Supporting Information

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SI Materials and Methods

ABR Hearing Screening. ABRs were measured with a tone burst stimulus (8, 16, and 32 kHz) using an ABR recording system (Intelligent Hearing System) as previously described (1). Mice were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg i.m.; Phoenix Urology of St. Joseph) and ketamine hydrochloride (40 mg/kg i.m.; Phoenix Urology of St. Joseph). Male mice were used for the WT and $Bak^{-/-}$, WT and $Bax^{-/-}$ mouse, and antioxidant tests, whereas female mice were used for the MCAT and age-matched WT mouse tests. Following the ABR hearing measurements, the same mice were used to conduct histopathological, TUNEL, oxidative damage, or QRT-PCR analyses.

Histopathological Analysis. The temporal bone was excised from the head and divided into cochlear and vestibular parts (1). The cochlea was then excised, immersed in a fixative containing 4% paraformaldehyde (Sigma-Aldrich) in PBS solution for 1 d, and decalcified in 10% EDTA for 1 week. The paraffin-embedded specimens were sliced into 4-μm sections, mounted on silane-coated slides, stained with H&E, and observed under a light microscope (Leica). The Rosenthal canal was divided into 3 regions—apical, middle, and basal—that were used for evaluation of cochlear histology (2). We used 5 mice per group for histopathological assessment. In each mouse, we evaluated every fifth modiolar section obtained from one unilateral cochlea for a total of 5 sections. The same animals were used for SG neuron counting, hair cell counting, or TUNEL staining.

SG Neuron Survival. SG neurons were counted in the apical, middle, and basal regions of the cochlear sections using a $\times$20 objective as previously described (2, 3). Type I and type II neurons were not differentiated, and cells were identified by the presence of a nucleus. The corresponding area of Rosenthal canal was measured on digital photomicrographs of each canal profile. The perimeter of the canal was traced with a cursor using ImageJ software (National Institutes of Health). The computer then calculated the area within the outline. The SG neuron survival was calculated as the number of SG neuron per mm^2. Five sections of the unilateral apical, middle, and basal turns were evaluated in one cochlea per mouse.

Hair Cell Survival. For $Bak^{-/-}$ and WT mouse studies, OH cells and IH cells were counted in the apical, middle, and basal regions of the cochlear sections using a $\times$40 objective. Hair cells were identified by the presence of a nucleus. The OH cell survival percentage was calculated as the number of intact OH cells present among the 3 OH cells that should be observed in each turn of one cochlea in tissue sections of mice with normal hearing. The IH cell survival percentage was calculated as the number of intact IH cells present of the one IH cell that should be observed in each turn of one cochlea in tissue sections of mice with normal hearing.

For MCAT and WT mouse studies, counts of the numbers of OH cells and IH cells were obtained over 0.1- to 0.18-mm intervals using a differential interference contrast microscope at a magnification of $\times$1,000 (oil immersion) (4). Hair cell survival percentage was determined on the basis of the hair cell density distribution in each mouse. Individual cochleograms were determined by plotting the percentage of missing OH cells and IH cells as a function of percent total distance from the apex of the cochlea.

Measurement of RNA and DNA Oxidation Levels Using HPLC-ECD. Total RNA and DNA oxidation levels of cochleae were analyzed simultaneously with a HPLC-ECD method (5, 6). This procedure is based on high-salt nucleic acid release from proteins, followed by removal of proteins/fats by organic solvents at neutral pH, all in the presence of the metal chelator deferoxamine mesylate [DFOM; affinity constant for Fe(III): log K, 30.8] at 0 °C. Briefly, the frozen cochleae were thawed and pulverized on slush ice in 0.5 mL guanidine thiocyanate buffer (3 M guanidine thiocyanate, 0.2% wt/vol; N-lauroyl sarcosinate, 20 mM Tris, pH 7.5) containing 10 mM freshly dissolved DFOM. After transferring the solution into a 2.0-mL phase-lock gel tube, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 6.7) was added and the samples were immediately vortexed, followed by a 10-min vortexing period at 0 °C to completely release nucleic acids as previously described (5, 6). After centrifugation (14,000 $\times$ g, 5 min, 0 °C), the aqueous phase was transferred into a new phase-lock gel tube and extracted against an equal volume of chloroform/isoamyl alcohol (24:1). The samples were hand-shaken and centrifuged, and the aqueous phase was collected and nucleic acids were precipitated with an equal amount of isopropanol at −80 °C overnight. After centrifugation (10,000 $\times$ g, 10 min, 0 °C), nucleic acids were washed in 70% (vol/vol) ethanol, dried, dissolved in DNase and RNase-free water containing 30 μM DFOM, and hydrolyzed using 4 U nuclease P1 and 5 U alkaline phosphatase in buffer (30 mM sodium acetate, 20 μM $ZnCl_2$, pH 5.3, final volume 100 μL) at 50 °C for 60 min. After filtration, the samples were analyzed by HPLC-ECD as described (5, 6).

