The molecular mechanisms of hearing loss in *tubby* mice

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Directed by Professor Chul Hoon Kim

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학위 과정에 진학하기 위해 이 곳 의과대학 약리학교실의 문을 두드린 지 어느덧 약 8년 이라는 시간이 흘렀습니다. 오랜 시간이 지났지만 돌이켜 보면 처음 이 곳에 와서 제가 사용할 실험 테이블을 닦고 정리하던 기억이 생생하게 떠오릅니다. 다니던 회사를 정리하고 이곳에서 다시 공부를 하겠다고 마음을 다지고 시작했지만, 일이 잘 안 풀릴 때마다 마음가짐이 흔들릴 때도 있었습니다. 하지만 많은 분들의 도움으로 여기까지 올 수 있었습니다. 여러 우여곡절이 있었지만 부족한 저를 물심양면으로 지원하고 지도해 주신 김철훈 교수님께 진심으로 감사의 말씀을 드립니다. 그리고 개인적인 고민과 상담을 들어 주시고 힘이 되는 조언과 함께 아낌없는 지원을 베풀어 주신 김동구 교수님께도 진심으로 감사의 말씀을 드립니다. 연구 방향에 대한 좋은 의견과 격려의 말씀을 아낌없이 해 주신 약리학교실 교수님들과 선생님들께도 감사의 말씀을 전합니다. 또한 현재 연구실에는 안계시지만, 연구실을 거쳐 가신 여러 선생님들께도 감사하다는 말씀 전하고 싶습니다.

본 프로젝트를 진행해 오면서 수많은 난관에 부딪혔지만, 많은 분들의 도움으로 좋은 성과를 이룰 수 있었습니다. 프로젝트를 위해 아낌없이 지원해 주신 복진웅 교수님, 문석준 교수님, 최재영 교수님, 신정오 박사님, 마지현 박사님께 정말 감사하다는 말씀 드리고 싶습니다.

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ABSTRACT

The molecular mechanisms of hearing loss in *tubby* mice

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Tubby mice are mutant mice which arose spontaneously that exhibit three major phenotypes: late onset obesity, retinal degeneration, and progressive hearing loss.[1-4] The point mutation in the splice site of the *tub* gene completely displaces the 44 amino acids of the C-terminus of the Tub protein with 24 completely different amino acids encoded by the intron, results in a complete loss of function of the Tub

protein.[5,6] It has long been suggested that Tub protein acts as a transcriptional regulator due to its structural characteristics such as nuclear localization sequence, DNA binding domain, and *in vitro* transcriptional activity.[4,7,8] However, it is not well known whether the Tub protein actually regulates the expression of specific genes *in vivo*, and what is the molecular mechanisms of *tubby* mice phenotype. In this study, I investigated the molecular mechanism of hearing loss phenotype in *tubby* mice. Hearing measurement and electron microscopy analysis data of tubby mice showed that the hearing loss of *tubby* mice originated from the cochlear outer hair cells (OHCs) dysfunction. Immunostaining results demonstrated that Tub protein is specifically located to the hair bundle of cochlear OHCs. Furthermore, Tub protein was found to be essential for the formation of stereociliary links, especially horizontal top connectors, in cochlear OHCs. Stereocilin is a protein also known to be essential for the formation of horizontal top connectors.[9] Stereocilin is expressed specifically in cochlear OHCs and located on the OHC hair bundles.[9] Notably, I found that stereocilin was disappeared from the mature OHC hair bundles of *tubby* mice. This suggests that the Tub protein on the OHC hair bundles is essential for the formation of a horizontal top connectors by stereocilin. These results suggest that, at least in the hearing loss phenotype of *tubby* mice, Tub protein plays a direct role in the formation of stereociliary links on the cochlear OHCs, rather than a transcriptional regulator. The results of this study also suggest that the hearing loss of *tubby* mice is due to dysfunction of cochlear sensory hair cells rather than cochlear synaptic function suggested in previous study.[10]

Key words: *tubby* mice, Tub protein, hearing loss, cochlea, outer hair cells, horizontal top connector, stereocilin

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I. INTRODUCTION

Tubby mice which have lost Tub protein's function due to spontaneous mutation on the *tub* gene show late onset obesity, retinal degeneration, and progressive hearing loss.[1-4] These phenotypes of *tubby* mice has overlapping spectrum with several phenotypes in ciliopathy such as Alstrom's syndrome or Bardet-Biedl syndrome.[11,12] And sensory neuronal degeneration of retina and cochlea are similar to the phenotypes of Usher syndrome.[13] However, the molecular mechanism for the phenotypes of *tubby* mice is largely unknown. Previous studies have reported that

there is defect in hearing ability in *tubby* mice before cochlear sensory hair cell degeneration begins.[2,3,13] This suggests that cochlear function of *tubby* mice is already abnormal before the histological phenotypes, such as sensory hair cells loss, are observed. In this study, I investigated the role of Tub protein in cochlear, and the mechanisms of hearing loss in *tubby* mice.

1. Tubby and tubby-like proteins

The *tub* gene was first identified in *tubby* mice.[5,14] The Tub protein encoded by *tub* gene has a highly conserved tubby domain on the carboxyl terminus.[4] The nuclear localization sequence and phosphoinositide binding region is also located on Tub protein.[7] This allows the Tub protein can localize to the plasma membrane and the nucleus.[4,7] Translocation of Tub protein from the plasma membrane to the nucleus is known to be triggered by G-protein coupled receptor (GPCR) signaling.[8] It is also known that the amino terminus of Tub proteins have transcriptional acitivity.[7] Although numerous studies suggest Tub proteins function as transcriptional regulators, the genes regulated by Tub proteins are currently unknown.

After the first *tub* gene was identified, additional tubby-like protein families were identified by sequence homology. Tubby-like proteins (Tulps) commonly have a highly conserved tubby domain at the carboxyl terminus. Each tubby-like protein

has a distinct tissue expression pattern and associated phenotypes. Tulp1 is mainly expressed in the retina, and knockout mice show retinal degeneration phenotype.[15] The mutation on the tulp1 gene in human cause retinitis pigmentosa type14.[16,17] Retinal degeneration patterns and electroretinograms found in tulp1 knockout mice are similar with that of *tubby* mice.[15] Tulp1 is known to play an important role in the rhodopsin transport, and double homozygote knockout mice of tubby and tulp1 shows more rapid degeneration retinal patterns than with each single knockout mouse.[18] Tulp2 is mainly expressed in testes, with a very small amount is expressed in retina.[19] The function of tulp2 is currently unknown and the phenotype of tulp2 knockout mice is also not reported yet. Tulp3 plays an important role in the formation of the neural system during the embryo development process. Tulp3 knockout mice die at embryonic day 14.5 due to the failure of neural tube closing and subsequent hemorrhaging.[20] Tulp3 is closely related with the sonic hedgehog signaling pathway, and known to be required for trafficking of ciliary GPCRs.[21-23] Tulp4 is the most distant member of the tubby-like protein family and has a less conserved tubby domain.[24] In humans, tulp4 has a broad tissue expression pattern, but in mice, tulp4 is mainly expressed in the brain and testes.[25] Cellular localization of tulp4 is cytoplasmic, but the cellular function of tulp4 and the phenotypes of tulp4 knockout mice are currently unknown.[25] However, in humans, there are some reports that genetic variations on the tulp4 gene have correlation with short statue and cleft palate.[26,27]

2. Phenotypes of *tubby* mice

Tubby mice have a single point mutation on the donor splice site of the *tub* gene. This mutation causes the substitution of the last 44 amino acids of the Tub protein into the 24 amino acids encoded by intron.[5,14] Tub protein has a highly conserved tubby domain on its carboxyl terminus and a specific tertiary structure consisting of 12-beta strands and a central alpha helix.[7] The mutation on the *tub* gene occurring in *tubby* mice cause structural disruption of a highly conserved tubby domain. Mutant Tub proteins are not detected in *tubby* mice because they are structurally unstable.[6] *Tubby* mice show three major phenotypes: late onset obesity, retinal degeneration and hearing loss.[1-6,13,14] The tubby null mice, in which *tub* gene was deleted, show almost the same phenotypes with original *tubby* mice.[6] Thus phenotypes of *tubby* mice are caused by loss of function in the Tub protein. However, the exact role of Tub protein *in vivo* is largely unknown.

