Supplement

I-Legends of supplemental Figures1-7

Figure S1

mTORC1 signaling was activated in the cochlear neurosensory epithelium in aged DBA and BALB/C mice.

A, B Western blot analyses of sensory epithelium shows increased P-S6 (235/236) levels without alterations in P-Akt (S473) levels in 12-month-old WT mice compared with 2-month-old WT mice. Protein lysates were obtained from sensory epithelial tissues from cochleae. β-actin served as the sample loading control; n=5. Data represent the mean ± SEM. *P<0.05 by 2-tailed student's t-test.

Figure S2

A，**B** ABR thresholds of the rapamycin-treated groups and control DBA mice (A) and BALB/C mice (B) to click stimuli at different ages. Rapamycin was injected from 6 months of age to 10 months of age every other day. The ABR thresholds in the rapamycin-treated mice at 8 and 10 months of age were significantly decreased compared with the age-matched wild-type mice, and the rapamycin-treated mice exhibited a similar ABR pattern to the wild-type mice at 6 months of age. Data represent the mean ± SEM. *P<0.05, **P<0.01, by 2-tailed student's t-test. n=5.

Figure S3

Degeneration of SGNs in *Tsc1-cKO* cochleae.

Transverse sections of 4-month-old C57BL/6J WT and *Tsc1-cKO* mice were stained with H&E. Severe degeneration of hair cells in the OC and SGNs was observed in *Tsc1-cKO* mice but not WT mice. SGN, spiral ganglion neuron; OC, organ of Corti; RM, Reissner's membrane. The red box indicates the organ of Corti. Scale bar=100 μm.

Figure S4

A Western blot analysis of cochlear sensory epithelia shows highly activated mTORC1 signaling in *Tsc1-cKO* mice and limited mTORC1 activation in *Pten-cKO* mice. **B** Increased levels of P-Akt were observed in *Pten-cKO* mice.

Figure S5

Representative images of HEI-OC1 cells transfected with GFP-Tsc1^{-PTS1} and stained with DAPI. Scale bar=10 μm.

Figure S6

Examination of the localization of Tsc1, Tsc2, Rheb in Subcellular fractionation of auditory cells.

Subcellular fractionation of auditory cells demonstrating the mTORC1 signalling node at the peroxisome. PMP70 was used as subcellular markers for the peroxisomes (p). LAMP1 and VDAC were used as markers for lysosomes (L) and mitochondria (M), respectively, N, nuclear.