QRT-PCR. Detailed protocols for QRT-PCR analysis have been described (3). The cochleae were dissected, frozen in liquid N_2, and stored at −80 °C. Total RNA was extracted from the frozen cochlear tissues using the TRIzol reagent (Invitrogen) as previously described (3). Detection of mRNA was carried out with the TaqMan EZ RT-PCR kit using an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems). β-Actin was used as an internal standard. Oligonucleotide primers and MGB fluorescent probes (TaqMan gene expression assays) were purchased from Applied Biosystems. Samples were run in duplicate and each sample consisted of the 2 cochleae pooled from one mouse.

Antioxidant Dietary Study. Starting at 4 months of age, B6 mice were randomly divided into 18 groups: control diet (AIN-93M formulation by Bio-Serv) and 17 antioxidant diet groups (Table S1). The antioxidant diet groups were fed the same caloric intake as controls, but were supplemented with the following, per kg diet: 500 mg acetyl-*L*-carnitine (Sigma-Aldrich), 150 mg LA (Sigma-Aldrich), 8,000 mg β-carotene (natural carotenoids from Cognis), 500 mg L-carnosine (Sigma-Aldrich), 50 mg CQ (Sigma-Aldrich), 500 mg curcumin (Sigma-Aldrich), 16.7 mg *d*-α-tocopherol (natural tocopherols from Cognis), 250 mg epigallocatechin gallate (Taiyo Chemical), 250 mg gallic acid (Sigma-Aldrich), 20 mg lutein (Kemin Industries), 250 mg lycopene (Sigma-Aldrich), 20 mg melatonin (Sigma-Aldrich), 500 mg NAC (Sigma-Aldrich), 309 mg proanthocyanidin (grapeseed extract; Kikkoman), 250 mg quercetin dehydrate (Sigma-Aldrich), 50 mg resveratrol (Sigma-Aldrich), and 250 mg tannic acid (Sigma-Aldrich) from 4 months of age until 15 months of age. Details on feeding control diets have been published elsewhere (7). At 15 months of age, ABR tests were conducted

and cochlea tissues were collected from the mice. Following the ABR measurements, the same mice were used to conduct histopathology, SG cell counting, and QRT-PCR analyses.

TUNEL Staining. TUNEL staining for apoptotic nuclei was performed using a DeadEnd Colorimetric TUNEL System (Promega) according to the manufacturer's instructions (3). Color development was accomplished with diaminobenzidine for 8 min. Duplicate sections were counterstained with hematoxylin and a cover glass was mounted to each slide with Permount mounting medium.

For tissue sections, TUNEL-positive cells were counted in the apical, middle, and basal regions of the cochlear sections using a ×20 objective. TUNEL-positive cells were identified by the presence of a brown-stained nucleus in the SG neuron regions. The corresponding area of the Rosenthal canal was measured on digital photomicrographs of each canal profile in the same manner as described in SG Neuron Survival (SI Text). The TUNEL-positive was calculated as the number of TUNEL-positive cells per mm^2. Five sections of the unilateral apical, middle, and basal regions were evaluated in one cochlea per mouse.

Primary Cochlear Cell Culture. The procedures for preparing primary cochlear cell cultures were similar to those described previously (8, 9). Cochleae were dissected out from the temporal bones of 4-d-old pups and dissociated in 0.5% collagenase (Sigma-Aldrich) and 0.25% trypsin (Sigma-Aldrich) in HBSS medium (Invitrogen) at 37 °C for 30 min. Male and female mice were not differentiated. The enzymatic reaction was terminated by adding DMEM (Invitrogen) containing 10% FBS (Invitrogen), 100 U/mL penicillin (Sigma-Aldrich), and 10 mM Hepes (Invitrogen; M10), and the cell suspension was spun down for 3 min at 386 × *g*. The cochlear cell suspension density was adjusted to a concentration of 250,000 cells/mL using M10 medium, and 200 μL of the cell suspension (50,000 cells) was plated in each well of a 96-well plate. The wells were pre-coated with 10 μg/mL fibronectin (1 h incubation at room temperature; Sigma-Aldrich) and 20 μg/mL polyL-lysine (1 h incubation at 37 °C; Sigma-Aldrich) before plating. The cells were then incubated at 37 °C in 5% COB_{2B}/95% relative humidity (RH) environment for 24 h before initiating the cell death tests. Cell death was induced by incubation with 0, 100, 200, 400, or 600 μM PQ (Sigma-Aldrich) in DMEM containing 5% FBS, 100 U/mL penicillin, 2 mM L-glutamine (Sigma-Aldrich), 100 μg/mL streptomycin (Sigma-Aldrich), 10 mM Hepes, and 1% N2 neuronal growth supplement (Invitrogen; M5) at 37 °C in 5% COB_{2B}/95% RH environment for 18 h.

For TUNEL studies, cell death was induced by incubation with 0 or 400 μM PQ in M5 medium as described earlier. The apoptosis-induced cells were dissociated in 0.25% trypsin in PBS solution for 10 min at room temperature. The enzymatic reaction was terminated by adding PBS solution and the cell suspension was spun down for 3 min at 386 × *g*. The cochlear cell suspension density was adjusted to a concentration of 10,000 cells/mL, and 1000 cells were plated to each polyL-lysine-coated slide. TUNEL staining for apoptotic nuclei in the cultured cells was performed using a DeadEnd Colorimetric TUNEL System (Promega) according to the manufacturer's instructions. TUNEL-positive cells were counted in each slide using a ×20 objective. Cells were identified by the presence of a brown-stained nucleus. The TUNEL-positive cells were reported as the number of TUNEL-positive cells per 1,000 cells. Each condition was run in duplicate.