About 8 to 12 wk after birth, *tubby* mice begin to increase in body weight and eventually become twice the weight of the wild-type mice.[1,6,28] With increasing weight, *tubby* mice show insulin resistance but are not diabetic.[1] There are various suggestions on why *tubby* mice show weight gain, such as increase of food intake, decrease in activity, and metabolic changes.[28-30] Tubby is mainly expressed in brain, especially the hypothalamus which orchestrates food intake and energy metabolism. It is reported that the expression of genes which associated with food intake is

changed in *tubby* mice.[31]

The reduction of photoreceptor cells is observable after 3 wk of age in the retina of *tubby* mice, and this degenerative process gradually proceeds until the photoreceptor cells do not exist.[3,13] An electroretinogram of *tubby* mice show abnormal wave forms, and it is completely lost after 6 mo of age.[13] These results demonstrate that the vision of *tubby* mice is significantly reduced. The *tub* gene is mainly expressed in the ganglion cell layer and inner segment of photoreceptor cells of the retina. Previously reported studies suggest that loss of photoreceptor cells in the retina of *tubby* mice is caused by increased apoptotic cell death at the outer nuclear layer of retina.[32]

Tubby mice show hearing loss after 3 wk of age.[13] A histological degenerative phenotype of cochlea in *tubby* mice first appears at 6 mo after birth.[2,3] After 6 mo of age, OHCs are mainly reduced at the cochlear basal turn of *tubby* mice, and inner hair cells (IHCs), spiral ganglion cells, and supporting cells are also reduced.[3,13] Notably unlike other phenotypes of the *tubby* mice, hearing loss phenotype of *tubby* mice is dependent on the background strain.[10,33] Hearing loss only appears in the C57BL6/J strain with the homozygote *tub* mutation. Other strains like AKR or CAST strains do not show hearing loss phenotype even though they have the homozygote *tub* mutation.[10,33] The difference in hearing loss phenotypes according to the mouse strain appear due to the polymorphism of microtubule-associated protein 1A (Map1A).[10,33] Only the C57BL6/J strain has different Map1A amino acid sequences, and these differences cause the weakening of interaction between Map1a

and synaptic protein PSD95.[10] Previous studies suggest that Tub protein has an important function at cochlear neurons based on these results.[10] However it is currently unknown that Tub proteins form a protein complex with Map1A and PSD95, and it is also unclear whether the hearing loss phenotype of *tubby* mice is due to neuronal defects.

3. Aims of study

In this study, I focused on the hearing loss phenotype of *tubby* mice. The purpose of this study is identifying the precise function of Tub protein in the cochlea, and demonstrating the mechanism of hearing loss in *tubby* mice. Previous studies reported that progressive degeneration of cochlear hair cells is the main cause of the hearing loss in *tubby* mice, and the cochlear hair cell loss is caused by apoptosis.[34] Furthermore, previous studies suggest that the hearing loss of *tubby* mice originates from neuronal problems based on the results that hearing ability of *tubby* mice is dependent on Map1a polymorphism. However, the actual function of Tub proteins on the cochlea and the molecular mechanisms of hearing loss in *tubby* mice are largely unknown. To investigate the mechanisms of hearing loss in *tubby* mice and the role of Tub protein in cochlea, I performed hearing measurements, hair cell morphology analysis using electron microscopy, and whole mount cochlear immunostaining.

II. MATERIALS AND METHODS

1. Animals

B6(Cg)-Tub^{tub}/J mice were purchased from Jackson laboratory. All animal care and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University College of Medicine. Mice were maintained in a temperature and humidity controlled environment and 12 hr light/12 hr dark lighting cycle. Genotypes of tubby mutation were identified by the method which based on Sanger sequencing. First, polymerase chain reaction (PCR) was performed using a pair of primers that amplify the region involving the tubby mutation site. Then, PCR products were purified using Megaquick-spin™ DNA purification kit (Intron, Korea). After purification, sequencing was performed using the primer that binds to PCR products. Wild-type and *tubby* mice at 3 wk were used for analysis of hearing ability, and mice at P5, P9, P14 and P21 were used for whole mount cochlea immunostaining.

2. Hearing ability measurements

To assess hearing abilities of wild-type and *tubby* mice, auditory brainstem

responses (ABRs) and distortion-product otoacoustic emissions (DPOAEs) were recorded at 3 wk of age. Auditory measurement was performed in a soundproof chamber. Before auditory testing, mice were anesthetized by an intraperitoneal injection of a mixture of Rumpun (0.4 mL/kg) and Zoletil (0.6 mL/kg) and boosted with one-fifth of the original dose as required. During auditory test, mouse body temperature was maintained with a heating pad.

A. ABR measurement

The evoked ABR thresholds were differentially recorded from the scalp. Responses were recorded using subdermal needle electrodes at the vertex, below the pinna of the left ear (reference), and below the contralateral ear (ground). Sound stimuli included click (100 μs duration; 31 Hz) and tone pips at 6, 12, 18, 24, and 30 kHz (1562 μs duration; $\cos^2$ shaping; 21 Hz). ABR measurements were taken using an Intelligent Hearing System (IHS) Smart EP System, running HIS High-frequency Software (ver. 5.10) and using IHS high-frequency transducers (HFT9911-20-0035, IHS, Miami, FL, USA). Acoustic stimuli were presented directly to the entrance of the ear canal. Stainless steel needle electrodes were placed subcutaneously at the vertex and over the bullae, with a reference electrode at the occiput. ABR thresholds were obtained for 5 ms duration clicks, and 5 ms duration tone bursts were presented at a rate of 50 s^{-1}. Tone bursts were gated using an exact Blackman envelope (2.5 ms rise/decay, 0 ms plateau) and presented in a decreasing intensity

series, beginning with levels that elicited distinct evoked potentials. Evoked potentials were amplified (x200,000), bandpass-filtered (100-3000 Hz), and averaged over 1024 sweeps. Recording epochs comprised the 12 ms following stimulus onset. Thresholds were determined by two investigators who were blind at the age and strain of the mice for a broad-band click and for 6, 12, 18, 24, and 30 kHz pure-tone stimuli by decreasing the sound pressure level (SPL) in 10 dB decrements until reaching the lowest level at which a distinct ABR wave pattern could be recognized by two of the investigators.

B. DPOAE measurement

DPOAEs were recorded using the SmartOAE system (v. 4.26; IHS, Miami, FL, USA). DPOAE measurements were conducted for pure tones of 6-32 kHz. SmartOAE software (ver. 5.10) was used for measurements. An Etymotic 10B+ probe was inserted into the external ear canal and used in conjunction with two different types of transducer, depending on the range of the stimulation frequency. An Etymotic ER2 stimulator was used for frequencies ranging from 6 to 16 kHz. For frequencies ranging from 16 to 32 kHz, an IHS high-frequency transducer was used. Stimulus response signals were sampled at a rate of 128 kHz using a 16-bit D/A converter. L1 amplitude was set to 65 dB SPL and L2 amplitude was set to 55 dB SPL. Frequencies were acquired with a ratio of frequency 2 (F2) to frequency 1 (F1) of 1.22. Five stimulation levels, ranging from 65 to 25 dB SPL in 10 dB steps, were used. In total, four blocks were acquired, each block consisting of 32 sweeps.

