Supporting information for

Activation of Rictor/mTORC2 signaling acts as a new pivotal strategy to protect against sensorineural hearing loss

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SI Materials and Methods
Materials and Methods:
Animal models
All animal experiments were approved by the Animal Ethics Committee of Shandong First Medical University. Animal experiments were conducted strictly in accordance with the standards of the Animal Ethics Committee of Shandong First Medical University.

Male and female C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. Atoh1-Cre mice were a gift from Lin Gan (University of Rochester, New York, New York, USA). Rosa26-TdTomato (no.007914), Pten⁻⁻ (no.005601).
Rictor<sup>fl/fl</sup> (no. 020649), mTOR<sup>fl/fl</sup> (no. 011009), and Raptor<sup>fl/fl</sup> (no. 013188) mice were procured from The Jackson Laboratory. p53<sup>-/-</sup> mice (no. 002101) back-crossed into the C57BL/6J mice background were a gift from Professor Chang-shun Shao. All Atoh1-Cre mice were first bred with Rosa26-tdTomato (no. 007914). The Pten<sup>fl/fl</sup>, Rictor<sup>fl/fl</sup>, Raptor<sup>fl/fl</sup>, P53<sup>-/-</sup>, and Atoh1-Cre mouse lines were backcrossed with the C57BL/6J line for 5 generations. We generated mTOR<sup>fl/fl</sup>Atoh1-Cre (mTOR-cKO), Rictor<sup>fl/fl</sup>Atoh1-Cre (Rictor-cKO), Tsc1<sup>fl/fl</sup>Atoh1-Cre (Tsc1-cKO), Pten<sup>fl/fl</sup>Atoh1-Cre (Pten-cKO), and Raptor<sup>fl/fl</sup>Atoh1-Cre (Raptor-cKO) mice, which were confirmed using PCR genotyping. The floxed mice without Cre were used as controls in our study. A detailed description of the three types of mice (Tsc1-cKO, Pten-cKO, and Raptor-cKO) can be found in our previous report (1). Next, we sequenced the Cdh23 gene from DNA obtained from the tails of young mice. Mice of either sex were used in all experiments. PCR genotyping primer sequences are listed in Supplemental Table 1. Mice of all genotypes were viable.

**In vivo auditory tests**

Auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) were measured using a previously described procedure (2).

**Auditory brain stem responses (ABRs)**

Briefly, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight) via intraperitoneal (i.p.) injection, and body temperature was maintained at 37°C using a heating pad in a sound-attenuating chamber. Three needle electrodes were inserted subcutaneously into the anesthetized mice: the active electrode was placed between the ears above the vertex of the skull, the ground electrode was placed between the eyes, and the reference electrode was placed underneath the left ear. Click and tone burst stimuli at frequencies of 4, 8, 16, 24, and 32 kHz were generated, and responses were recorded using a Tucker-Davis Technologies System (TDT, USA) workstation running SigGen32 software (TDT, USA). Auditory thresholds (dB SPL) were defined by reducing the sound intensity in 5-dB steps from
90 dB to 10 dB. The ABR threshold was defined as the lowest sound intensity sufficient to clearly elicit the first wave. We used 10-15 mice per group for ABR threshold assessment.

Distortion product otoacoustic emissions (DPOAEs)

DPOAE responses of 2f1-f2 were measured with two primary tone frequencies (f1 and f2, with f2/f1=1.2 and the f2 level 10 dB<f1 level) to predict auditory thresholds. DPOAE response thresholds were recorded across a range of frequencies (4 kHz, 8 kHz, 12 kHz, 16 kHz, 24 kHz and 32 kHz) within the acoustic microphone probe and the TDT system. F1 and f2 emissions stimulated the cochlea and passed through a multifunction processor (TDT) to a computer-controlled programmable attenuator, buffer amplifier, and earphone. Stimuli were generated digitally, and the maximum level of stimulation for DPOAE was 80 dB SPL. The 2f1-f2 DPOAE amplitude and surrounding noise floor were extracted. For each f2/f1 primary pair, levels were swept from 5 dB SPL to 80 dB SPL (for f2). Hearing thresholds were defined as the average signal for each identified frequency tested compared with the corresponding frequency in the controls; the threshold was defined as the f2 level required to produce a DPOAE at 5 dB SPL.