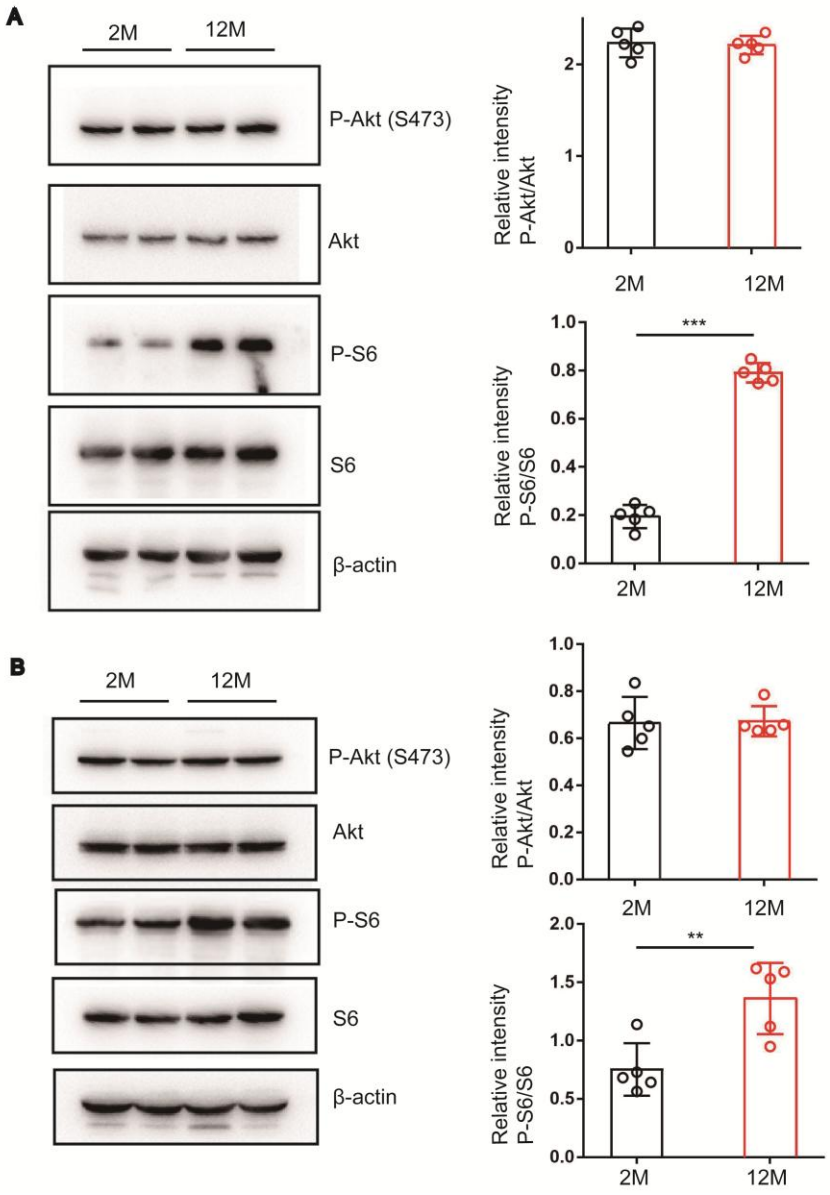
Figure S7

The mTORC1 signaling functions in the peroxisome in response to ROS.

Western analysis of organ of Corti treated with 50 µM Wy-14643 or vehicle (DMSO) for mTORC1 signaling proteins ((pS6 (S235/236), S6, P-P70S6K and P70S6K)). The mTORC1 activation (indicated by the level of P-P70S6K and P-S6) was decreased in Wy-14643-treated organ of Corti.

II- Supplemental Figures 1-5

Supplemental Figure 1



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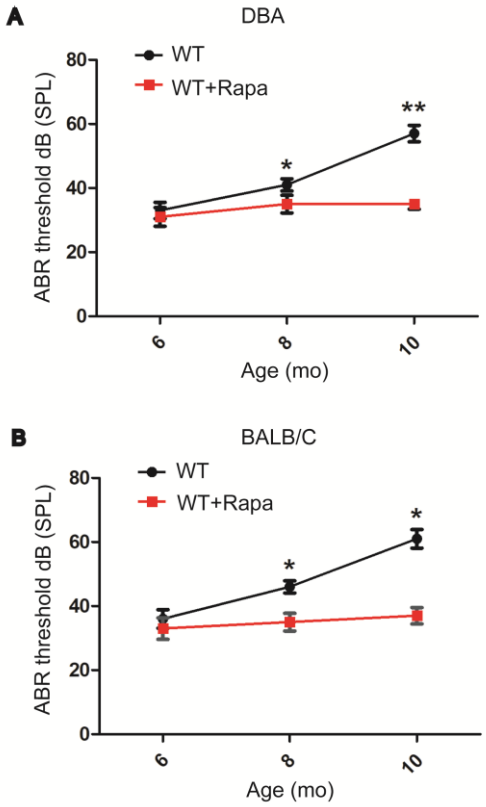