3. Scanning electron microscopy

The morphology of hair bundles of OHCs was investigated by scanning electron microscopy (SEM). For fixation, the basilar papilla is dissected in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 hr at room temperature. The tissue is further incubated overnight in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer with 3.5% sucrose solution and 2 mM $CaCl_2$. Post-fixation is performed using the OsO_4/thiocarbohydrazide (OTOTO) protocol. After post-fixation, the samples are dehydrated through gradient concentrations of ethanol solutions (20%, 40%, 60%, 70%, 80%, 90%, 95%, and 100%), and the dehydrated specimens in isoamyl acetate are dried following the method of critical point drying (HCP-2, Hitachi, Japan). The samples are mounted on a stub holder and coated with platinum in 5 nm thickness for SEM imaging. The coated samples were observed using schottky type field emission gun SEM (JSM-7001F, JEOL, Japan) operated at 15kV.

4. Transmission electron microscopy

For transmission electron microscopy (TEM), the inner ear of wild-type and *tubby* mice (P21) were isolated from euthanized mice and fixed overnight with 2.5%

glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for overnight. The specimens were washed three times for 30 min in 0.1 M phosphate buffer. The specimens were post-fixed with 1% OsO_4 dissolved in 0.1 M PB for 2 hr and dehydrated using a graded ethanol and infiltrated with propylene oxide. Specimens were embedded by Poly/Bed 812 kit (Polysciences, Warrington, PA, USA). After pure fresh resin embedding and polymerization at 60°C electron microscope oven (TD-700, DOSAKA, Japan) for 24 hr 350 nm thick section were initially cut and stained with toluidine blue for light microscope. 80 nm thin sections were double stained with 7% (20 min) uranyl acetate and lead citrate for contrast staining. These sections were cut by LEICA EM UC-7 Ultra-microtome (Leica Microsystems, Austria). All of the thin sections were observed by TEM (JEM-1011, 80Kv, JEOL, Japan) at the acceleration voltage of 80 kV.

5. FM1-43 dye uptake assay

To demonstrate whether hair cells in the cochlea are viable and maintains their transductive functionality of ion channels during the experiments, the viability of the samples were analyzed using FM1-43 (N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide, ThermoFisher Scientific, Waltham, MA, USA), as described in a previous study. Briefly, the tissue prepared from the cochlea was incubated in 5 μM FM1-43 for 10 sec and washed three times in HBSS.

Then, the tissue was mounted in ProLong® Gold antifade reagent (ThermoFisher Scientific, Waltham, MA, USA) and is covered by a coverslip (EMS, Thickness #0). The cochlea was observed using a fluorescence microscope (Axio Imager M2, Carl Zeiss, Jena, Germany) with a 20X oil immersion lens.

6. Generation of tubby and stereocilin antibodies

A his tagged fusion protein involving the amino acids 1 to 189 of rat Tub protein was expressed in E. coli, purified and injected into rabbits for generation of polyclonal anti-tubby antibody. Stereocilin antibody was generated by the previously reported method.[9] The synthetic peptide CFLSPEELQSLVPLSD (amino acids 970-985) derived from mouse stereocilin amino-acid sequence was injected into rabbits for generation of polyclonal anti-stereocilin antibody. All antibodies were affinity purified and verified by immunoblotting and immunostaining.

7. Immunolabeling studies

Immunolabeling of cochlea OHC hair bundles were performed as described[9], with the following modifications. Inner ears were dissected and immediately fixed by 4% paraformaldehyde in PBS pH 7.4 for 30 min at room temperature with gentle

agitation. After three times rinse with wash buffer (0.02% triton X-100 in PBS), cochlear sensory areas were micro-dissected and re-fixed in 4% paraformaldehyde in PBS pH 7.4 for 30 min at room temperature with gentle agitation. After fixation, the cochlea were rinsed three times with wash buffer. The fixed cochlea were blocked with PBS containing 20% normal goat serum for 1 hr at room temperature with gentle agitation. After three times rinse, cochlea were permeabilized with PBS containing 0.5% triton X-100 for 30 min at room temperature with gentle agitation. After three times rinse with wash buffer, primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and incubated for overnight at 4°C with gentle agitation. After three times rinse with wash buffer, Alexa-Fluor-488-conjugated goat anti-rabbit IgG antibody was used for immunofluorescence detection. Fluorescence tagged secondary antibody and phalloidin-Alexa-Fluor-568 were diluted in PBS containing 1% BSA and incubated for 1 hr at room temperature with gentle agitation. After three times rinse with wash buffer, whole mount cochlear preparations were placed on slide glass, and mounted with ProLong Gold anti-fade reagent (ThermoFisher Scientific, Waltham, MA, USA). The immunostained cochlea were observed using LSM710 confocal microscope (Carl Zeiss, Jena, Germany) with a 100X oil immersion lens.

8. Semi-quantitative real-time polymerase chain reaction (PCR)

Inner ears of wild-type and *tubby* mice were quickly dissected in a cold DEPC-treated PBS and immediately immersed in Trizol reagent (ThermoFisher Scientific, Waltham, MA, USA), and homogenized using Precellys®24 (Bertin Corp., Rockville, MD, USA) system. Total RNA were isolated using RNeasy Mini Kit (Qiagen, Germany). cDNA were synthesized by PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan) with oligo dT primers. Real-time PCR was performed using SYBR® Premix Ex Taq (Takara, Japan) and analyzed by Applied Biosystems 7500 Real-time PCR Instrument Systems (ThermoFisher Scientific, Waltham, MA, USA).

9. Statistical analysis

All data are expressed as mean ± SEM. Statistical differences of ABRs, DPOAEs, real-time PCR data were evaluated using Student's *t*-test.

III. RESULTS

1. *Tubby* mice show elevated ABR threshold and no DPOAE responses

To compare hearing ability of wild-type and *tubby* mice, I performed ABR and DPOAE experiments. ABR is the widely used method for measuring hearing ability.[35,36] ABR is the type of auditory evoked potential detected by electrodes installed on the scalp. The ABR appears in seven positive wave forms, among these the first five waves (wave I to V) which are analyzed for access hearing ability. ABR thresholds of P21 wild-type and *tubby* mice were measured to check hearing abilities. As reported in previous studies, elevated ABR threshold values were observed in *tubby* mice when compared to that of wild-type.[13] The result showed that *tubby* mice had 40 to 50 dB elevated ABR threshold values than wild-type mice (Figure 1A). These elevated ABR threshold values represent severe hearing loss of *tubby* mice. DPOAE is also a widely used method for hearing ability measurement. DPOAE is reflected sound signals from the cochlea when stimulated by two pure tone frequencies. It is known that cochlear amplification is important for DPOAE. DPOAE measurement can detect the problems in cochlea function.[37,38] To further confirm the results and figure out the cause of hearing loss, DPOAE experiments were conducted using P21 wild-type and *tubby* mice. DPOAE levels reflect cochlea amplification ability by measuring otoacoustic emissions. If amplification ability of

the cochlea is normal, specific frequency ($2f_1 - f_2$) of otoacoustic emission responses will be detected by microphone sensors when two different frequencies (f_1, f_2) of pure tone stimuli were given. Wild-type mice showed DPOAE components above the noise floor when sound stimuli were given, however *tubby* mice did not show any detectable DPOAE response (Figure 1B). These results indicate that hearing loss of *tubby* mice are originated from disability of cochlea amplification.

