kHz, *p < 0.05 for 12 kHz. D The HCs of the apical and middle turns of the basilar membrane of the “AAV group” and “Vehicle group” were immunostained with parvalbumin (red) at P40, and loss of OHCs was significantly decreased in the “AAV group”, n = 3. Scale bar: 100 μm. ***p < 0.001
specific role in the low-frequency region. We wondered what would cause the apical OHCs to be more vulnerable in Klc2-null mice, and we first hypothesized that different expression levels of Klc2 in the basilar membrane are the main reason for this phenotype. However, we did not find any significant differences in mRNA levels between the different turns of the cochlea by q-PCR analysis, and we were unfortunately unable to obtain antibodies suitable for immunolabelling to study its expression pattern at the protein level. We then hypothesized that changes in the overall morphological structure of the cochlea account for this and that HCs might suffer from squeezing stress of cupula cochleae. We examined WT and Klc2-null cochleae at P40 and found no obvious differences between them. Thus, more factors need to be considered, like the microenvironment, different types of efferent nerve receptors, or other Klc2-related genes that have distinct expression patterns. These factors might play a role alone or together, and they might collectively determine the survival of HCs in the different turns of the cochlea.

Mitochondrial transport is mediated by kinesin and relies on an intact microtubule network [67, 68]. In neurons, the motor/adaptor complex consisting of two core proteins called Miro (also known as RhoT1 and RhoT2) and Milton (also known as TRAK1 and TRAK2) connect the mitochondria to kinesin (or dynein), which allows the mitochondria to be transported along the microtubules [69–72]. In our mouse model, mitochondrial distribution was changed in HCs, and we therefore speculate that mitochondria might use a similar kinesin-mediated transport mode in HCs. Supplying ATPs from the respiratory chain, mitochondria are concentrated in areas with strong metabolism, and abnormal distribution of mitochondria may lead to cell dysfunction. Bavister et al. found that the distribution of mitochondria changes in different groups of mammals during oocyte maturation or fertilization [73], while Pathi et al. showed that oxygen controls the intracellular distribution of mitochondria in muscle fibers, and a more asymmetric mitochondrial distribution presumably results in increased ROS production [46]. In the HCs of the cochlea, mitochondria are concentrated along the plasma membrane, under the cuticular plate, and around the nucleus [45] in order to provide energy for stereocilium movement, ion channels, and synaptic activity. In our model, deficiency of Klc2 caused abnormal mitochondrial distribution and ultimately led to HC death. This study proves that mitochondrial transport in cochlear HCs relies on Klc2, and it provides new evidence that mitochondria need to be transported to the proper intracellular position in order to maintain cell survival.

As one of the motor protein subunits, Klc2 is widely expressed, but loss of Klc2 only results in hearing loss. To investigate the reason for OHC death, we checked the mitochondria’s known functions and found that OHC death was not related to apoptosis, autophagy, or excessive ROS production, at least suggesting that the above function of mitochondria is not affected when its localization was changed. After ruling out the above mitochondrial function, we thought that two possible reasons might explain why only HC death is seen in Klc2-null mice. The first is that other Klcs (Klc1, Klc3, or Klc4) cannot compensate for Klc2’s function in the cochlea, which we showed to be the case. The online database https://shield.hms.harvard.edu/ showed that Klc2 has the highest expression ratio in HCs compared to other types of cells in the cochlea, which implies that Klc2 plays a major role in HCs compared to other KLCs, and in other tissues, the other KLCs might compensate for the functions of Klc2. Second, we thought that mitochondria transport might be more sensitive in HCs than in other types of cells. In addition to maintaining basic life activities, mitochondria also supply more energy for special activities like stereocilium motion for sound signal transmission, synapase activity for both information transfer and protective efferent suppression (like GABA as discussed below) [8], all of which require timely energy production from the mitochondria. Insufficient energy supply will impede the normal functions of HCs, causing the loss of cellular communication or even influencing basic metabolism, eventually resulting in cell death. In addition, we performed the AAV rescue experiment to re-express Klc2 only in the cochlea and observed recovery of function, indicating that loss of HCs is autonomous. Taken together, these results suggest that Klc2 is essential for HC function in the cochlea, but not in other tissues.

We further performed RNA-seq to investigate the mechanism behind the HC death seen in Klc2-null mice. Of the changes in gene expression, we focused on the GABAA receptor, which is an inhibitory neurotransmitter receptor that is associated with hearing protection through the olivocochlear efferent system. A total of 21 receptor subunits have been identified, but most GABAA receptors are pentamers composed of 5 subunits – 2 α, 2 β, and 1 γ subunit – and the different spatial expression patterns and distinct properties of these subunits allow the GABAA receptor to play different roles [74]. GABA receptor, which expression and function in cochlea were wildly reported, is thought to transmit inhibitory messages that protect HCs from over-excitation. Whitlon et al. reported that GABA-positive fibers are expressed along the entire length of the basilar membrane but that innervation of OHCs is restricted to the apical and middle turns [75], which conforms to the pattern of OHC death in our model. However, one study using different immunologic markers indicated the presence of GABAergic terminals on OHCs throughout the whole cochlea [76]. It is noteworthy that deletion of Gabrb3 (GABAA receptor subunit β3) caused low-frequency hearing loss, similar to the phenotype of Klc2-null mice [51]. In our study, deficiency of
Klc2 resulted in decreased expression of multiple GABAA receptor subunits, which might lead to the inhibition of cochlear efferent nerve protection and eventually lead to the death of OHCs. Moreover, it is generally acknowledged that mitochondria are essential for synapses. Mitochondria buffer cytoplasmic calcium and provide energy to ensure the function and plasticity of synapses [77, 78]. Strikingly, Verstreken et al. reported that Drp1-knockout flies showed defects in mitochondrial localization, and lacking of energy supply then leads to synaptic depression [79]. And another interesting study recently showed that mitochondria can sequester GABA to reduce GABA neurotransmission and even cause social behavioral impairments in Drosophila [80]. Taken together, we presumed that, in our model, reduced expression of GABAA receptor subunits may on account of some abnormal mitochondria activities or functions. However, we cannot exclude the possibility that whether changes in GABAA receptor were directly effect of Klc2 deletion; thus, the relationship between GABAA receptor and mitochondria in HCs remains to be studied next.

In summary, we present the role of the novel deafness-related gene Klc2, which caused unusual low-frequency hearing defects both in mice and human, and we discuss the reasons for hearing loss in Klc2-null mice. We provide new evidence that abnormal mitochondrial distribution can affect normal cellular activities, and our work lays the foundation for research on the role of motor proteins in the auditory system.

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Authors’ Contributions Renjie Chai and Jiangang Gao designed and supervised the project. Xiaolong Fu, Yachun An, Peipei Li, Jing Lin, Jia Yuan, Rongyu Yue, and Yecheng Jin performed most experiments and acquired the data. Hongyang Wang screened and analyzed clinical data. Xiaolong Fu, Yachun An, Peipei Li, Jing Lin, Jia Yuan, Rongyu Yue, and Yecheng Jin analyzed and interpreted the experiments results. Xiaolong Fu, Yachun An, and Renjie Chai wrote the manuscript.

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Data Availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval We performed all animal procedures according to protocols that were approved by the Animal Care and Use Committee of Southeast University and that were consistent with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. We made all efforts to minimize the number of animals used and to prevent their suffering.

Consent to Participate Informed consent to participate in the study was obtained from all participants.

Consent for Publication All authors consent to the publication of current data.

Conflict of Interest The authors declare no competing interests.

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