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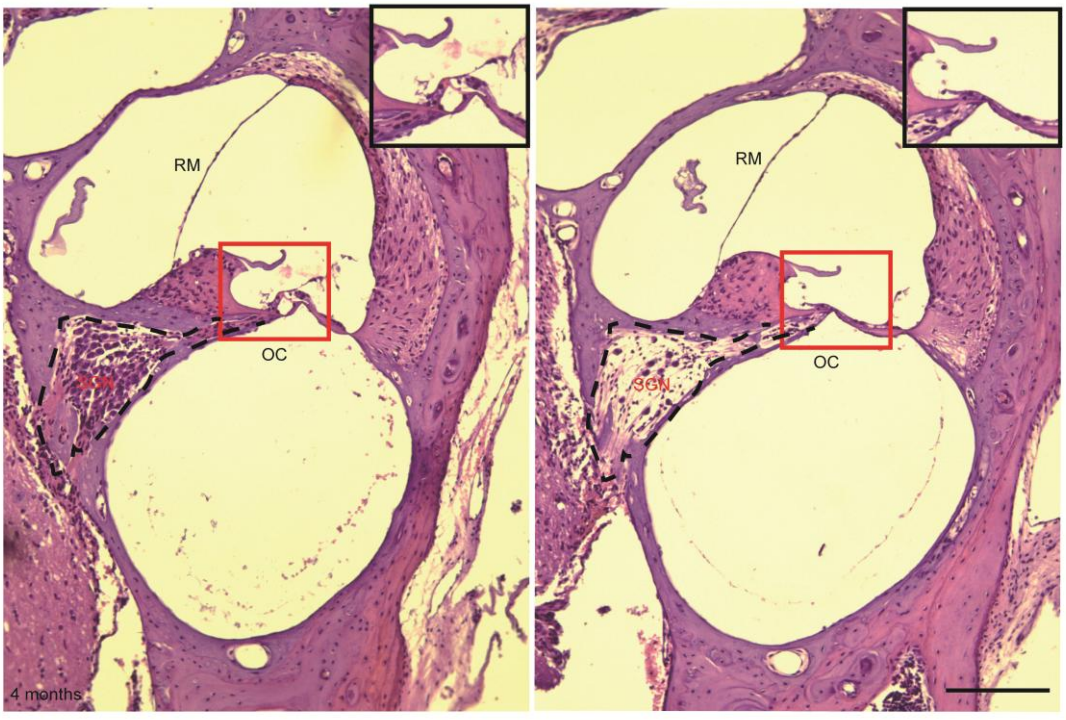
103 **Supplemental Figure 3**



WT *Tsc1-cKO*

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105 **Supplemental Figure 4**

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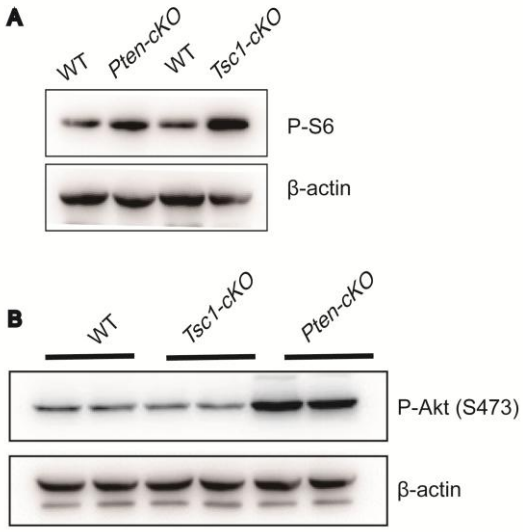

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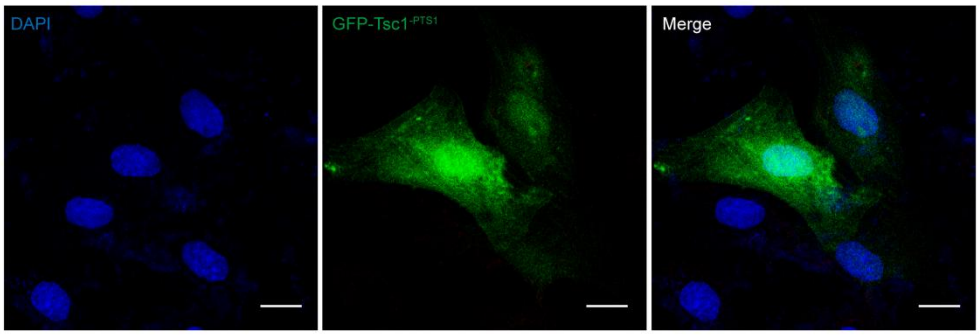