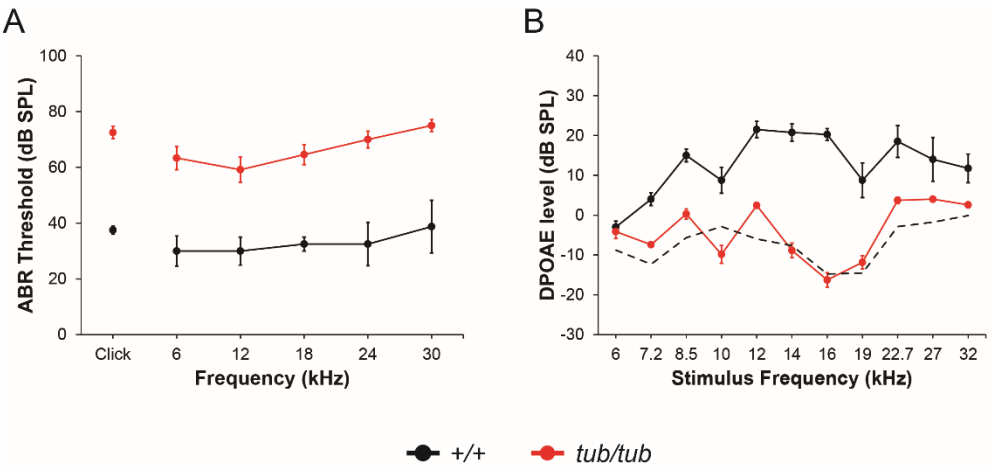

Figure 1. Auditory phenotypes of wild-type and *tubby* mice. (A) ABR threshold of wild-type (+/+, black line) and *tubby* (*tub/tub*, red line) mice. ABR threshold values were measured at 6, 12, 18, 24, 30 kHz and Click sound. ABR threshold values are significantly different ($P < 0.05$). +/+ (n=4), *tub/tub* (n=12). (B) DPOAE level of wild-type (black line) and *tubby* (red line) mice. Dashed black line represents noise level. DPOAEs above 7.2 kHz stimulus frequency are significantly different ($P < 0.05$) +/+ (n=3), *tub/tub* (n=7).

2. Horizontal top connectors and lateral links are disappeared in OHC hair bundles of *tubby* mice

Previous studies showed that cochlea in *tubby* mice are progressively degenerate, however the detailed morphology of hair bundles has not yet been reported.[2,3] In hearing tests, I found that the DPOAE responses of the *tubby* mice are almost lost, which means that the OHCs of *tubby* mice are not functioning properly. Through SEM image analysis, I found that the OHC hair bundles array is slightly disrupted in *tubby* mice (Figure 2A, B). I next analyzed stereociliary link formation using high magnification SEM images. High magnification images of wild-type OHC hair bundles showed horizontal top connectors and other types of lateral links (Figure 2C). However, in *tubby* mice, there are no horizontal top connectors or any other lateral links at OHC hair bundles (Figure 2D). Notably, tip links still existed in *tubby* mice (Figure 2D). TEM images showed corresponding results with SEM images. In wild type OHC hair bundles, horizontal top connectors and tip links were shown in TEM images (Figure 2E). However, I could not observe any lateral links including horizontal top connectors in TEM images of *tubby* mice while tip links existed (Figure 2F). These results suggest that hearing loss of *tubby* mice originated from functional failure of OHCs caused by incomplete stereociliary links. It is known that OHC hair bundles maturation is completed by 3 wk of age.[39] At P5, there were no significant morphological differences between wild-type and *tubby* mice's hair bundle of OHCs (Figure 3A, B). However, the OHC hair bundles array

was slightly disturbed in *tubby* mice after P14 (Figure 3C-F). This result suggests a morphological defect of OHC hair bundles in *tubby* mice appeared after horizontal top connectors generated.

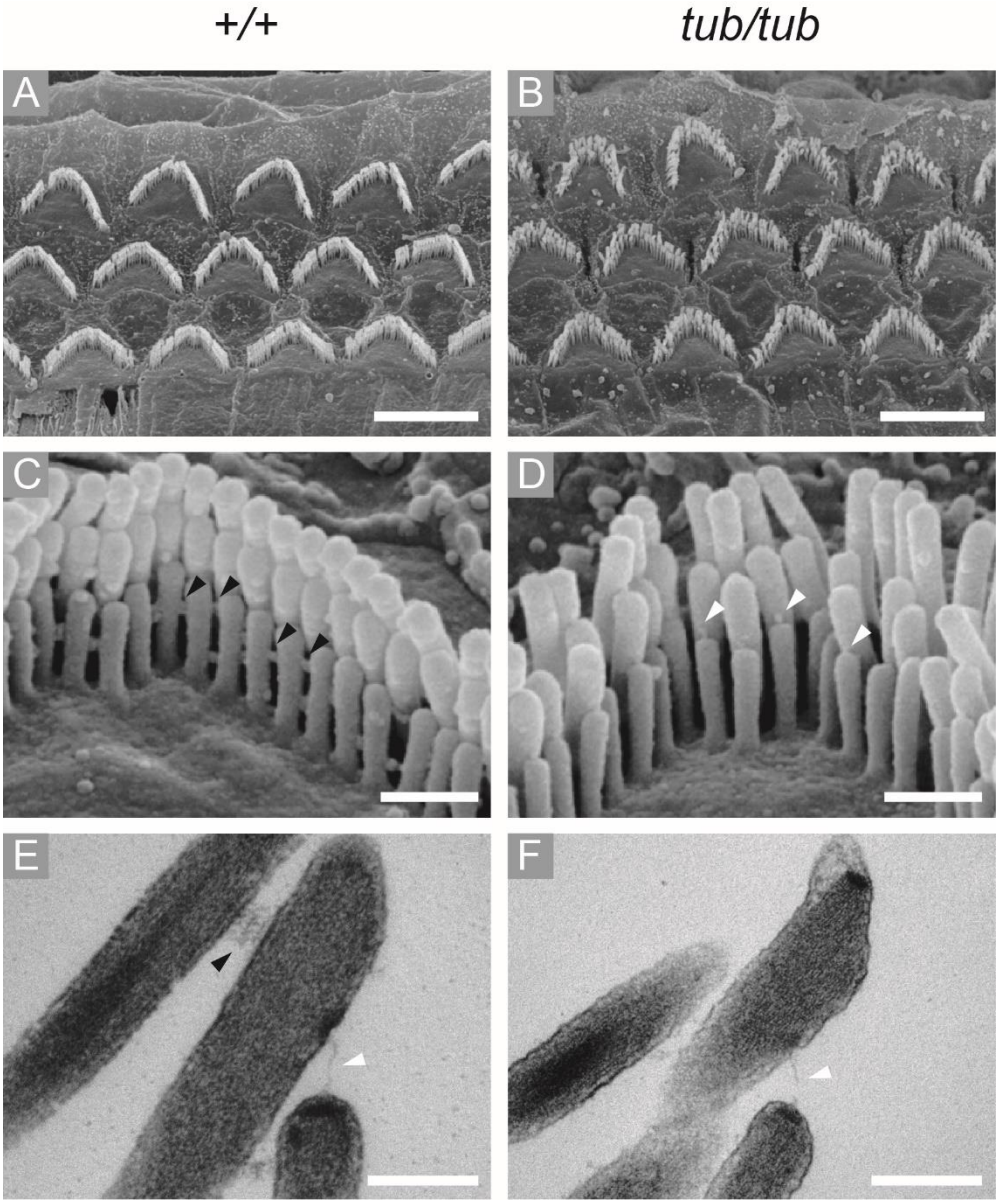

Figure 2. OHCs hair bundle morphology of wild-type and *tubby* mice. SEM and TEM images of wild-type (A, C, E) and *tubby* mice (B, D, F). Low magnification SEM images show 3 rows of OHCs, wild-type (A) and *tubby* mice (B). High magnification SEM images show horizontal top connectors in wild-type OHCs hair

bundles (C, black arrowhead). In *tubby* mice, there are no horizontal top connectors and other lateral links between stereocilia, but tip links are present (D, white arrowhead). TEM images also shows that horizontal top connectors (E, black arrowhead) and tip link (E, white arrowhead) are exist in wild-type OHCs hair bundle. But in *tubby* mice, only tip-links (F, white arrowhead) are exist. Scale bars : 5 μm (A, B), 0.5 μm (C, D), 0.2 μm (E, F).