SGN counting

SGNs were counted in the apical, middle, and basal regions of the cochlear sections using a 40× objective. The corresponding area of the Rosenthal canal was measured in digital photomicrographs of each canal profile. The perimeter of the canal was traced with a cursor using ImageJ software. The computer then calculated the area within the outline. The number of neurons was calculated as the number of SGN per mm². Five discontinuous sections of the unilateral apical, middle, and basal turns were evaluated in one cochlea per mouse. SGN counting was performed for three mice per group.

Immunolocalization studies
Paraffin-embedded sections were deparaffinized and immersed in unmasking solution for antigenic retrieval and heated in an autoclave (121°C) for 5 min. Sections were then incubated with a blocking reagent for 30 min, followed by incubation with one of the following primary antibodies overnight at 4°C: Ctbp2 (BD Transduction LaboratoriesTM, 1:200, 612044), Prestin (Santa Cruz, 1:400, sc-22694), P-S6 (Ser235/236) (CST, 1:400, 4858T), Myo7A (Proteus Biosciences, 1:400, #25-6790), NF200 (Abcam, 1:10,000, ab4680), SOX2 (CST, 1:400, 3579), Spectrin beta-II (Millipore, 1:400, MABT1364), Parvalbumin (Sigma, 1:2000, MAB1572), Otoferin (Abcam, 1:500, ab53233), GluR2 (Abcam, 1:100, ab133477), P-Akt (S473) (CST, 1:400, 4060), Foxo1 (CST, 1:100, 2880). After three washes with 10 mM PBS, samples were incubated at room temperature (RT) for 1 hour in secondary antibodies diluted in 10 mM PBS. Then, the samples were stained with phalloidin (a specific marker of cellular F-actin) and DAPI (a marker used to stain nuclei). Images were acquired using a Leica LSM 700 laser scanning microscope or a Nikon TE2000 fluorescence microscope. For whole mount staining, cochleae were exposed to the sensory epithelium and dissected into basal, middle, and apical segments. Samples were blocked for 30 min with blocking reagent, followed by overnight incubation with primary antibodies at 4°C. After three washes with 10 mM PBS, samples were incubated at RT for 1 hour in secondary antibodies diluted in 10 mM PBS. Then, the samples were stained with phalloidin and DAPI. The numbers of missing OHCs and inner hair cells (IHCs) in the base and apex of the cochleae were counted. The percentage of missing-to-whole hair cells (HC) was analyzed. At least five samples for each genotype and condition were examined.

**Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies**

For SEM, inner ears were dissected and fixed (2.5% glutaraldehyde in PBS). A hole was poked in the apex of the cochlea. The fixative was flushed through the round window, and then the sample was fixed for 8 hours at 4°C. Cochleae were dissected to expose the organ of Corti and then post-fixed in 1% osmium tetroxide for 2 hours.
The samples were dehydrated through an ethanol series, critical-point dried using CO2 as the transitional fluid, and sputter-coated with gold. The samples were examined in a QUANTAFEG 250 scanning electron microscope at an accelerating voltage of 5 KV. Images were obtained from five controls and four mutant mice. For TEM, cochleae from mutant mice and wild-type mice were fixed with glutaraldehyde as described for SEM. Then, the sensory epithelium of the middle turn of the cochlear duct was dissected and post-fixed for 2 hours in 1% osmium tetroxide. The samples were dehydrated and embedded in Epon 812 resin after post-fixation. Ultra-thin (70 nm) sections were cut on the ultramicrotome. The sections were placed on formvar-coated 1×2-mm copper slot grids and post-stained with uranylacetate/lead citrate following standard protocols. The sections were examined on a JEOL-1200EX electron microscope.

