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ORIGINAL ARTICLE

GRAIL gene knockout mice protect against aging-related and noise-induced hearing loss

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Abstract

Background: Hearing loss is a global health issue and its etiopathologies involve complex molecular pathways. The ubiquitin-proteasome system has been reported to be associated with cochlear development and hearing loss. The gene related to anergy in lymphocytes (*GRAIL*), as an E3 ubiquitin ligase, has not, as yet, been examined in aging-related and noise-induced hearing loss mice models.

Methods: This study used wild-type (WT) and *GRAIL* knockout (KO) mice to examine cochlear hair cells and synaptic ribbons using immunofluorescence staining. The hearing in WT and KO mice was detected using auditory brainstem response. Gene expression patterns were compared using RNA-sequencing to identify potential targets during the pathogenesis of noise-induced hearing loss in WT and KO mice.

Results: At the 12-month follow-up, *GRAIL* KO mice had significantly less elevation in threshold level and immunofluorescence staining showed less loss of outer hair cells and synaptic ribbons in the hook region compared with *GRAIL* WT mice. At days 1, 14, and 28 after noise exposure, *GRAIL* KO mice had significantly less elevation in threshold level than WT mice. After noise exposure, *GRAIL* KO mice showed less loss of outer hair cells in the cochlear hook and basal regions compared with WT mice. Moreover, immunofluorescence staining showed less loss of synaptic ribbons in the hook regions of *GRAIL* KO mice than of WT mice. RNA-seq analysis results showed significant differences in C-C motif chemokine ligand 19 (*CCL19*), C-C motif chemokine ligand 21 (*CCL21*), interleukin 25 (*IL25*), glutathione peroxidase 6 (*GPX6*), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (*NOX1*) genes after noise exposure.

Conclusion: The present data demonstrated that *GRAIL* deficiency protects against aging-related and noise-induced hearing loss. The mechanism involved needs to be further clarified from the potential association with synaptic modulation, inflammation, and oxidative stress.

Keywords: Aging; Cochlea; GRAIL; Hearing loss; Noise; RNF128; Ubiquitin-proteasome system

1. INTRODUCTION

Hearing loss affects over 20% of the world's population. The annual additional investment attributed to hearing care amounted to US$ 750 billion, especially in low- and middle-income countries.[1] The term sensorineural hearing loss (SNHL) refers to problems that involve transmission at and after the cochlea.[2] The etiologies of SNHL included genetic causes, aging, ototoxic drug therapies, loud noise exposure, and underlying medical conditions. Aging-related hearing loss (ARHL) and noise-induced hearing loss (NIHL) are the most common forms of SNHL.[3]

There are several hypotheses regarding the mechanisms associated with SNHL. Dominant sites of pathological change associated with SNHL are generally in the cochlea, particularly metabolic and structural changes and decrease in vascular supply due to interference with the transport of ions, depolymerization of actin filaments, and decrease in cochlear blood flow.[2] Acoustic overstimulation could potentially cause oxidative stress in hair cells, which leads to inflammation and further mitochondrial damage associated with cochlear synaptopathy.[4,5] Noise exposure and aging could lead to damage of the synaptic connections leading to degeneration of the spiral ganglions and a permanent threshold shift.[6–8] Excessive noise exposure may directly contribute to inflammatory response and alter the molecular mechanisms, according to the transcriptome analyses of the cochlear tissues in the NIHL.[9] There is no doubt that hearing loss involves complex molecular and multifactorial events.

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Ubiquitination is a highly selective posttranslational modification that involves the covalent conjugation of ubiquitin to a substrate protein. The ubiquitin-proteasome system (UPS) has been reported to be associated with cochlear development and hearing loss.[10] As an important regulator of synaptic plasticity[11–13] and being activated in the cochlea following excessive noise exposure,[14] the UPS is essential for cochlear development and maintenance.

The gene related to anergy in lymphocytes (*GRAIL*) is a type I transmembrane protein that participates in the endocytic system. *GRAIL* plays a crucial role in T cell anergy through the degradation of proteins involved in CD4+ T cell activation which suppresses the adaptive immune response.[15–17] A recent study has shown that *GRAIL*-deficient mice are highly susceptible to lipopolysaccharide-induced sepsis. *GRAIL* deletion potentiates macrophage activation and organ injury during sepsis.[18] Moreover, *GRAIL* is involved in the adipocyte differentiation process, and the deletion of *GRAIL* protects against diet-induced obesity.[19] Another study also demonstrated that *GRAIL* could regulate the hepatic steatosis through the inhibition of sirt1.[20] *GRAIL* has also been found to be one of the gene targets of signal transducer and activator of transcription 3 (STAT3) and can eventually reduce alternative activation of macrophage.[21]

GRAIL has been identified as an E3 ligase that controls the last step of ubiquitination in the UPS.[22] However, the *GRAIL* gene has not yet been examined in hearing-related issues. It is hypothesized that *GRAIL*-related gene expression may be involved in the mechanism of ARHL and NIHL. This study used wild-type (WT) and *GRAIL* knockout (KO) mice to investigate the biological function of *GRAIL* in ARHL and NIHL using cochlear surface preparation, immunofluorescence staining, auditory brainstem response (ABR), and RNA-sequencing analysis.