For QRT-PCR studies, cell death was induced by incubation with 0 or 400 μM PQ in M5 medium as described earlier. The treated cells were dissociated in 0.25% trypsin in PBS solution for 10 min at room temperature. The enzymatic reaction was terminated by adding PBS solution and the cell suspension was spun down for 3 min at 386 × *g*. Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen) as previously described (7) and detection of mRNA was carried out as described in the QRT-PCR section. Each condition was run in duplicate.

Cell Viability Test (Neutral Red Uptake Assay). To perform the cell viability assay, at the conclusion of treatment to induce apoptosis, the cells were washed twice in PBS solution, then incubated in DMEM composed of 50 μg/mL neutral red (Sigma-Aldrich) at 37 °C in 5% CO_2/95% RH environment for 3 h. After the incubation, the cells were rinsed with PBS solution, and 200 μL of a neutral red destain solution composed of 50% ethanol, 49% deionized water, and 1% glacial acetic acid (Sigma-Aldrich) was added to each well. The 96-well plate was placed on a plate shaker for 10 min and the OD of the neutral red extract in each well was measured at 540 nm in a microtiter plate reader spectrophotometer. Each condition was run in duplicate.

Neuron Viability Test. To perform the neuron viability assay, the cochlear cell suspension density was adjusted to a concentration of 62,500 cells/mL using M10 medium, and 400 μL of the cell suspension (25,000 cells) was plated on a coverslip in each well of a 24-well plate. The coverslips were pre-coated with 10 μg/mL fibronectin and 20 μg/ml polyL-lysine. The cells were incubated at 37 °C in 5% CO_2/95% RH environment for 24 h before cell death tests. Cell death was induced by incubation with 0 or 400 μM PQ in M5 medium as described in TUNEL Staining (SI Text). The cells were then rinsed with PBS solution, fixed with 4% paraformaldehyde in PBS solution for 30 min at room temperature, incubated in 3% H_2O_2 (Sigma-Aldrich) in PBS solution for 10 min, incubated in blocking buffer (Pierce) for 60 min, and incubated in a moist chamber for 60 min at 25 °C with primary mouse monoclonal anti-neurofilament antibody (Sigma-Aldrich) diluted 1:1,000 in the blocking buffer. This was followed by incubation with biotinylated mouse secondary antibody (Vector) diluted in 1:300 in PBS solution for 30 min. The cells were then treated with ABC solution (Vector) for 30 min and incubated with DAB substrate (Vector) for 8 min. The coverslip was mounted to each slide with Permount mounting medium. Anti-neurofilament-positive (i.e., neuron-positive) cells were counted in each slide, and each condition was run in duplicate.

1. Yamasoba T, et al. (2006) Cochlear damage due to germanium-induced mitochondrial dysfunction in guinea pigs. *Neurosci Lett* 395:18–22.
2. Keithley EM, Canto C, Zheng QY, Fischel-Ghodsian N, Johnson KR (2004) Age-related hearing loss and the ahl locus in mice. *Hear Res* 188:21–28.
3. Someya S, Yamasoba T, Prolla TA, Tanokura M (2007) Genes encoding mitochondrial respiratory chain components are profoundly down-regulated with aging in the cochlea of DBA/2J mice. *Brain Res* 1182:26–33.
4. Ding D, Jiang H, Wang P, Salvi R (2007) Cell death after co-administration of cisplatin and ethacrynic acid. *Hear Res* 226:129–139.
5. Hofer T, Seo AY, Prudencio M, Leeuwenburgh C (2006) A method to determine RNA and DNA oxidation simultaneously by HPLC-ECD: greater RNA than DNA oxidation in rat liver after doxorubicin administration. *Biol Chem* 387:103–111.
6. Xu J, Knutson MD, Carter CS, Leeuwenburgh C (2008) Iron accumulation with age, oxidative stress and functional decline. *PLoS One* 3:e2865.
7. Pugh TD, Klopp RG, Weindruch R (1999) Controlling caloric consumption: Protocols for rodents and rhesus monkeys. *Neurobiol Aging* 20:157–165.
8. Nicotera TM, Ding D, McFadden SL, Salvemini D, Salvi R (2004) Paraquat-induced hair cell damage and protection with the superoxide dismutase mimetic m40403. *Audiol Neurootol* 9:353–362.
9. Cheng AG, et al. (1999) Calpain inhibitors protect auditory sensory cells from hypoxia and neurotrophin-withdrawal induced apoptosis. *Brain Res* 850:234–243.