**Extraction of proteins from formalin-fixed sensory epithelia and Western blot analysis**

The extraction of proteins from formalin-fixed sensory epithelia was performed as described previously (1). Briefly, cochleae were rapidly removed and perfused with formalin through the cochlear duct (scala media) and incubated at RT for 2 hours. After decalcification, the dissected sensory epithelia from eight mice were placed in a collection tube with 100 μL of extraction reagent (FFPE Total Protein Extraction Kit, Sangon Biotech). The tubes were sealed with a sealing clip and vortexed for seconds. After a brief centrifugation, the tissue was ground down with a grinding rod. The samples were incubated on ice for 20 min, followed by repeated vortexing. The tubes were then incubated for 20 min at 100°C in a water bath. After this incubation, the samples were incubated for 2 hours at 60°C in a metal bath. Finally, the samples were centrifuged at 12,000xg at 4°C for 20 min. The supernatant containing the extracted proteins was transferred to a new clear tube. The protein concentration was measured using a BCA kit. The samples were mixed with loading buffer and heated at 100°C for 5 min and stored at -20°C. Protein samples (10 μg) were assessed by Western blot and probed with anti-P-S6 (Ser235/236) (CST, 1:2,000,
4858T), anti-S6 (CST, 1:3,000, #2217S), ant-Akt (CST, 1:1,000, #9272S), anti-P-Akt (Ser473) (CST1:2,000, 4060P), anti-Raptor (24C12) (CST, 1:1,000, 2280T), P70S6K (Proteintech, 1:3,000, 14485-1-AP), anti-P-P70S6K (CST, 1:3000, #9204S), anti-P70S6K151 (Proteintech, 1:3,000, #13038), anti- Rictor (CST, 1:1000, #2140S), anti- mTOR (CST, 1:1000, #2972S), anti- P-Foxo1(CST, 1:1000, #9464T), anti- NDRG1 (CST, 1:1000, #5196S), anti-P-NDRG1(CST, 1:1000, #5482T), anti- Foxo1 (CST, 1:1000, #2880S), anti- GSK3β (CST, 1:1000, #12456S), anti- P-GSK3β (CST, 1:1000, #5558T), anti-P- mTOR (CST, 1:1000, #5536P), anti- Raptor(CST, 1:1000, #2280T), and an anti-rabbit HRP-conjugated secondary antibody (CST, 1: 10,000, 7074) or anti-mouse HRP-conjugated secondary antibody (CST, 1: 10,000, 7074). The same sample was loaded into a second well (two columns with the same label) in our experiments, and three or more samples (n ≥3, mice) were used for each group.

**Histological analysis**

Cochlea samples were fixed and decalcified using a procedure similar to that used for the immunostaining assay (1), dehydrated using a gradient ethanol series ranging from 30–100%, and embedded in paraffin. Sections were deparaffinized in xylene, rehydrated using an ethanol series, and stained using Hematoxylin & Eosin (H&E). The sections were then dehydrated, cover-slipped, and analyzed using a light microscope (Nikon YS100).

**Drug Preparation**

Sirolimus (Sigma-Aldrich) was dissolved in 100% methanol to yield a 20 mg/mL solution and stored at −20°C until further use. A working solution was prepared using 0.25% Tween-80 and 0.25% polyethylene glycol-400 diluted in phosphate-buffered saline (PBS). Postnatal treatment to 6-month-old C57BL/6J mice was commenced using an i.p. injection at doses ranging from 1–20 mg/kg every other day for 2 weeks. Cocaine hydrochloride (Sigma-Aldrich) was dissolved in saline, the drug was injected i.p. at a dose of 20 mg/kg.
Cisplatin was purchased from Sigma (479306). For *in vivo* experiments, cisplatin (16 mg/kg) was administered intraperitoneally (IP) over 30 min using an infusion pump in mice anesthetized with pentobarbital sodium. There was no evidence of middle-ear effusion or infection in these animals. Post-treatment ABRs were measured 72 h after cisplatin administration. The cochleae were dissected and used for total RNA determination; they were perfused with 2.5% glutaraldehyde for scanning electron microscopy (SEM) studies or with 4% paraformaldehyde for immunohistochemical analyses. For *in vitro* experiments, 100 mM cisplatin solution in pure water was prepared and diluted using the culture medium to a final concentration of 20 µM, which is commonly used in *in vitro* studies.