2. METHODS

2.1. Animals and experimental design

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan (IACUC-22-021). Animal care complied with all institutional guidelines and regulations. *GRAIL* KO mice were generated by the Transgenic Mouse Models Core (Taipei, Taiwan) using the CRISPR-Cas9 technology, whereas *GRAIL* KO mice were generated using the C57BL/6J background as in a previous study.[19] The mice were housed under a regular 12 hours light/dark cycle for 2 weeks before the experiments. A total of 57 C57BL/6 male mice and 49 *GRAIL* KO C57BL/6 male mice aged from 6 to 8 weeks were used.

2.2. Cochlear surface preparations and immunofluorescence staining

Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The mice were sacrificed at day 60 after noise exposure in NIHL experiments. Then the mice were flushed with pre-warmed phosphate-buffered saline (PBS) and then transcardially perfused with 4% paraformaldehyde. The deeply anesthetized mice were decapitated and the cochleae were removed. The oval window and the cochlear apex were opened to facilitate immediate perfusion with 4% paraformaldehyde in PBS. The samples were then immersed in the same fixative solution for 3 hours at room temperature (RT) to allow diffusion through the whole cochlea. Then the cochleae were decalcified (0.1 M ethylenediaminetetraacetic acid [EDTA]) for 3 days. After washing with PBS, the bony capsule surrounding the cochlea, the cochlear lateral wall, and Reissner's membrane were removed. The remaining part of the cochlea was permeabilized with 2% Triton X-100 for 1 hour at room temperature, followed by incubation with the following primary antibodies combined: (1) mouse monoclonal anti-C-terminal binding protein 2 (CTBP2) antibody (BD Biosciences Inc., East Rutherford, NJ) at 1:500; and (2) rabbit antimouse Myosin VIIa (Novus Biologicals, Littleton, CO) at 1:200. Primary incubations were followed by two sequential 60-minute incubations at 37°C in species-appropriate secondary antibodies (coupled to Alexa Fluor dyes) with 1% Triton X. Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI). All images were taken from the immunostaining slides by a confocal microscope camera (Carl Zeiss LSM880). The numbers of outer hair cells and synapse ribbons were calculated.

2.3. Noise exposure

Mice were anesthetized, placed in a soundproof booth with a loudspeaker (V12 HP; Tannoy Ltd., Coatbridge, UK) mounted above the center of the cage, and exposed to 115 dB sound pressure level (SPL) white noise for 3 hours. A specially designed and separated wire cage was used in order to avoid inappropriate exposure to noise caused by the animals congregating during noise stimulation. The noise level was measured using a sound level meter (Rion NL-52, Tokyo, Japan), and the difference in noise level within the booth between the center and edge of the cage was <1 dB.

2.4. ABR recording

Auditory function was evaluated by recording ABR in anesthetized mice. Specific stimuli (clicks and 8-, 16-, 24-, 28-, 32-kHz tone bursts) were generated using the SigGen software (Tucker-Davis Technologies, Gainesville, FL) and delivered to the external auditory canal. The average responses from 1024 stimuli for each frequency were obtained by reducing the sound intensity in steps of 5 dB until threshold. The resulting ABR thresholds were defined as the lowest intensity at which a reproducible deflection in the evoked response trace could be recognized.

2.5. Western blot

Aliquots of cochlea homogenates were separated on 8% sodium dodecyl sulfate polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), blocked with 5% skimmed milk in phosphate-buffered saline Tween-20 (PBST) (0.2 M Tris-base, 1.37 M NaCl, and 0.1% Tween 20), probed with *GRAIL* primary antibody (1:300; a gift from Dr. Ying-Chuan Chen, Department of Physiology & Biophysics, National Defense Medical Center, Taipei 11490, Taiwan) at 4°C overnight, washed with PBST, and incubated with anti-rabbit horseradish peroxidase-linked whole antibody (1:10 000; GE Healthcare, Chicago, IL) for 1 hour at RT. The immunoreactive bands were stained using a light-emitting nonradioactive method (ECL; Millipore).