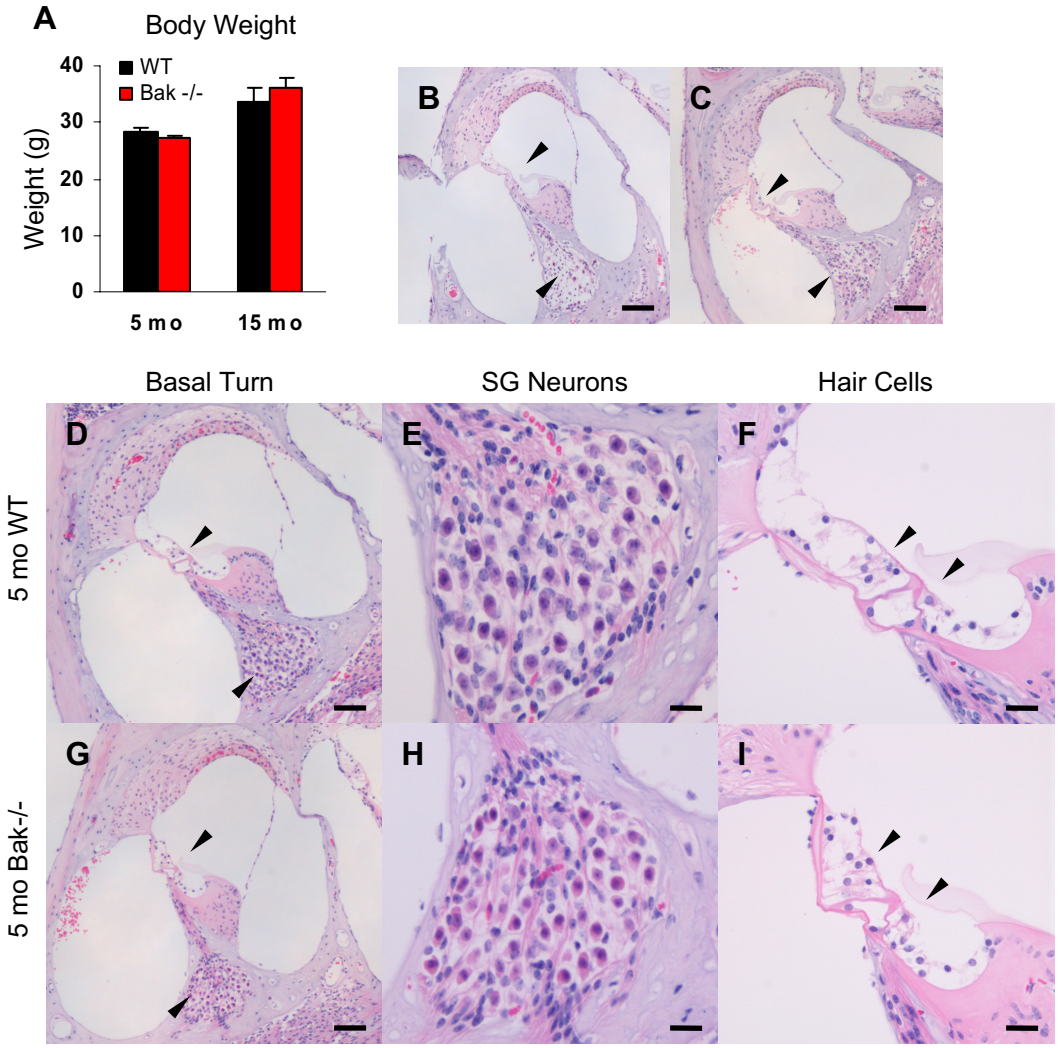

Fig. S1. Body weight and histopathology. (*A*) Body weight was measured from WT and *Bak*$^{-/-}$ mice at 5 and 15 months of age ($n = 10$). Basal cochlear regions from 15-month-old WT (*B*) and *Bak*$^{-/-}$ (*C*) mice. Arrows indicate the SG neuron and hair cell regions. (Scale bar, 100 μm.) Basal cochlear regions from 5-month-old WT (*D–F*) and *Bak*$^{-/-}$ (*G–I*) mice. Arrows in the lower-magnification photos (*Left*) indicate the SG neuron and hair cell regions, which are also shown at higher magnification (*Middle* and *Right*, respectively). (Scale bar, 100 μm, *Left*; 20 μm, *Middle* and *Right*.) Error bars represent SEM.