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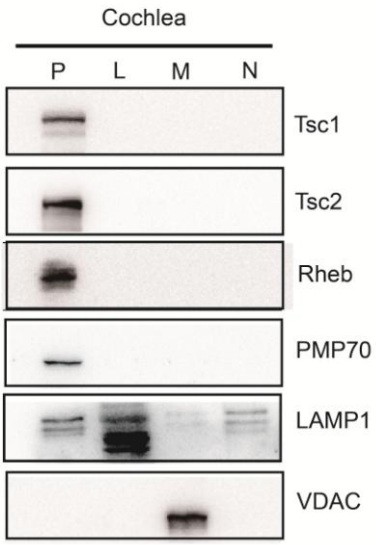
110 **Supplemental Figure 6**



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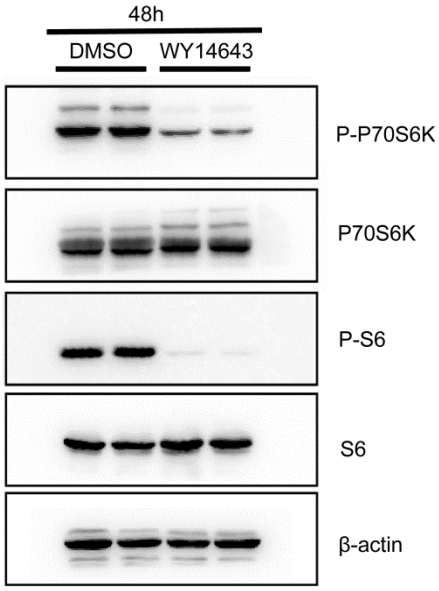
112 **Supplemental Figure 7**



III- Supplemental Tables 1&2

Table S1. Primers used for genotyping

Gene name	Forward primer	Reverse primer
*Tsc1*1 (mutant)	5'-AGGAGGCCTCTTCTGCTACC-3'	5'- CAGCTCCGACCATGAAGTG-3'
Pten(mutant)	5'-CAAGCACTCTGCGAACTGAG-3'	5'-AAGTTTTTGAAGGCAAGATGC-3'
Raptor (mutant)	5'-GAGGCAGTCTTTTGATCAGTTG-3'	5'-ATGAGCTTTGCTTAAGATATCG-3'
		5'-GCAGCGCATCGCCTT CTATC-3'
tdTomato (wildtype)	5'-AAGGGAGCTGCAGTGGAGTA-3'	5'-CCGAAAATCTGTGGGAAGTC-3'
tdTomato (mutant)	5'-GGCATTAAAGCAGCGTATCC-3'	5'-CTGTTCCTGTACGGCATGG-3'
Atoh1-Cre (mutant)	5'-TACTGACGGTGGGAGAATG-3'	5'-CTGTTTCACTATCCAGGTTACG-3'
LC3-GFP (wildtype)	5'-TCCTGCTGGAGTTCGTGACCG-3'	5'-AGCCGTCTTCATCTCTCTCTCGC-3'
LC3-GFP(mutant)	5'-TGAGCGAGCTCATCAAGATAATCAGGT-3'	5'-GTTAGCATTGAGCTGCAAGCGCCGTCT-3'

Table S2. The primers sets of Real-time PCR used were as follows:

Gene name	Forward primer	Reverse primer
GAPDH	5'-TGCGACTTCAACAGCAACTC-3'	5'-CTTGCTCAGTGTCCTTGCTG-3'
Gsr	5'-TATGTGAGCCGCCTGAACA-3'	5'-GTGGCAATCAGGATGTGTGG-3'
Nqo1	5'-ACTTCAACCCCATCATTTCCAG-3'	5'-TATCACCAGGTCTGCAGCTT-3'
Cat	5'-AGCGGATTCCTGAGAGAGTG-3'	5'-GACTGTGGAGAATCGAACGG-3'
Lpo	5'-CTGGACCAGAAGAGATCCATG-3'	5'-TCACCAGGTGGGAACATGATGG-3'
Alox15	5'-GACTTGGCTGAGCGAGGACT-3'	5'-CTTGACACCAGCTCTGCA-3'
Gpx2	5'-GAACAACTACCCGGGACTAC-3'	5'-GTCGGACATACTTGAGGCTG-3'

IV- Supplemental Methods

In vivo auditory tests

Auditory brain stem responses (ABRs)

ABR measurement was performed as described previously(67). 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 ℃ 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 ℃) 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 ℃: Ctbp2 (BD Transduction Laboratories™, 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), PMP70 (SIGMA-ALDRICH, 1:200, SAB4200181), NF200 (Abcam, 1:10,000, ab4680), SOX2 (CST, 1:400, 3579), 4-HNE (Abcam, 1:100, ab46545), 3-NT (Abcam, 1:100, ab110282), and Tsc1 (Abcam, 1:200, ab32936). 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 ℃. 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 microcopy (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 ℃. 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 CO_2 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 *Tsc1-cKO* mice.

For TEM, cochleae from *Tsc1-cKO* and wild-type mice (P40, P60) 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 uranyl acetate/lead citrate following standard protocols. The sections were examined on a JEOL-1200EX electron microscope.

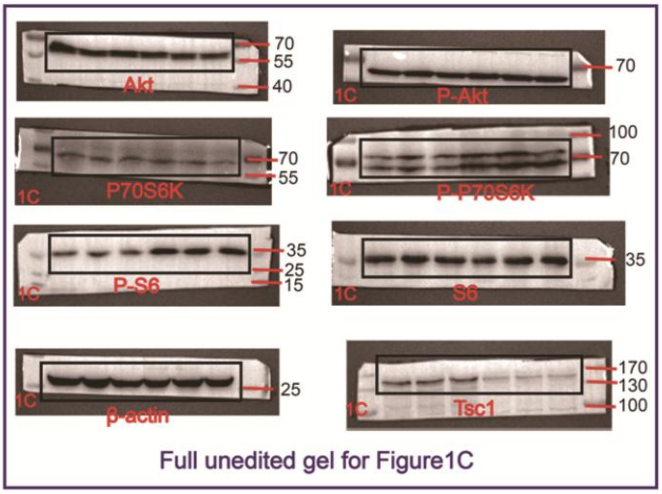
Extraction of proteins from formalin-fixed sensory epithelia and Western blot analysis

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 ℃ in a water bath. After this incubation, the samples were incubated for 2 hours at 60 ℃ in a metal bath. Finally, the samples were centrifuged at 12,000xg at 4 ℃ 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 ℃ for 5 min and stored at -20 ℃. 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) (CST, 1:2,000, 4060P), anti-Tsc1 (CST, 1:1,000, 4906S), anti-Tsc2 (CST, 1:2,500, 4308P), anti-Raptor (24C12) (CST, 1:1,000, 2280T), anti-P62 (Abcam, 1:1,000, ab56416), anti-LC3B (Proteintech, 1:3,000, 18725-1-AP), P70S6K (Proteintech, 1:3,000, 14485-1-AP), LAMP1 (Sigma, 1:1000, L1418), VDAC (Proteintech, 1:1000, 10866-1-AP) and anti-β-actin (Proteintech, 1:8000, 20536-1-AP) 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.