A-443654 was purchased from APExBio. For the *in vivo* experiments, A-443654 was dissolved in saline and then injected intraperitoneally at a dose of 2.5 mg/kg. For *in vitro* experiments, pifithrin-a (PFT-a; Tocris Bioscience) was dissolved in 100% DMSO and further diluted in the culture medium to a final concentration of 100 µM using 0.2% DMSO.

**Trans-tympanic administration of Torin1**

Mice (6 months of age) were anesthetized using an i.p. injection of avertin and their body temperature was maintained using a heating pad during the surgical procedure. The tympanic membrane was visualized using a surgical stereomicroscope. A retroauricular incision was made to provide an approach to the temporal bone. Using a 30G cannula, 8 µL of Torin1 in 0.5% DMSO in PBS, or alternatively, 0.5% DMSO in PBS alone was gently injected through the tympanic membrane. The surgical stereomicroscope was used to confirm that the solution was instilled in the cavity of the middle ear. Either ear of each mouse was injected with either the test compound or control solution in random order. After drug delivery, the puncture was covered by the surrounding muscles. No seal was necessary to prevent leakage. The skin incision was closed using tissue adhesive.

**Cochlear explant cultures**
The cochleae of neonatal mice at postnatal day 3 (P3) to P5 were extracted using a dissection medium containing ice-cold PBS and placed on coverslips pre-coated with poly-D-lysine (Sigma-Aldrich, St Louis, MO, USA). Cultures were maintained in DMEM/F-12 medium (Gibco, Carlsbad, CA, USA) supplemented with N2 and B27 (both from Invitrogen, Carlsbad, CA, USA) at 37 °C in an atmosphere of 5% CO₂. Fetal bovine serum (5%; Invitrogen) was added to the medium for only the first 24 h of culture to facilitate cell attachment.

**Isolation and collection of hair cells**

Twenty Atoh1-Cre/mTOR/Rosa26-tdTomato mice (P4), Atoh1-Cre/pten/Rosa26-tdTomato mice, or Atoh1-Cre/Rictor /Rosa26-tdTomato mice of either sex were used for this experiment. The basilar membrane was isolated and transferred to a prewarmed digestion system (0.125% trypsin/EDTA). After digestion (37°C, 5 min), soybean trypsin inhibitor was added to terminate the reaction, followed by mechanical trituration using blunt tips and pipetting up-and-down about 50–100 times. The suspended cells were percolated through a 40-μm cell strainer before FACS analysis. tdTomato-positive hair cells were sorted in a BD FACSAriaIII (BD Biosciences) using the tdTomato channel.

**Single-cell isolation and cDNA synthesis**

Apical and medial half-turns of the organ of Corti from 30-day-old WT and Rictor-cKO mice were dissected and fixed on a coverslip. Inner hair cells (IHCs) and outer hair cells (OHCs) were separately harvested using micropipettes (30 IHCs and 80 OHCs) using fast flow of a physiological solution (120 mM Na-glucanate, 35 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 0.9 mM MgCl₂, 0.7 mM Na₂HPO₄, 10 mM HEPES, and 5.6 mM glucose; pH 7.35; 320 mOsm/kg) to preserve hair cells. Cells were immediately frozen in liquid nitrogen and cDNA synthesis was performed using SuperScript III (Vazyme, China) following the manufacturer’s instructions.