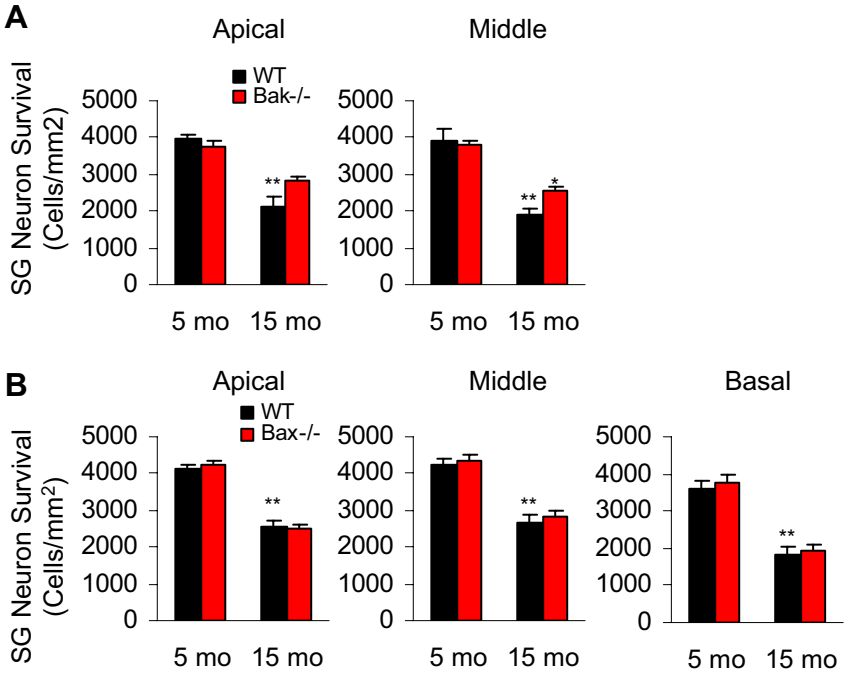

Fig. S2. SG neuron survival. (*A*) SG neuron survival (i.e., SG neuron density) of apical and middle cochlear regions was measured from WT and *Bak*$^{-/-}$ mice at 5 and 15 months of age ($n = 5$). (*B*) SG neuron survival (i.e., SG neuron density) of apical, middle, and basal cochlear regions was measured from WT and *Bax*$^{-/-}$ mice at 5 and 15 months of age ($n = 5$). *Significantly different from 15-month-old WT mice ($P < 0.05$). **Significantly different from 5-month-old WT mice ($P < 0.05$). Error bars represent SEM.

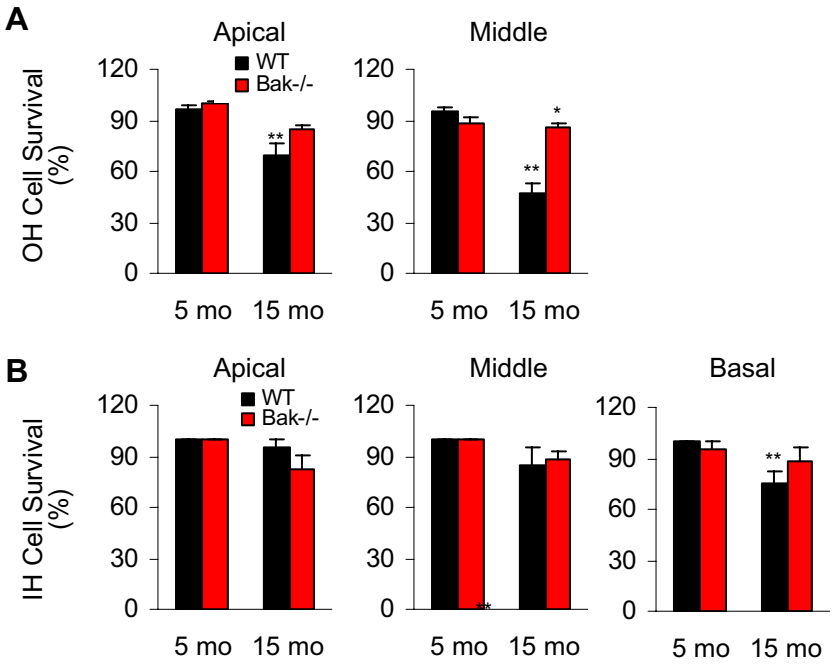

Fig. S3. OH cell and IH cell survival. (*A*) OH cell survival (%) of apical and middle cochlear regions was measured from WT and *Bak*$^{-/-}$ mice at 5 and 15 months of age ($n = 5$). (*B*) IH cell survival (%) of apical, middle, and basal cochlear regions was measured from WT and *Bak*$^{-/-}$ mice at 5 and 15 months of age ($n = 5$). *Significantly different from 15-month-old WT mice ($P < 0.05$). **Significantly different from 5-month-old WT mice ($P < 0.05$). Error bars represent SEM.