Statistical analysis

Data are expressed as the mean ± SEM from at least 3 independent experiments. Statistical analysis of the data was performed using a two-tailed distribution Student's t-test or one-way ANOVA followed by Bonferroni's multiple comparison correction using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA). For all tests, a value of $P<0.05$ was considered statistically significant.



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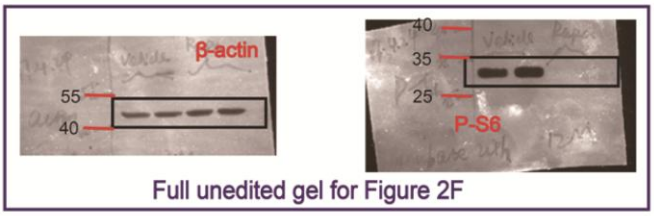

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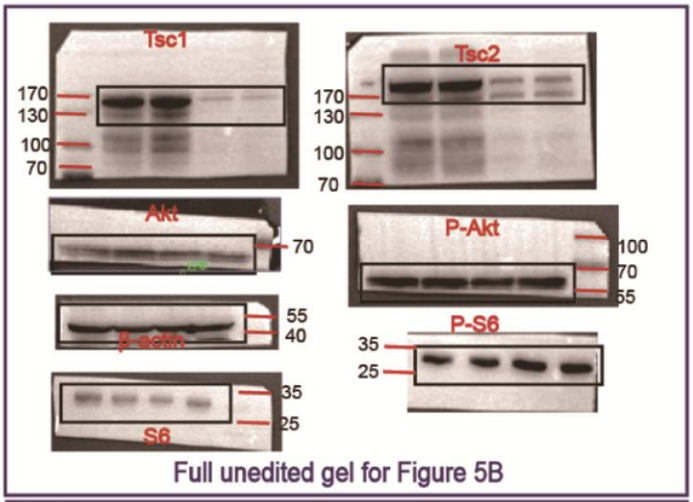

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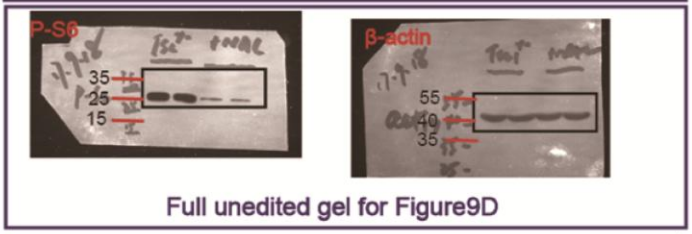

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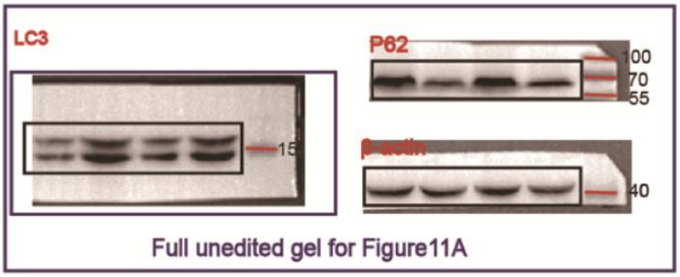

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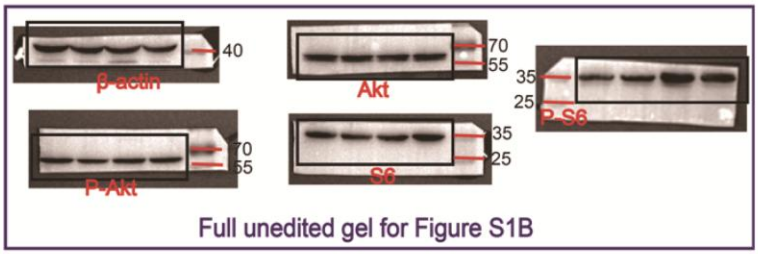