2.6. RNA-extraction and RNA-sequencing

Mice were anesthetized and then decapitated. The cochleae were extracted from the temporal bones of the mice. Total RNA was isolated from cochleae using TRIzol reagent (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Cochlear RNA at 300 ng was used for RNA-sequencing. mRNA library was prepared using the Illumine TruSeq Stranded mRNA Library Prep kit, and sequenced using S4 chip on Illumine NovaSeq 6000 System. Sequencing data were analyzed using QIAGEN CLC Genomics Workbench and Ingenuity Pathway Analysis. Differentially expressed

transcripts were identified using the CLC software. Transcripts with p-adjust <0.05, |log2 (fold change)|>2 were defined as differentially expressed genes.

2.7. Statistical analysis

Statistical analysis was performed using a two-tailed Student's t test. Results were expressed as mean ± SEM. Differences at $p < 0.05$ were considered significant.

3. RESULTS

3.1. Hearing threshold of *GRAIL* WT and *GRAIL* KO mice in ARHL and NHIL

First, the expression level of *GRAIL* in cochleae of WT and KO mice was investigated. No *GRAIL* protein was detected in KO mice (Fig. 1).

Next, how *GRAIL* KO mice would respond to ARHL was examined. C57BL/6J mice have generally been used in ARHL animal models. Hearing thresholds were determined every 3 months using recorded ABR (Fig. 2). As shown in Fig. 2, the hearing of *GRAIL* WT mice at 12 months had elevated to 80 to 90 dB, whereas that of *GRAIL* KO mice ranged between 50 and 80 dB at high frequencies (24, 28, and 32 kHz), indicating statistically significant differences when compared with *GRAIL* WT mice.

How *GRAIL* KO mice would respond to NIHL was also examined with 8-week-old male WT and KO mice exposed to 8 to 32 kHz octave-band noise at 115 dB SPL for 3 hours. Hearing thresholds before and at days 1, 14, 28 after noise exposure were determined using ABR recorded. All genotypes demonstrated elevated thresholds, but with significantly less elevation in threshold levels observed in *GRAIL* KO mice (Fig. 3).

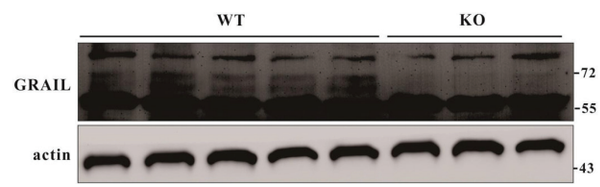

Fig. 1 The expression level of *GRAIL* protein in cochleae of WT and KO mice. No expression of *GRAIL* protein was shown in KO mice. *GRAIL* = gene related to anergy in lymphocytes; KO = knockout; WT = wild-type.

3.2. Cochlear surface preparation and immunofluorescence staining showed protection against aging and noise exposure in *GRAIL* KO mice

Aging and noise exposure could reduce the numbers of outer hair cells and synaptic ribbons. The cochlear surface preparation and immunofluorescence staining showed less loss of outer hair cells and synaptic ribbons in the hook region in *GRAIL* KO mice than *GRAIL* WT mice in both AIHL and NIHL (Figs. 4 and 5).

3.3. RNA-sequencing showed different gene changes after noise exposure between *GRAIL* WT and *GRAIL* KO mice

In this study, 20 gene expression levels were determined using the heat map. The up-regulated genes, including the CC-chemokine genes, were significantly elevated in *GRAIL* WT mice at day 1 after noise exposure as compared with unexposed *GRAIL* WT mice ($p < 0.0001$). However, significant downregulation of these gene expressions was detected at day 1 after noise exposure in *GRAIL* KO mice compared with unexposed *GRAIL* KO mice.