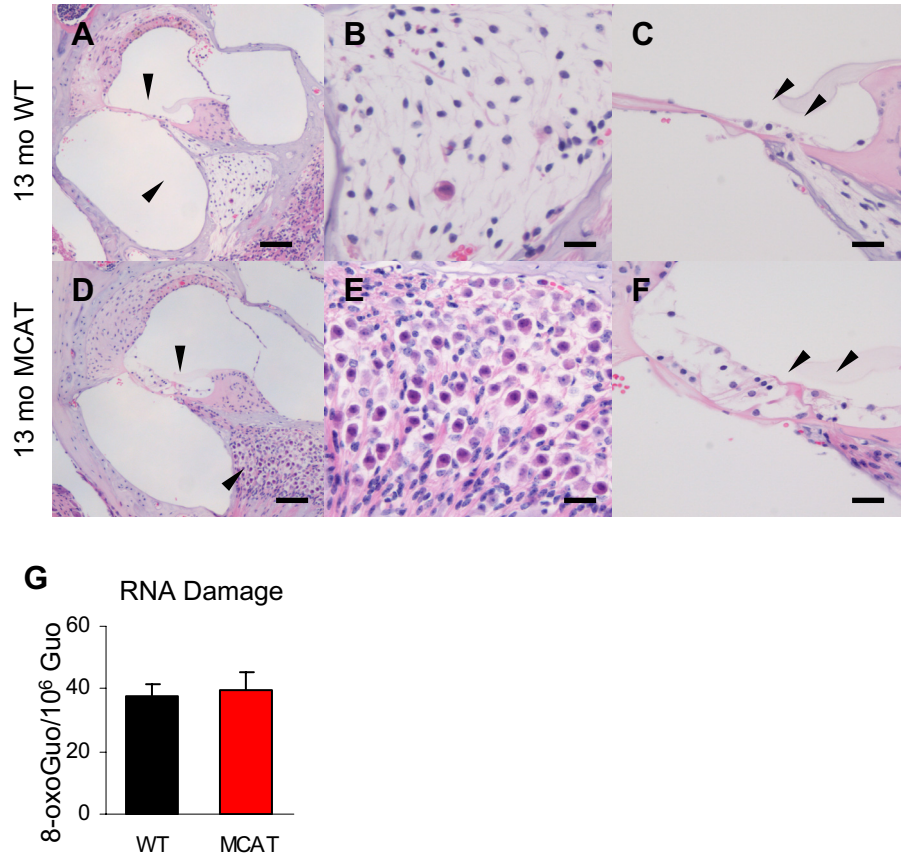

Fig. S4. Histopathology and oxidative RNA damage. Basal cochlear regions from 13-month-old WT (*A–C*) and MCAT (*D–F*) mice. Arrows in the lower-magnification photos (*Left*) indicate the SG neuron and hair cell regions, which are also shown at higher magnification (*Middle* and *Right*, respectively). (Scale bar, 100 μm, *Left*; 20 μm, *Middle* and *Right*.) (*G*) Oxidative damage to RNA (8-oxoGuo) was measured in the cochleae of WT and MCAT mice at 13 months of age (n = 5–10). Error bars represent SEM.

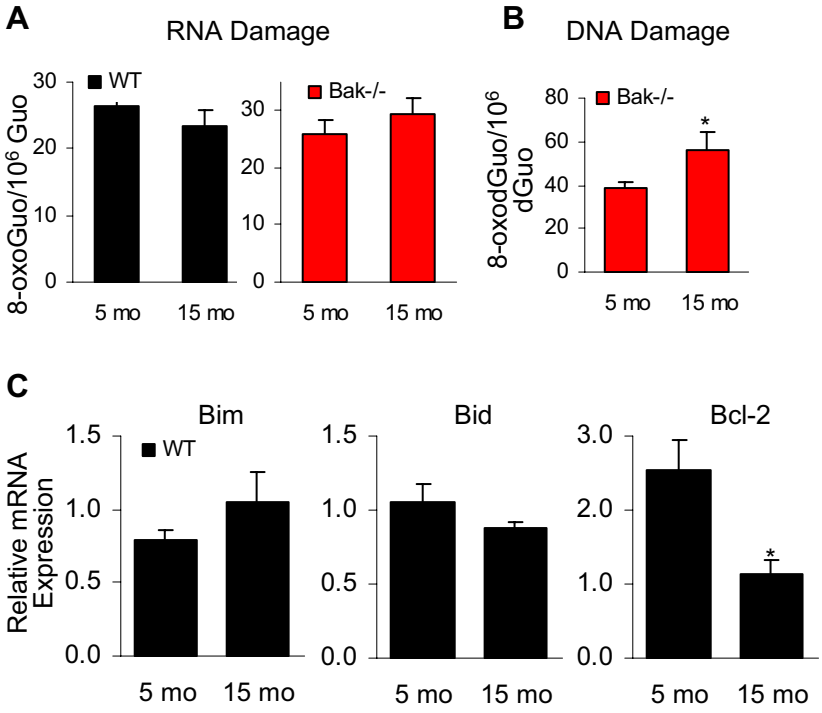

Fig. S5. Oxidative RNA and DNA damage and relative mRNA levels of Bcl-2 family members. (*A*) Oxidative damage to nuclear RNA was measured in the cochleae of WT and *Bak*$^{-/-}$ mice at 5 and 15 months of age ($n = 5$–10). (*B*) Oxidative damage to DNA (8-oxodGuo) was measured in the cochleae from *Bak*$^{-/-}$ mice at 5 and 15 months of age ($n = 5$–10). (*C*) Relative cochlear mRNA expression of *Bim*, *Bid*, and *Bcl-2* was measured in the cochleae from WT mice at 5 and 15 months of age ($n = 5$). ⁎Significantly different from 5-month-old WT mice ($P < 0.05$). Error bars represent SEM.