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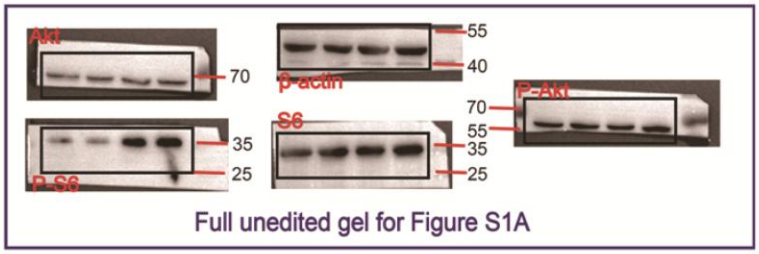

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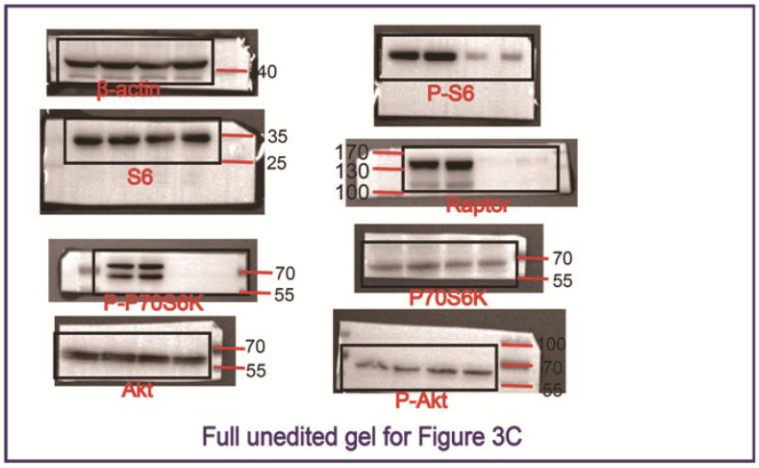

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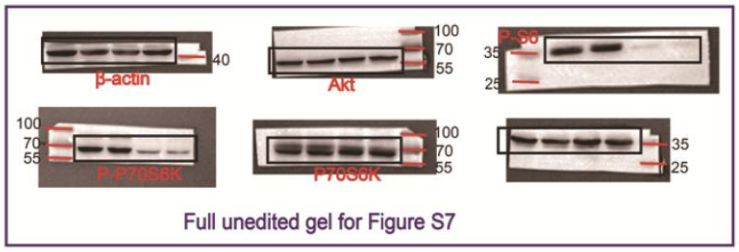

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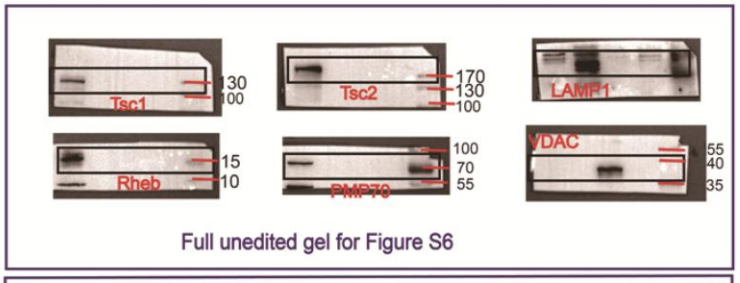

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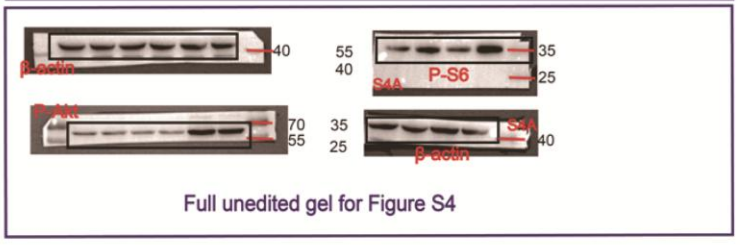

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