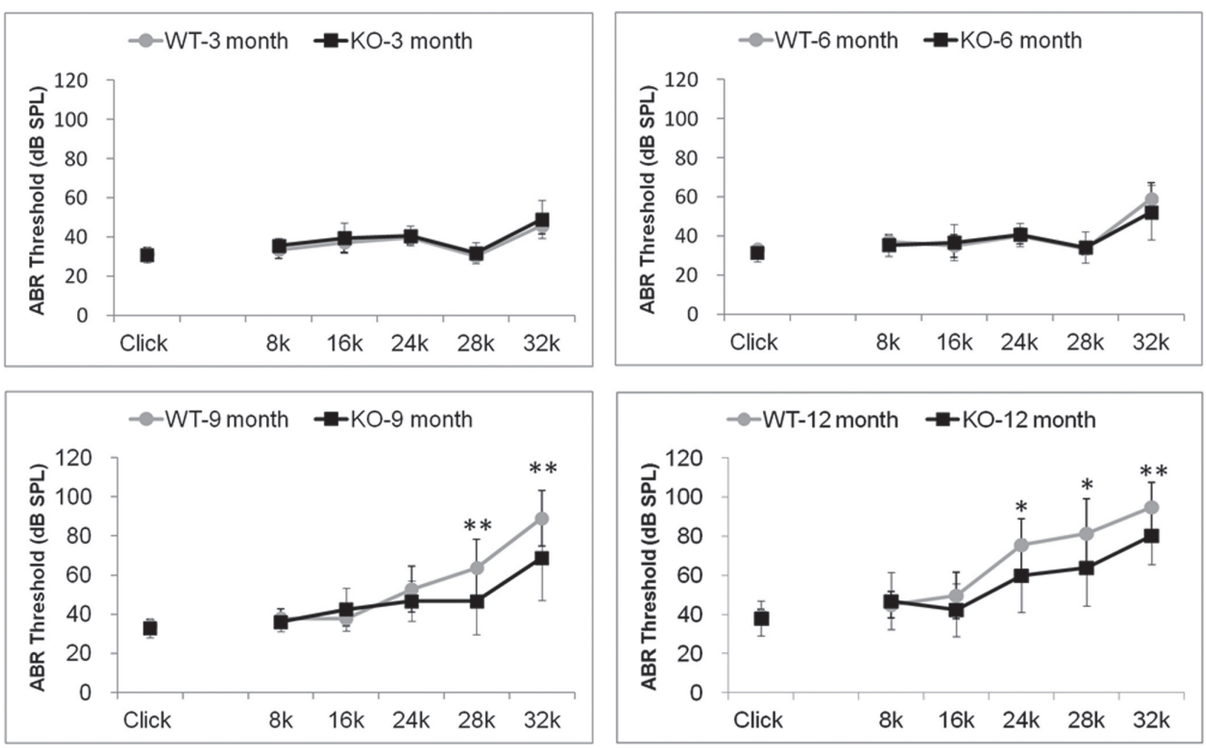

Fig. 2 Aging *GRAIL* KO mice had less elevation in ABR threshold levels. The ABR was recorded from one ear in each animal. The results are expressed as mean ± SEM (n > 6 [refers to >6 measured ears from >6 different animals]). *$p < 0.05$; **$p < 0.01$. ABR = auditory brainstem response; *GRAIL* = gene related to anergy in lymphocytes; KO = knockout; SPL = sound pressure level; WT = wild-type.