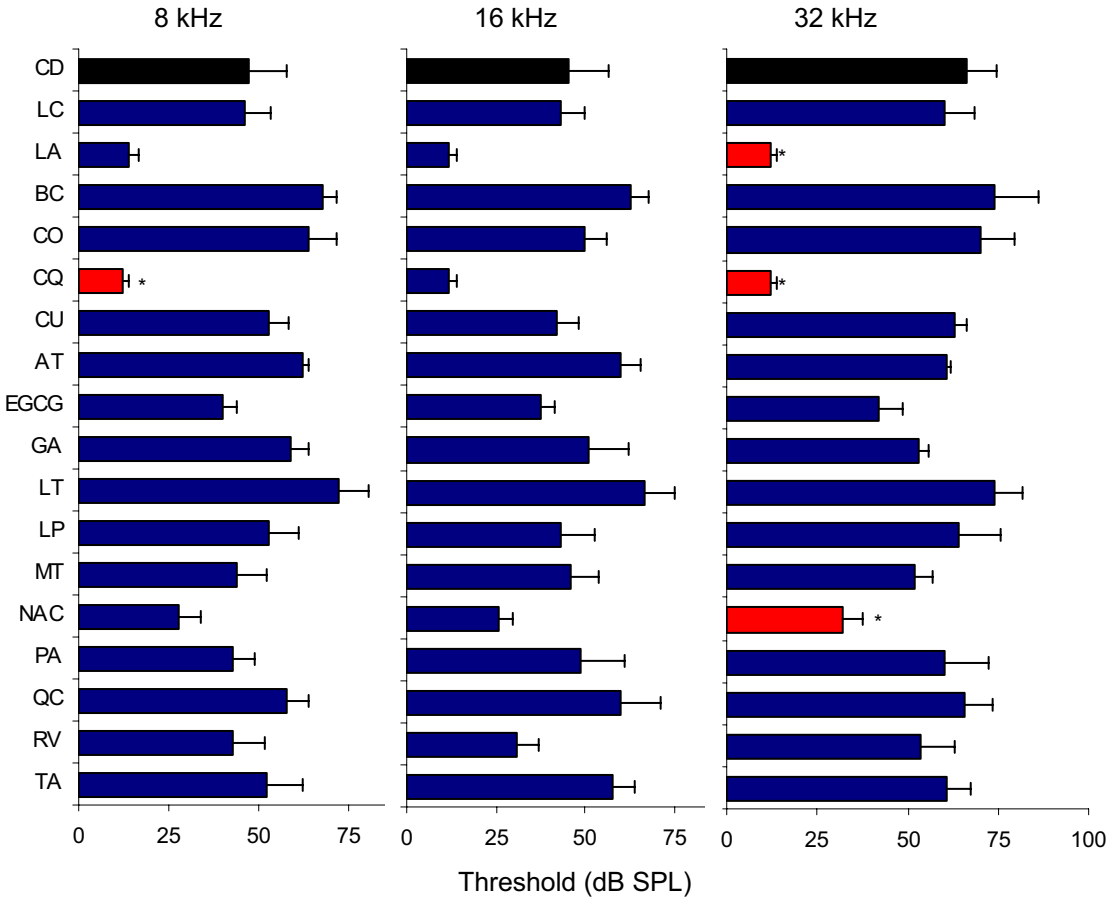

Fig. S6. Supplementation of antioxidants that target mitochondria delays the onset of AHL. ABR hearing thresholds were measured at 8, 16, and 32 kHz in 15-month-old mice fed control diet or diets supplemented with one of 17 antioxidant compounds ($n = 5–7$). AT, *d*-α-tocopherol; BC, β-carotene; CC, quercetin; CD, control diet; CO, carnosine; CQ, coenzyme Q_{10}; CU, curcumin; EGCG, epigallocatechin gallate; GA, gallic acid; LC, acetyl-L-carnitine; LP, lycopene; LT, lutein; MT, melatonin; PA, proanthocyanidin; RV, resveratrol; TA, tannic acid. *Significantly different from control diet or WT mice ($P < 0.05$). Error bars represent SEM.