**Quantitative real-time PCR**
Quantitative real-time PCR was performed using the SYBR Premix Ex Taq reagent system (TakaRa, Japan) according to the manufacturer’s instructions and a BioRad Sequence Detection System. The primer sequences are listed in Supplemental Table 2. Each reaction was performed in triplicate. The mRNA levels were determined relative to the housekeeping gene, β-actin, and analyzed using the $2^{-\Delta\Delta Ct}$ method.

**Statistical analysis**

Data are expressed as the mean ± SEM or SD from at least three independent experiments. Statistical analysis of the data was performed using a 2-tailed-distribution Student’s t-test or one-way ANOVA followed by Bonferroni’s multiple-comparison correction using GraphPad Prism 7.0 (GraphPad Software). For all tests, a P value less than 0.05 was considered statistically significant.
**Fig.S1. Inhibition of mTORC1 by sirolimus prevents mice from developing cocaine-induced hearing loss.** (A) Cocaine stimulates S6 phosphorylation in C57BL/6J mice (2-month-old) cochleae. Cocaine (20mg/kg) was injected intraperitoneally and cochleae were dissected 3 days after injection. S6 phosphorylation was detected using western blot. GAPDH served as the sample loading control. Relative intensities of phosphorylated S6 to total S6 are quantified on the right side (B); n=6. Western blotting shows increased p-S6 (235/236) levels in cocaine-treated mice cochleae compared to vehicle-treated WT controls. (C) ABR hearing thresholds are increased in cocaine-treated C57BL/6J mice compared to vehicle-treated WT mice. However, no significant differences can be observed between the control and cocaine plus sirolimus. (D, E) sirolimus blocks cocaine-induced mTORC1 activation. n=6. Data are presented as the mean ± SD. **P < 0.01, ***P < 0.001, using 2-tailed Student’s t-test. “n.s.” denotes no significant difference between two groups.

**Fig.S2.** (A) The mice injected with high concentration sirolimus showed weight loss, n=6. (B) No morphological difference of Heart, Kidney, Liver, and Spleen was found between different doses of sirolimus treated groups. Scale bar: 10µm
**Fig. S3.** mTORC2 was decreased after treatment with high doses of sirolimus in mice cochleae (A) Western blotting of whole cochleae showed a reduction of P-P70S6K and P-S6 (235/236) levels in both 2 and 10 mg/kg sirolimus-administered mice. P-P70S6K and P-S6 (235/236) levels are quantified on the right side (B, C). No significant differences are observed between the two groups after treatment with different concentrations of sirolimus. GAPDH was used as the sample loading control; n=6. (D) Raptor-cKO mice (6 months of age) treated with 10 mg/kg sirolimus
showed significant elevation of the ABR threshold whereas no significant differences were observed between the Raptor-cKO mice treated with 10 mg/kg siromycin and WT mice treated with 10 mg/kg siromycin. n=6. (E) Schematic view of the signaling mechanisms downstream of mTORC1 and mTORC2. (F) Immunoblotting analysis confirmed that mTORC2 activity was decreased, as demonstrated by the decreased expression of P-Akt (S473), and PKCa-S657. Phosphorylation of Akt at T308, Rictor, and pT1135-Rictor was not affected by mTORC2 inactivation. The two columns with the same label indicate that the same sample was loaded onto a second well; n=6 for each group. P-Akt (S473) (G), P-Akt (T308) (H), and PKCa-S657 (I) levels are quantified on the right side. (J) Effects of 2 and 10 mg/kg of sirolimus on the integrity of mTORC1 and mTORC2. mTOR was immunoprecipitated from cochleae and immunoblotted to determine Raptor and Rictor (subunits of mTORC1 and mTORC2, respectively). Data are presented as the mean ± SD. **P < 0.01, ***P < 0.001, using 2-tailed Student’s t-test.
Fig. S4. Loss of mTOR decreases mTORC1 and mTORC2 activity simultaneously. (A) Schematic overview of the procedure for the isolation of tdTomato-positive cells using FACS. (B) FACS collected hair cells at P4 were digested and the PCR analysis was performed, then successful verify of the deletion of mTOR in mTOR-cKO was tested using PCR. (C) Western blotting of tdTomato-positive cells revealed a considerable decrease in mTOR expression and inactivation of both mTORC1 (decreased expression of P-S6 and P-4EBP1) and mTORC2.
decreased expression of P-Akt (S473) in mTOR-cKO/tdTomato mice. The two columns with the same label indicate the same sample loaded onto a second well; n=6 for each group. mTOR, P-4EBP1, P-S6 (235/236), and P-Akt (S473) levels are quantified and shown in (D), (E), (F), and (G), respectively. (H) No morphological difference was found between WT and mTOR-cKO cochleae at P14. Images from confocal microscopy show that P-S6 (235/236) (I) and P-Akt (S473) (J) are considerably decreased in the hair cells of mTOR-cKO mice. Scale bar: 20 µm. Data are represented as the mean ± SD. **P < 0.01, ***P < 0.001, using 2-tailed Student’s t-test.