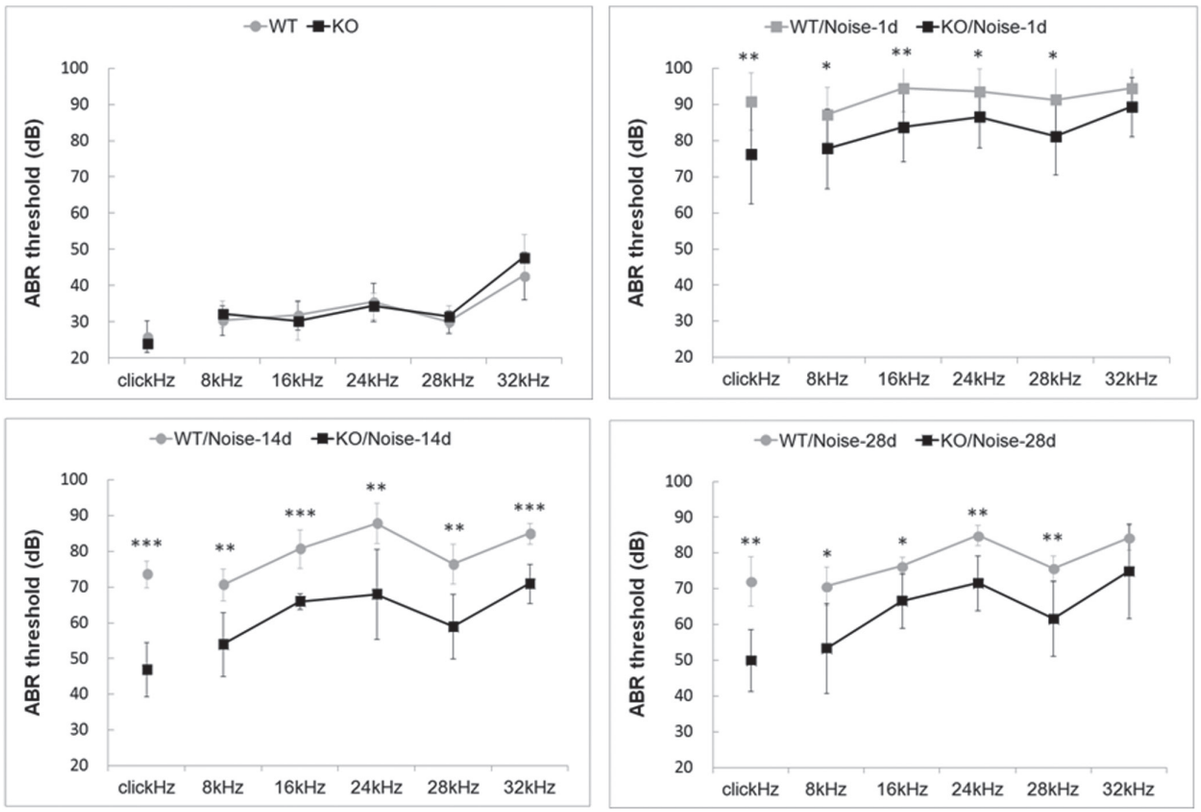

Fig. 3 *GRAIL* KO mice showed less elevation in ABR threshold levels after noise exposure. The ABR was recorded from one ear in each animal. The results are expressed as mean ± SEM (n > 6 [refers to >6 measured ears from >6 different animals]). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ABR = auditory brainstem response; *GRAIL* = gene related to anergy in lymphocytes; KO = knockout; WT = wild-type.

A trend toward reduced activation of inflammasome-associated genes in the *GRAIL* KO as compared with the *GRAIL* WT cochleae was found (Fig. 6). The expression of inflammation-related interleukin 25 (*IL25)*, C-C motif chemokine ligand 21 (*CCL21),* C-C motif chemokine ligand 19 *(CCL19)*, and Serum amyloid A1 (*SAA1*) genes were downregulated. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes (NADPH oxidase 1 [*NOX1*]) were also downregulated while glutathione peroxidase 6 (*GPX6*) was upregulated.

4. DISCUSSION

Protein ubiquitination and deubiquitination involved in the UPS have played many roles in cell biological developments and functions. Ubiquitination is a three-step process with ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) involved respectively in each step. Pouyo et al[10] have recently reviewed the major roles of UPS actors in cochlear development and function, otoprotection and mouse, or human deafness and presbycusis. However, related research is still scarce and the precise role of the UPS in hearing loss remains unclear.

E3 ligases are categorized into three prominent families, namely, really interesting new gene (RING), homologous to E6-AP terminus (HECT), and RING between RING (RBR) families.[23] The RING E3 ligases constitute the most prominent family of ubiquitin ligases. E3 ligases have been associated with deafness and hearing. F-Box protein 2 (Fbx2) is an E3 ligase and a member of the SKP1-cullin-F-box (SCF) complex. KO of Fbx2 in mice results in hearing loss due to cochlear cell death.[24] RING finger protein 8 (RNF8) is an E3 enzyme of the RING family and deletion of RNF8 leads to increased DNA damage in the cochlea and causes ARHL.[25] Mouse double minute 2 homolog (Mdm2) is a RING-Type E3 ligase and deletion of Mdm2 induces degeneration of the outer hair cells.[26] Most aforementioned studies have reported that deletion of E3 ligases may have a negative impact on hearing and cochlear development. This study showed a novel E3 ligase with protective effects against ARHL and NIHL although the *GRAIL* gene (also known as RNF128) was deleted.

The mechanism behind the protective effects of *GRAIL* KO against hearing loss remains unclear. As shown in Figs. 4 and 5, *GRAIL* KO mice lose less presynaptic ribbons after noise exposure and at 12-month follow-up, suggesting that the E3 ligase may be associated with noise-induced cochlear synaptopathy and aging-related cochlear synaptopathy.[8,14] Deletion of Mdm2 might lead to the accumulation of a postsynaptic protein, PSD95, whose level correlates with the synaptic strength and maturation.[27] It would be important to study the effect of E3 ligase deletion on synaptic function in the cochlea to elucidate the mechanism involved.

RNA-seq analysis further showed significant differences between inflammation- and oxidative stress–related genes after noise exposure in the potential targets of the *GRAIL* gene-related hearing loss. *CCL21* and *CCL19* are genes encoding the pro-inflammatory chemokine ligands that play an essential role in recruiting normal lymphocyte or activated T cells from the peripheral blood to the local injured area in traumatic and acute noise stresses.[28] *IL25* is a pro-inflammatory cytokine favoring the Th2-type immune response.[29] *SAA1* is a major acute-phase