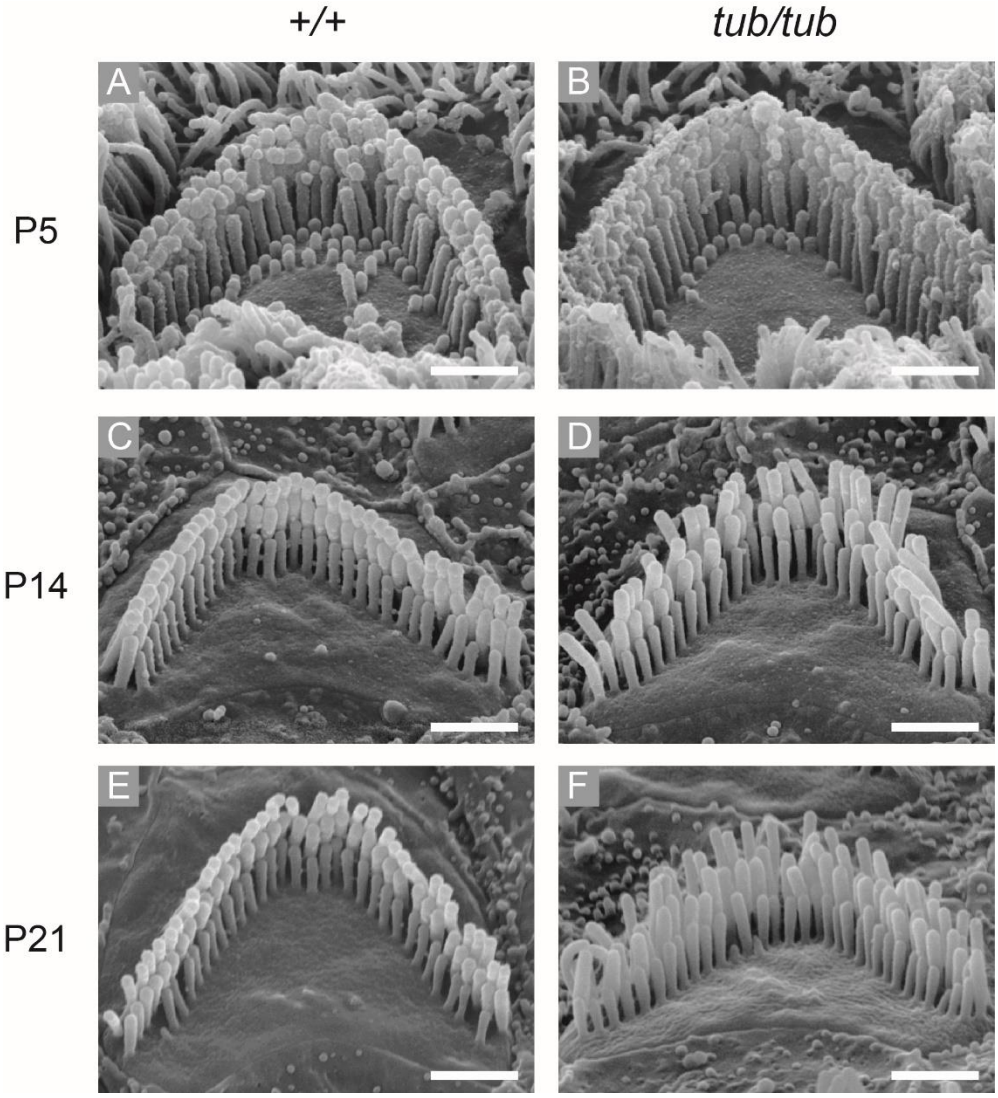

Figure 3. OHCs hair bundle morphology of wild-type and *tubby* mice during OHCs hair bundle maturation. At P5, there are no morphological differences between wild-type and *tubby* mice OHC hair bundles (A, B). After P14, OHC hair bundles array of wild-type mice is well aligned until P21 (C, E), while OHC hair bundles array is slightly disturbed after P14 in *tubby* mice (D, F). Scale bars : 1 μm (A-F).

3. MET channels of OHCs are functionally normal in *tubby* mice

FM1-43 styryl dye is widely used for identifying actively firing neurons and studying vesicular recycling.[40-42] In the inner ear, FM1-43 dye is quickly and specifically enter the sensory hair cells through MET channels.[43,44] For testing the MET channels are properly working in *tubby* mice, an FM1-43 dye uptake assay was performed using P6 cochlea. The result showed that both OHCs of wild-type and *tubby* mice are strongly labeled by FM1-43 dye (Figure 4A-D). This result indicates that MET channels are normally open in *tubby* mice. It is known that functional tip-links is important for the opening of MET channels.[45] In the morphological analysis of OHC hair bundles, I found that tip-links still exist in *tubby* mice (Figure 2D, F). The FM1-43 dye uptake assay result also suggests that tip-links in *tubby* mice have a normal physical property.