**Fig. S5.** mTOR-cKO mice show normal hearing development. Images from confocal microscopy show no significant differences in the distribution of phalloidin (A,B,M,N,O) and key proteins, including NF-20 for nerve fibers (C,D), Prestin for OHCs (E,F), Otoferin for IHCs (E,F), Ctbp2 and GluR2 for ribbon synapse (I,J), and Sox2 for supporting cells (K,L) between wild-type (WT)mice and mTOR-cKO mice at P14. FM1-43 uptake by auditory hair cells at P7 confirmed their normal function (G,H). n=3. Data are expressed as the mean ± SD. Scale bar: 20 µm.
**Fig. S6.** Deletion of Rictor decreases mTORC2 activity whereas mTORC1 is unchanged. (A) Western blots showing that Rictor protein is considerably decreased in the NSE of Rictor-cKO mice. Results from immunohistochemistry (B) and immunofluorescence staining (C) show that the expression of Rictor and P-Akt(S473) is decreased in the hair cells of Rictor-cKO mice compared to control mice. (D) Immunoblots showing decreased mTORC2 activity and unchanged mTORC1 activity.
as demonstrated by decreased P-Akt (S473) and P-mTOR (S2481). P-S6 (235/236), P-4EBP1 (T37/46), and P-Tsc2 were not affected by decreased mTORC2 expression in Rictor-cKO mice compared to that in WT mice; P-Tsc2 (E), P-4EBP1 (T37/46) (F), P-Akt (S473) (G), P-mTOR (S2481) (H), p-S6 (235/236) (J), and P-S6K1 (T389) (K) levels are quantified in the right subpanel. Data are presented as the mean ± SD. ***P < 0.001, using 2-tailed Student’s t-test. Scale bars: 20 μm.