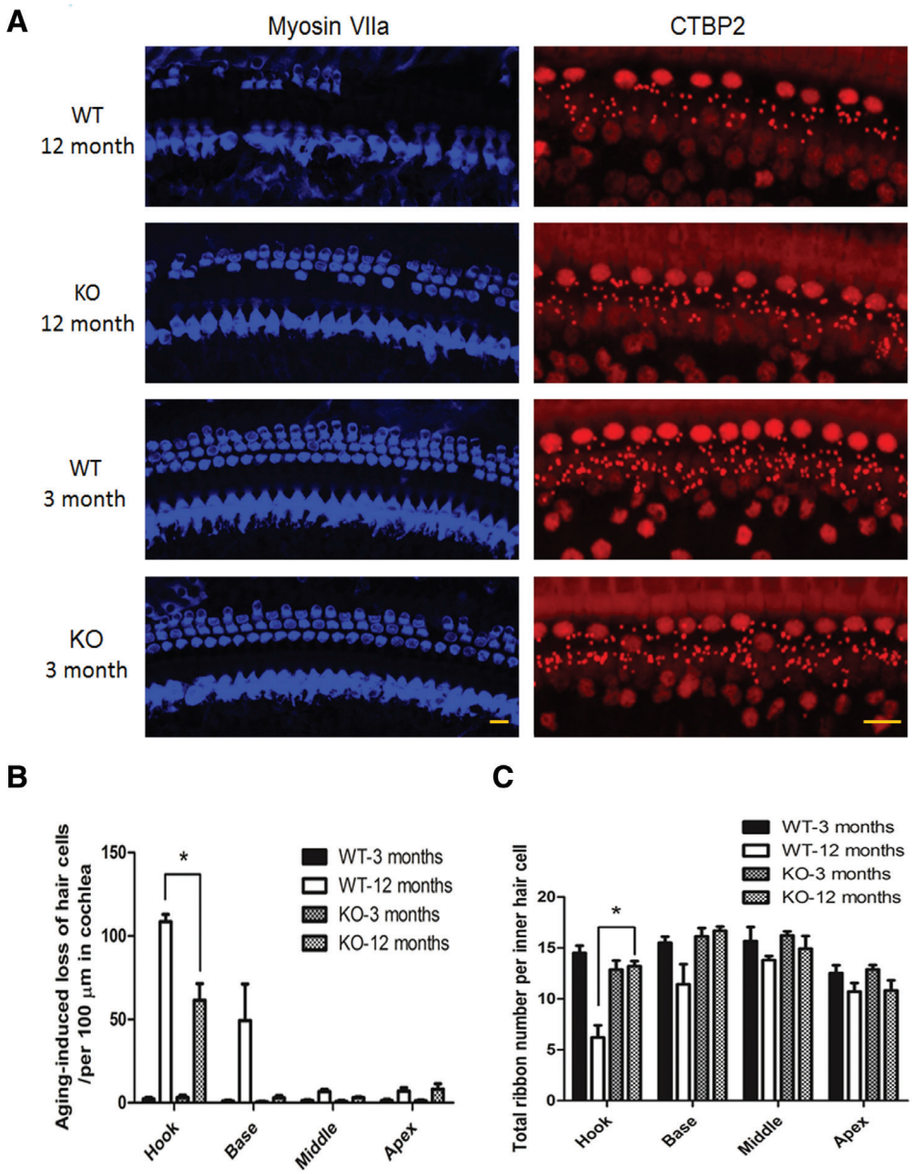

Fig. 4 A, Cochlear surface preparation and immunofluorescence staining with synaptic ribbons of *GRAIL* WT and *GRAIL* KO mice aged 3 and 12 mo. B, The numbers of lost outer hair cells in hook regions of KO and WT mice were calculated and compared. C, Representative images and quantification of synapse ribbons in hook regions. Cochlear synaptic ribbons in the hook region of KO mice were significantly maintained compared with WT mice. Scale bars, 10 μm. *$p < 0.05$. *GRAIL* = gene related to anergy in lymphocytes; KO = knockout; WT = wild-type.

protein that is triggered by an inflammation response associated with the stimulation of cytokine/chemokine, including IL8, IL6, CXCL5, and CCL2 production and knockdown of *SAA1*, resulting in a significant reduction of the pro-inflammatory cytokines and chemokines.[30] Downregulation of *CCL21*, *CCL19*, *IL25*, and *SAA1* may disrupt sending signals to the immune cells and decrease the induction of inflammatory cytokines so as to recruit inflammatory cells to the noise-injured cochlea against NIHL in *GRAIL* KO mice.

The expression of *GPX6*, which catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, and lipid hydroperoxides, and thereby protects the cells against oxidative damage. NADPH oxidases (*NOX1*) are enzymes that transport electrons across the plasma membrane and generate superoxide radicals from molecular oxygen. Postexposure inhibition of NADPH oxidases by diphenyleneiodonium, a broadly selective NADPH oxidase inhibitor, mitigated NIHL.[31] Upregulation of *GPX6* and downregulation of *NOX1* may protect against NIHL in *GRAIL* KO mice.