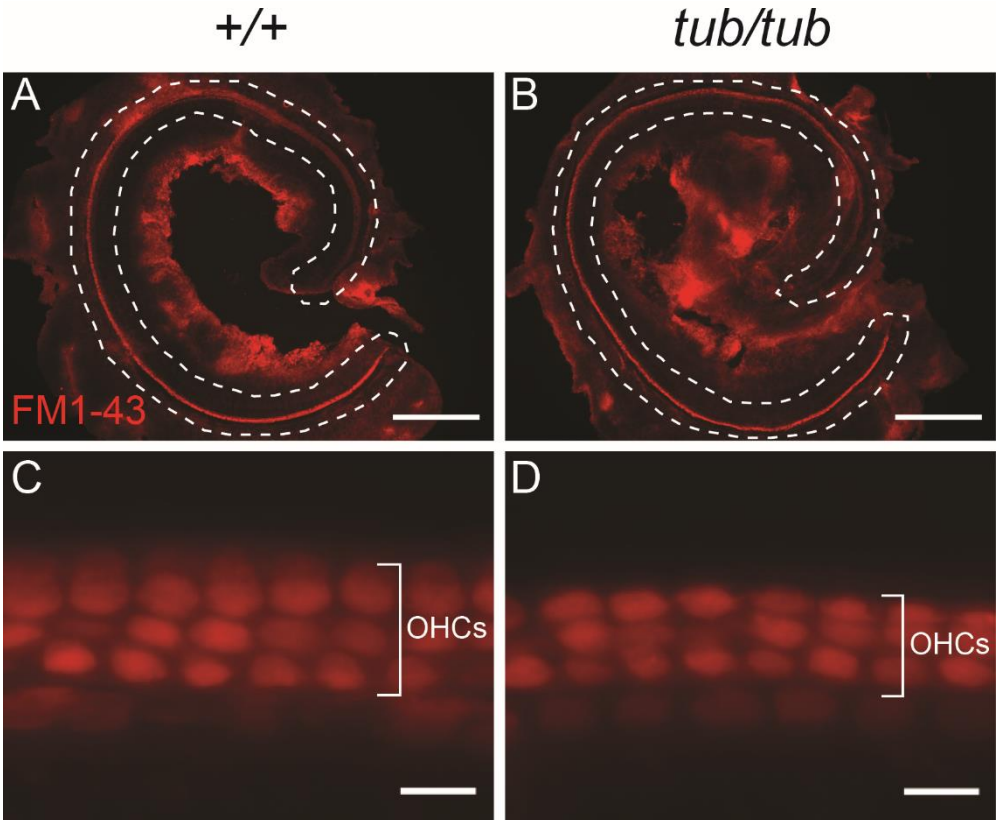

Figure 4. FM1-43 dye enters OHCs of *tubby* mice through MET channels. (A, B) Low magnification images of cochlea of wild-type (A) and *tubby* mice (B). From apex to base, FM1-43 dye uptake is observed in cochlea OHCs of wild-type and *tubby* mice (dashed line). (C, D) High magnification images of cochlear basal region of wild type (C) and *tubby* mice (D). FM1-43 dye is uptake to 3 rows of OHCs in both wild-type and *tubby* mice cochlea. Scale bars : 10 μm (A, B), 0.5 μm (C, D).

4. Tub proteins are localized to the stereocilia tip of OHC hair bundles

Previous studies revealed that Tub proteins are expressed in OHCs, IHCs, supporting cells and spiral ganglia neurons, and there are no differences in expression pattern of the Tub protein between wild type and *tubby* mice.[33] To check the localization of Tub proteins at the cochlea, I conducted whole mount cochlear immunostaining with polyclonal antibody specifically binds to Tub protein. Whole mount cochlear immunostaining of P21 wild-type mice with tubby antibody showed enriched Tub protein localization at the stereocilia tip of OHCs (Figure 5A), however the Tub protein was not observed in the OHC stereocilia of *tubby* mice (Figure 5B, I). Tub protein localization at the stereocilia tip of IHCs was not observed (Figure 5C). To monitor the changes in Tub protein localization during development of OHCs hair bundle links, cochlea of P5, P9, P14 and P21 wild-type mice were immunostained by tubby antibody. I found that Tub protein was not localized at OHC hair bundles until P5 (Figure 5D). Tub protein was detected at the tallest stereocilia tip of OHC hair bundle at P9 (Figure 5E), and after P14, Tub proteins were localized at all 3 rows of stereocilia tip of OHC hair bundles (Figure 5F, G, H). The time point of localization of Tub protein at the stereocilia tip is similar to that of formation of horizontal top connectors.[39]

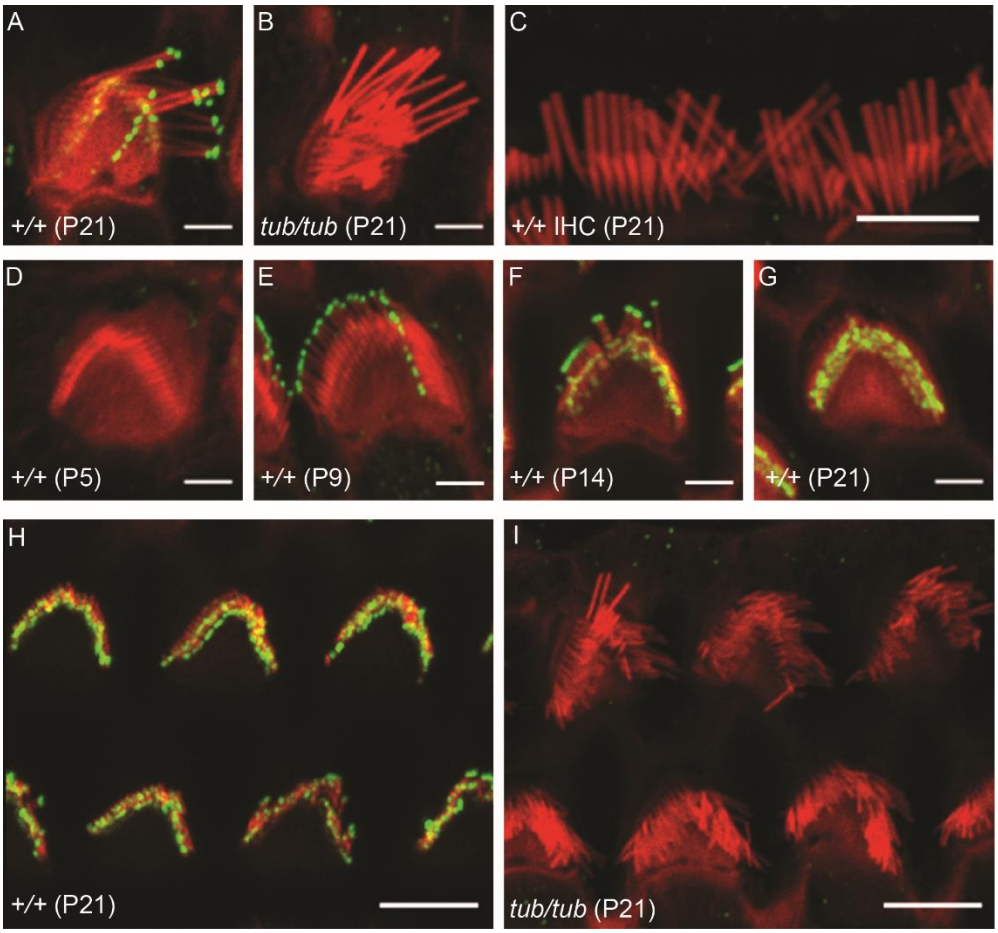

Figure 5. Localization of Tub protein in cochlear OHCs. Confocal microscope images of OHC hair bundles stained for Tub (green) and F-actin (red). (A, B) Tub protein is localized to stereocilia tip of OHC hair bundles (P21) in wild-type mice but not in *tubby* mice. (C) In hair bundles of IHCs (P21), there are no Tub protein signals. Tub protein is only localized to OHC hair bundles. (D) At P5, there are no Tub proteins in OHC hair bundles. (E) At P9, Tub protein is localized to tip of tallest stereocilia of OHC hair bundles. (F, G) At P14 and P21, Tub protein is

localized to tip of all 3 rows of stereocilia tip of OHC hair bundles. (H, I) Low magnification images of OHC hair bundles (P21) in wild-type (H), and *tubby* mice (I). Scale bars : 2 μm (A, B, D-G), 5 μm (C, H, I).