Fig. S7. (A) ABR thresholds of Rictor-cKO and WT mice (both at 14 days after birth) to click stimuli were measured. n=10. No significant differences were observed between genotypes. (B) Normal pattern of the hair cells (Parvalbumin staining) and supporting cells (Sox2 staining) in Rictor-cKO mice compared to mice in P14. n=3. Scale bars: 20μm (C) Representative confocal image of β II-spectrin signals in the HCs in apical turns from WT and Rictor-cKO mice at P30. n=3. Scale bars: 20μm. (D) Images from confocal microscopy show that Rictor-cKO mice began to lose hair cells at three weeks of age (n=3) and loss of SGNs (E) was shown in 9 months of age.
**Fig. S8.** Hair cells of *Rictor-cKO* mice show characteristics of apoptosis. (A) Immunofluorescent staining for TUNEL among different groups at P30. The basilar membrane is co-stained with DAPI to identify the nucleus. Arrows indicate apoptotic hair cells. Scale bar: 20 μm (B) TEM analysis revealing the morphologies of cochlear hair cells in WT and *Rictor-cKO* mice at P30. The nucleus shows a large and round morphology in WT mice, whereas that of mutant mice shows chromatin condensation and deep staining. Scale bar: 5 μm for the upper row. Scale bar: 3 μm for the lower row. (C) Quantitative real-time PCR analysis of inner hair cells indicating a significant increase in the expression levels of the pro-apoptotic genes, Bax, Caspase 3, Caspase 8, and Caspase 9, and a significant decrease in the levels of the anti-apoptotic gene, Bcl2, in *Rictor-cKO* mice compared to the WT controls. n=6. (D) Z-VAD-FMK (1.25 mM) delayed the death process of hair cells derived from the *Rictor-cKO* mice to a certain extent, n=8.
Fig. S9. mTORC2 deficiency is the main cause of hair cell death and hearing loss in mTOR-cKO mice. (A) Age-related Click ABR threshold of mTOR-Rictor-DKO mice largely resembles that of Rictor-cKO mice. (B) The already small cerebellum in Rictor-cKO mice shows a further decrease in size in mTOR-Rictor-DKO mice compared to that in wild-type control mice at P30. Scale bar: 100 μm. (C) SEM images show that many hair cells disappear in the middle turn of the cochlea in Rictor-cKO mice; however, no obvious differences are seen in mTOR-Rictor-DKO mice compared to Rictor-cKO mice at P30. n=3. Scale bar: 10 μm. (D) ABR results indicate no significant differences between control Rictor-cKO mice and 10 mg/kg sirolimus-injected 4-week-old Rictor-cKO mice for three consecutive days.
**Fig. S10.** (A) Click ABR threshold was shown in WT mice, Tsc1-cKO mice, and Tsc1-Pten-DKO mice. n=8. (B) Western blot results show that P-NDRG1 and P-aPKC were decreased in Pten-cKO mice compared with the WT controls. n=3. P-NDRG1 and P-aPKC levels are quantified on the right side (C, D). (E) Go6976 injection blocks the expression level of a-PKC in mice. n=3. P-aPKC levels are quantified in (F). (G) ABR threshold results show no significant difference between Go6976 injected mice and control mice. n=10.

**Fig. S11.** (A) Cisplatin injection does not change the level of P-Akt(S474) and downstream P-GSK3β in the whole cochleae. P-Akt(S474) and P-GSK3β are quantified on the right side (B, C) n=6. (D) Cisplatin injection does not change the mTORC1 activity in mice. n=3. Scale bars: 20um. (E) No significant difference of the
hair cell pattern was shown in four mice lines (WT, Rictor-cKO, Rictor-Pten-DKO, Pten-cKO) at P14. Scale bars: 20um.

**Fig. S12. Activation of mTORC2 enhances hair cell survival during acoustic trauma (A-D)** Compared to that in WT control mice, mTORC2 activity is decreased in Rictor-Het mice but increased in A-443654 treated mice. (A) Western blots of P-Akt (S473) in the cochleae NSE of Rictor-Het and WT control mice. (B) Densitometric analysis of blots showing the ratios of P-Akt (S473) to Akt (n=3) in Rictor-Het and WT control mice. (C) Western blots of P-Akt (S473) in the cochlea of A 443654 (2.5mg/kg) and vehicle-treated mice. (D) Densitometric analysis of blots showing the ratios of P-Akt (S473) to Akt (n=3) in the cochlea of A 443654 and vehicle-treated mice. (E) ABR thresholds were comparable in Rictor-Het mice and A-443654 injected mice compared with wild-type controls at each time shown above. n=6. (F) Experimental design for noise exposure and ABR test: Mice at P30 were exposed to noise at 105 dB for 4 h. ABR thresholds were tested at the following time points: 2 h before, 2 h after, and 14 d after noise exposure. (G) No significant changes in the number of ribbon synapse among Rictor-Het, A-443654 injected, and control mice.
(H) ABR threshold shift of mice at 2 d before, 2h after noise exposure, and 2 weeks after noise exposure. n=6. (I) Amplitudes of ABR wave I at 2 d before, 2h after noise exposure, and 2 weeks after noise exposure. n=6. Data are expressed as the mean ± SD. *P<0.05, **P <0.01, ***P < 0.001, using 2-tailed Student’s t-test.