In conclusion, the present results provided additional evidence of a new E3 ubiquitin ligase (*GRAIL* [RNF128]) in the UPS association with protection against ARHL and NIHL. The findings also suggested that the synaptic modulation, expression of inflammation-associated genes (*CCL19*, *CCL21*, *IL25*), and oxidative stress–related genes (*GPX6*, *NOX1*) may play a role in protecting *GRAIL* KO mice against NIHL. While further validation tests are required, these genes could potentially be used as therapeutic targets for preventing ARHL and NIHL and

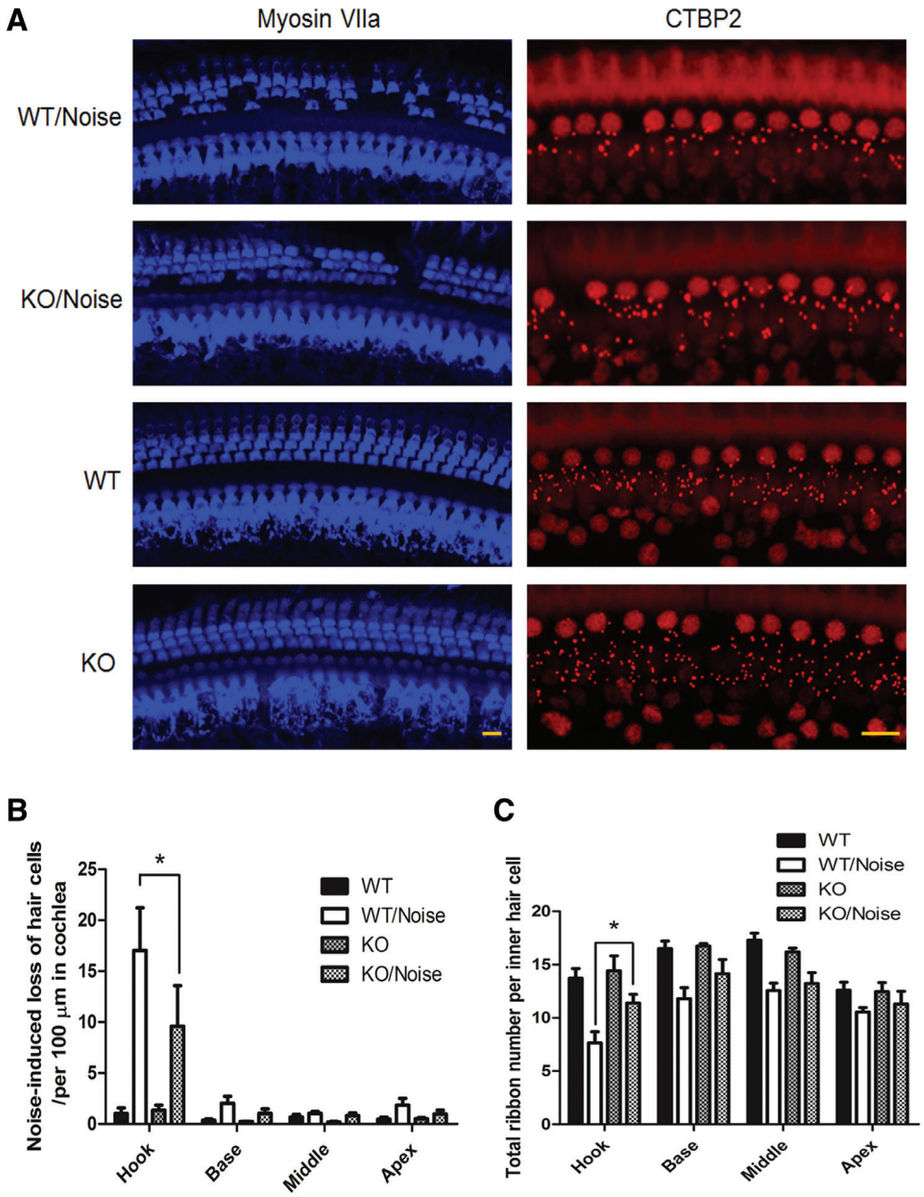

Fig. 5 A, Cochlear surface preparation and immunofluorescence staining with synaptic ribbons of four groups: *GRAIL* WT, *GRAIL* KO, *GRAIL* WT at day 60 after noise exposure (WT/noise), *GRAIL* KO at day 60 after noise exposure (KO/noise). B, The numbers of lost outer hair cells in hook regions of KO/noise and WT/noise mice were calculated and compared. C, Representative images and quantification of synapse ribbons in hook regions. Cochlear synaptic ribbons in the hook region of KO/noise mice were significantly maintained compared with WT/noise mice. Scale bars, 10 μm. *$p < 0.05$. CTBP2 = C-terminal binding protein 2; *GRAIL* = gene related to anergy in lymphocytes; KO = knockout; WT = wild-type.

might also provide insight into the underlying pathophysiological mechanism.