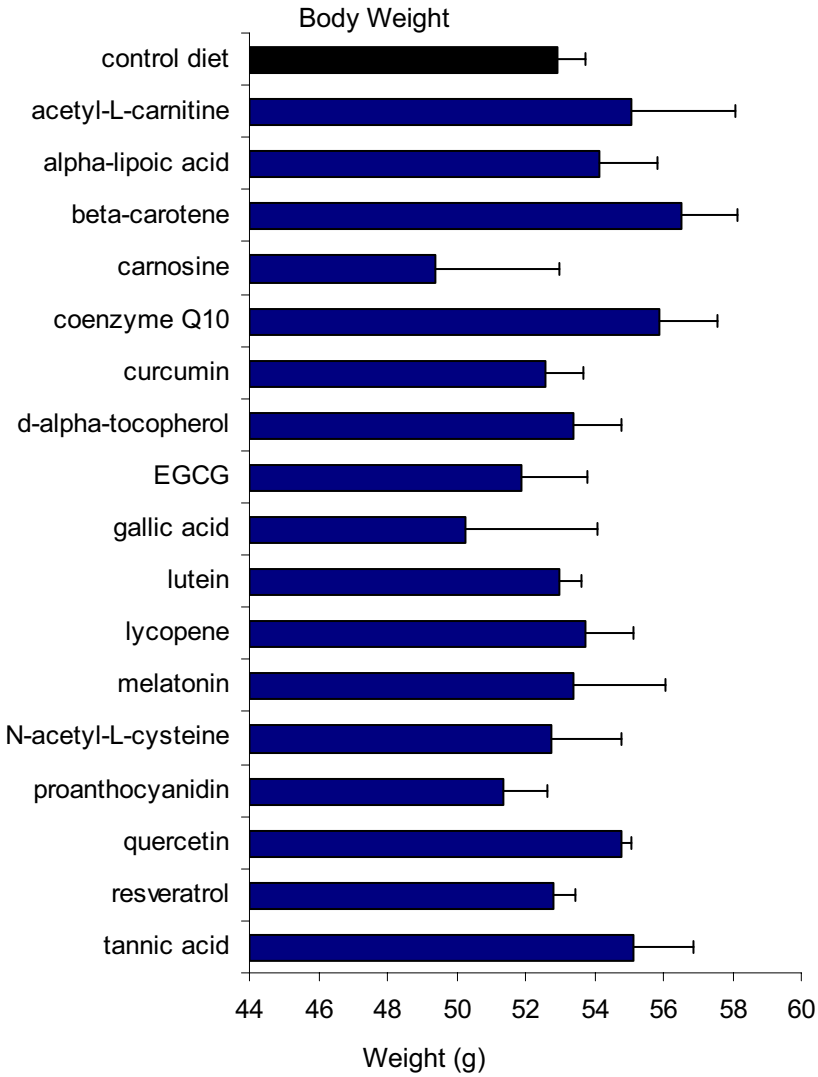

Fig. S7. Body weight. Body weight was measured from control and antioxidant diet-fed mice at 15 months of age (n = 5–8). Error bars represent SEM.

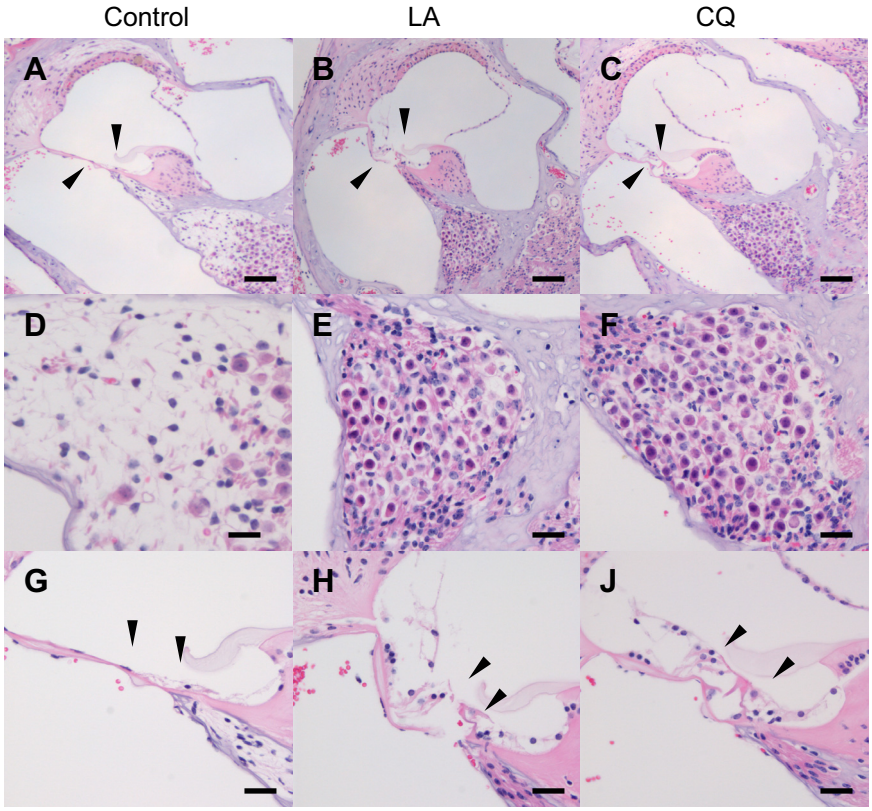

Fig. S8. Histopathology. Basal cochlear regions from 15-month-old control (*A*, *D*, and *G*), LA (*B*, *E*, and *H*), and coenzyme Q_{10} (*CQ*) (*C*, *F*, and *J*) diet-fed mice. Arrows in the lower-magnification photos (*Upper*) indicate the hair cell regions, which are also shown at higher magnification (*Lower*). (Scale bar, 100 μm, *Left*; 20 μm, *Middle* and *Right*.)

Table S1. List of antioxidants and dosages

Name	Dose, mg/kg of diet
Acetyl-L-carnitine	500
α-Lipoic acid	150
β-Carotene	8,000
Carnosine	500
Coenzyme Q_{10}	50
Curcumin	500
d-α-Tocopherol	17
EGCG	250
Gallic acid	250
Lutein	20
Lycopene	250
Melatonin	20
N-acetyl-L-cysteine	500
Proanthocyanidin	309
Quercetin	250
Resveratrol	50
Tannic acid	250