Fig. S13. Deleting P53 activity rescued the hearing phenotype in Rictor-cKO mice. (A) Representative western blots using antibodies against P-P53, P53, P21, and GAPDH in control and cisplatin-exposed whole cochlear extracts of P30. Relative P-P53, P53, and P21 levels are quantified and shown in (B). (C) Representative western blots of P-P53, P53, and GAPDH in control and Rictor-cKO mice NSE. Relative P-P53, P53 levels are quantified and shown in (D). (E) Confocal microscopy images showing the middle region of the organ of Corti cultures treated with PTF-a, Torin1, and PTF-a in combination with Torin1 for 4 days and immunolabeled for Myo7A (red). Scale bar: 20 μm. (F) ABR thresholds were recorded in WT, Rictor-cKO, and Rictor-cKO; P53−/− mice. n=8. (G) Cochleograms of 5-month-old WT, Rictor-cKO, and Rictor-cKO; P53−/− mice. Graph showing the percentage loss of OHCs as a function of percentage distance from the apex. n=8. Data are presented as the mean ± SD. *P<0.05, **P <0.01, ***P < 0.001, using 2-tailed Student’s t-test.
### Table 1. List of Primers

| Gene      | Forward primer (5′-3′)          | Reverse primer (5′-3′)            |
|-----------|--------------------------------|----------------------------------|
| *Pten*fl/fl| CAAGCACTCTGCGAAGTGC            | AAGTTTTTGAAGGCAAGATG             |
| *Rictor*fl/fl| CAAGCATCATGAGCTCTTC       | TCCCAGAAATTCCAGGCTTA            |
| *mTOR*   | CAGTTGTCATGGGAAATGGG       | TCTCCCTCAGTCTGCTGAT             |
| *Raptor*fl/fl| CTCAGTATGTTATGTGCT   | GGGTACAGTATGTCAGCACA             |
| *p53*    | TGGATGGGATATACAAGGA         | CAGCTCTGTCTCCACATACA             |
|          | AGGCTTAGAGGGTGAAGCTG        |                                  |
| *Atoh1-Cre* | GGCAGCCGCCTTCAGCAAC  | GCCCAATGTGCTGATAG                |
| *Tsc1*fl/fl| GTCACGACCGAGAGAGAAGGAC    | GAATCAACCCCACAGGAGCAT            |
| *Rosa26-tdTomato*| AAGGGAGCTGACTGGAGGTGA  | CCGAAAATCTGTGGGAAGTC             |
|          | GGCATTTAAAGCAGCGGTATCC     | CTGTTCCTGTACGGCATGG             |

### Table 2. List of Primers

| Gene       | Forward primer (5′-3′)          | Reverse primer (5′-3′)            |
|------------|--------------------------------|----------------------------------|
| *Bax*      | TGAAGACAGGGGCGCTTTTTTG         | AATTCGCGGAGACACTCG              |
| *Caspase3* | AATCATGGAGCTTCGAGGAGGAGG      | CTCAGTATGCTGCAGGAGGAGAGG       |
| *Caspase8* | AGCCTATGCCACCTAGTGAT           | GGAGAGCTGTAACCTGTCGC            |
| *Caspase9* | CCAAGCATCGTGCTCAAGG            | ACCGCTTGGCAAAGAGGTAAG            |
| *Bcl2*     | GGTGAAGCTGCGGGGGAATG          | AGAGGCTGATTGCTGCC               |
| *GAPDH*    | GCAAGAGAGAGGCGCTCAG           | TGTGAGGGAGATGTGCTGAGT            |

### Reference

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2. X. Fu, L. Zhang, Y. Jin, X. Sun, J. Gao, Loss of Myh14 Increases Susceptibility to Noise-Induced Hearing Loss in CBA/CaJ Mice. *Neural Plasticity* **2016**, 1-16 (2016).