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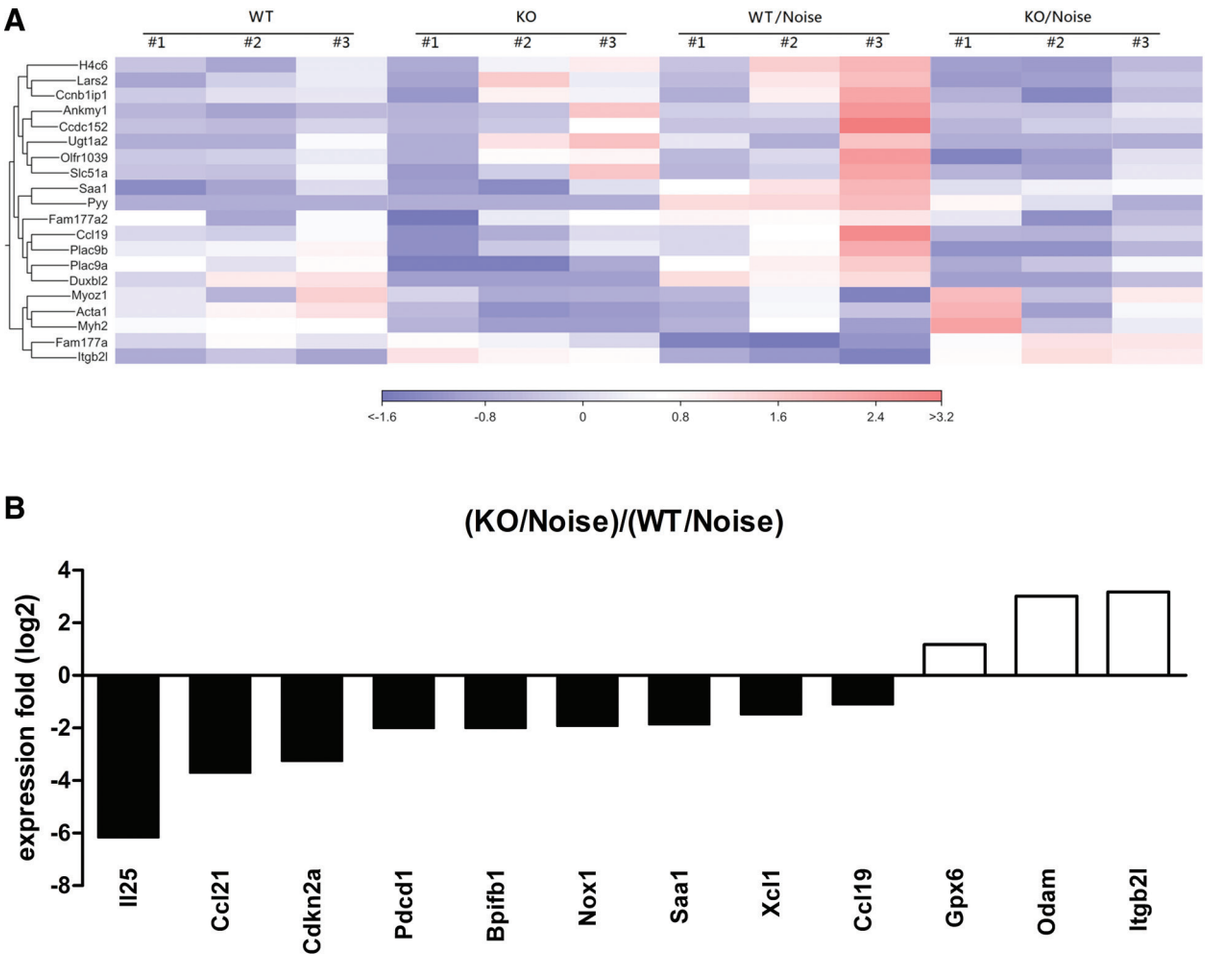

Fig. 6 A, Heat map showing hierarchical clustering of differentially expressed mRNAs in four groups: *GRAIL* WT, *GRAIL* KO, *GRAIL* WT at day 1 after noise exposure (WT/noise), *GRAIL* KO at day 1 after noise exposure (KO/noise). Red indicates upregulation and blue represents downregulation. B, The regulatory genes associated with inflammation and oxidative stress were shown, selected with *p*-adjust <0.0001 between two groups was considered significantly enriched. CTBP2 = C-terminal binding protein 2; *GRAIL* = gene related to anergy in lymphocytes; KO = knockout; WT = wild-type.

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