5. The OHC hair bundle localization of stereocilin is disrupted in *tubby* mice

Stereocilin is a protein specifically expressed in OHCs. This protein is located at the OHC hair bundles, especially at horizontal top connectors and TM attachment crowns.[9,46] The features of the OHC hair bundles morphology shown in *tubby* mice, such as the less clearly aligned OHC hair bundles and disruption of horizontal top connectors are very similar with the OHC morphological phenotypes of stereocilin null mice.[9,46] Furthermore, the OHC hair bundle localization pattern of Tub protein during P5 to P21 is also similar to that of stereocilin.[46] These results strongly suggest that tubby might be closely related with stereocilin. To confirm the localization of stereocilin at cochlea of *tubby* mice, I performed a cochlear whole mount immunofluorescence imaging assay with stereocilin antibody. As reported in previous studies, stereocilin was localized to the OHC hair bundles in wild-type mice (Figure 6A). However, in *tubby* mice, stereocilin was not localized to OHC hair bundles (Figure 6B). Stereocilin is the main component of the horizontal top connectors and is essential for its formation.[9,46] This immunostaining result supports the phenomenon that horizontal top connectors had been disappeared in *tubby* mice. Previous study shows that stereocilin is located at kinocilium before it appears at OHC hair bundles.[46] To test kinociliary localization of stereocilin in *tubby* mice, I performed whole mount cochlea immunostaining with P5 cochlea. In wild-type, stereocilin is localized to kinocilium and hair bundles of OHCs (Figure 6C). Interestingly, stereocilin is also localized to kinocilium and OHC hair bundles of

tubby mice (Figure 6D). This result suggests that trafficking of stereocilin to kinocilium may be conducted via a tubby-independent pathway. In addition, this result also suggests that initial localization of stereocilin to the OHC hair bundles do not requires Tub protein. In other words, Tub protein plays an important role in maintaining the OHC hair bundle localization of stereocilin.

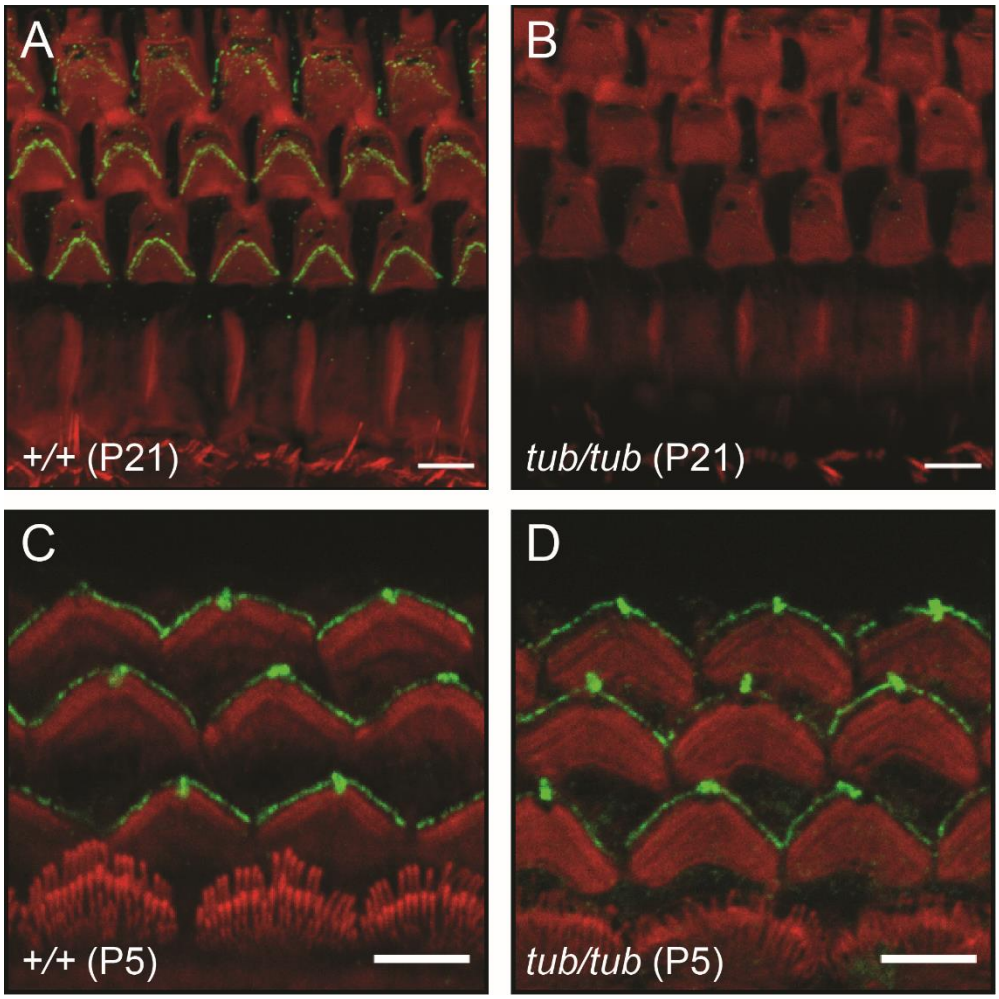

Figure 6. Disrupted OHCs hair bundle localization of stereocilin in *tubby* mice. Confocal microscope images of OHC hair bundles stained for stereocilin (green) and F-actin (red). At P21, stereocilin is localized to OHC hair bundles of wild-type OHCs (A) but not in *tubby* mice (B). At P5, stereocilin is localized to kinocilium and OHC hair bundles of wild-type (C) and *tubby* mice (D). Scale bars : 5 μm.

6. Expression of stereocilin mRNA in cochlea of *tubby* mice is normal

Since Tub protein has a DNA binding domain at carboxy terminal and nuclear localization sequence at the amino terminal, the possibility of its role as transcriptional regulator has been proposed by previous studies.[4,7] To test whether stereocilin gene expression level is reduced in cochlea of *tubby* mice, expression of stereocilin mRNA was analyzed by semi-quantitative real-time PCR. As a result, there were no significant differences on expression level of stereocilin mRNA between wild-type and *tubby* mice (Figure 7). This result suggests that stereocilin is expressed normally in cochlea of *tubby* mice but has defects in stereociliary localization.

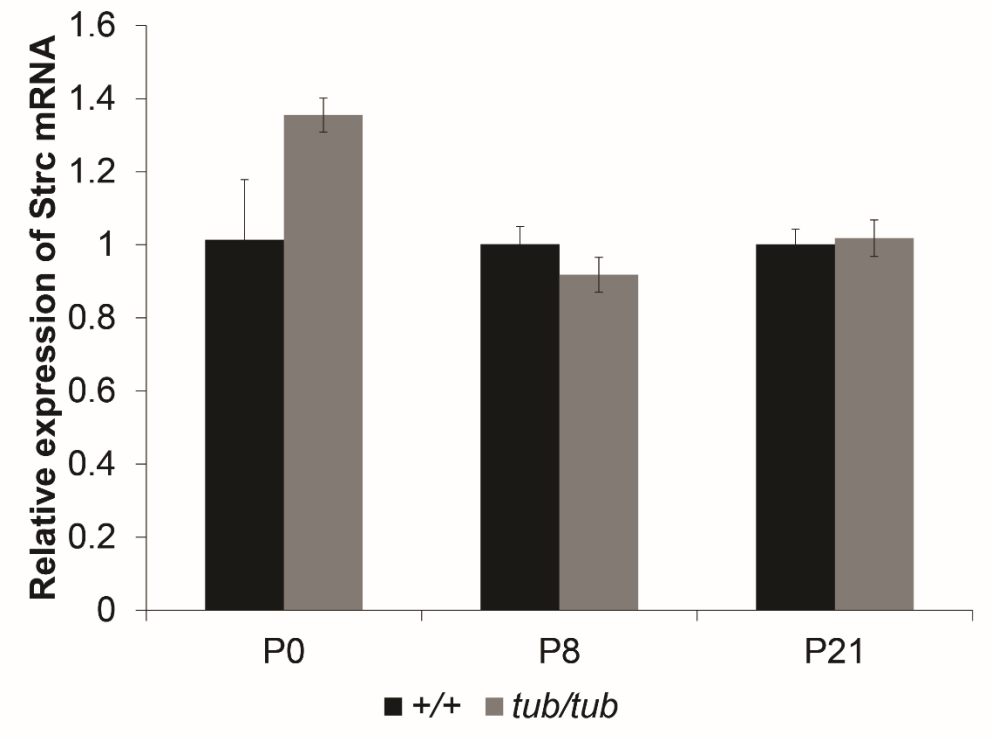

Figure 7. Gene expression level of stereocilin in cochlea of wild-type and *tubby* mice. The graph shows relative expression of stereocilin mRNA. Quantitative real-time PCR was performed. There are no significantly differences in expression level of stereocilin mRNA between wild-type (+/+) and *tubby* (*tub/tub*) mice cochlea ($P > 0.05$).

IV. DISCUSSION

In this study, I attempted to figure out the cause of hearing loss in *tubby* mice. At 3 wk of age, *tubby* mice showed elevated ABR threshold value at every tested frequency, and DPOAE response almost completely lost. According to previous reports, cochlear degenerative phenotypes like apoptotic cell death had been detected after 6 mo of age of *tubby* mice.[2,3] However, hearing ability of *tubby* mice is reduced before the onset of cochlear degeneration. This result suggests that cochlea of *tubby* mice is functionally disabled. The results of ABR and DPOAE measurement suggest that there is a defect in the cochlear amplification of *tubby* mice. The OHCs are known to play an important role in cochlear amplification.[38,47,48] This suggests that OHCs of *tubby* mice is functionally defect.

The gross morphology of the OHC hair bundles of *tubby* mice analyzed by electron microscopy was not seems to make a big difference compared to wild-type. However, the alignment of OHC hair bundles was slightly disturbed in *tubby* mice. High resolution image analysis showed that the horizontal top connectors were completely lost in the OHC hair bundles of *tubby* mice. Interestingly, however, tip-links were normally exist in the OHC hair bundles of *tubby* mice. And these tip links were tightly connected to MET channels, which was confirmed by FM1-43 fluorescent dye experiment. The horizontal top connector serves to connect each stereocilia in the OHC hair bundles, and it is known to be important for the stiffness

of OHC hair bundles, and DPOAE response.[9,46] The disturbance of OHC hair bundles array in *tubby* mice appeared after P10, which is the time point when the horizontal top connectors are formed. This suggests that the morphological phenotypes of the OHC hair bundles in *tubby* mice is due to the absence of the horizontal top connectors.

Previous study has reported that Tub proteins are present in sensory hair cells, supporting cells, and spiral ganglia neurons in the mice cochlea.[33] In this study, cochlear whole mount immunostaining results showed that the Tub protein was specifically localized in the stereocilia tip of OHCs. Many previous studies have suggested that tubby and other tubby family proteins might be closely related to microtubule cytoskeleton system such as primary cilium.[21,24,49,50] Surprisingly, however, the results of this study showed that the Tub protein is present on the stereocilia which composed of the F-actin structure. Stereociliary tip localization of Tub protein was completely absent in OHC hair bundles of *tubby* mice. In this study, it is currently not known that what is the role of Tub protein in the OHC stereocilia tip, but it seems clear that the Tub protein is closely related to the actin cytoskeleton.

Stereocilin gene is located at DFNB16 locus, and its mutation cause non-syndromic hearing loss in human.[51] Previous studies reported that stereocilin knockout mice have no DPOAE response, progressive hearing loss, and disruption of horizontal top connectors.[9,46] Although many genes affecting stereociliary link formation are known, stereocilin is the only known gene that affects the horizontal top connector.[52] OHC hair bundles of streocilin knockout mice have no horizontal

top connectors, but tip link remain, as seen in *tubby* mice. Based on the similarity of the phenotypes of tubby and stereocilin knockout mice, and the similarity of protein localization of tubby and stereocilin on OHC hair bundles, I expected that tubby is closely related to stereocilin. And the cochlear whole mount immunostaining with anti stereocilin antibody showed that there are no stereocilin in the OHC hair bundles of *tubby* mice as I expected. This suggests that the morphological phenotypes of *tubby* mice OHC hair bundle were due to the absence of stereocilin. Stereocilin is known to be also present in kinocilia, although it is not known about the role of stereocilin at kinocilia.[46] Kinocilium is microtubule based cilium structure which disappeared during hair bundle development process.[39] Interestingly stereocilin was present in the kinocilia of *tubby* mice during the early hair bundle developmental stage. These results suggest that there might be distinct mechanisms of stereocilin trafficking to kinocilia and stereocilia. In addition, at this time point, when the kinocilia remain, stereocilin was transiently present in the OHC hair bundles of *tubby* mice. This suggests that Tub protein is not required for initial stereociliary trafficking of stereocilin. However, as the OHC hair bundles are gradually maturated, stereocilin disappears from the OHC hair bundles of *tubby* mice, suggesting that the Tub protein plays an important role in the maintenance of OHC hair bundle localization of stereocilin.

Previous studies have shown that Tub protein is likely to act as a transcriptional regulator.[4,7] However, at least in the cochlear OHCs, Tub proteins do not seem to play a major role in transcriptional regulation. Because stereocilin gene expression

in cochlea of *tubby* mice was not different from that of wild-type mice. This suggests that, unlike the hypothesis that Tub protein will function as a transcription factor, it may play a totally different role *in vivo*.

Stereocilia are composed of filamentous actin fiber and distinct stereociliary membrane components.[48] Molecular trafficking in stereocilia is known to be carried out by unconventional myosins. Myosin IIIa and XVa are known to transport the cargo protein to the tip of OHC stereocilia.[53-55] It is however uncertain that these unconventional myosins are essential for stereociliary trafficking of tubby and stereocilin. To figure out stereociliary trafficking mechanism of tubby and stereocilin, further investigation is needed.

Overall results demonstrate that hearing loss phenotype of *tubby* mice is caused by disrupted stereociliary localization of stereocilin and loss of horizontal top connectors. It is clear that this would be the primary cause of hearing loss mechanism in *tubby* mice, but there are some reports that sequence variation of microtubule associated protein 1A (Map1a) affect *tubby* mice hearing ability.[10,33,56] These reports suggest possible roles of Tub protein at the ganglia neuron, but functional role of Tub protein at ganglia neuron and the relationship between tubby and Map1a is still elusive, and needed further studies. In human, it has been reported that homozygous mutation in *tub* gene is associated with obesity and retinal degeneration, but not associated with hearing loss.[57] It is currently not known why hearing defect does not appear in the people with *tub* gene mutation, but it is possible that human *tub* was affected by other genes, as in case of Map1a in mice.

Tub protein binds to PIP_2 through the PIP_2 binding domain present in the tubby domain, and this binding is very specific and strong, even to be used as a PIP_2 imaging tool in cellular experiments.[8,58] In Drosophila, dINPP5E mutant fly, it is known that the ciliary localization of drosophila tubby homolog (dTulp) is altered due to the change of the PIP_2 composition of the chordotonal cilia membrane, resulting in hearing defects in this mutant fly. This means that the localization of the Tub protein is dependent on the PIP_2 composition of the cell membrane. In addition, although not cochlear hair cells, it has been reported that PIP_2 is concentrated in the stereocilia of vestibule hair cells.[59] Based on these facts, it is likely that the enrichment of the Tub protein specifically to the stereocilia of OHCs is closely related to the PIP_2 composition of the stereocilia membrane. Further studies are needed to determine whether OHC hair bundles localization of Tub protein is associated with PIP_2 composition of stereocilia membrane.

V. CONCLUSION

Overall results demonstrate that the hearing loss of *tubby* mice results from the dysfunction of OHCs due to the loss of the horizontal top connector in the OHC hair bundles. And the loss of the horizontal top connectors observed in *tubby* mice is caused by stereocilin mislocalization. Stereocilin is present in the OHC hair bundles of *tubby* mice at P5, but it disappeared from after P9. This suggests that stereocilin does not require a Tub protein for initial trafficking to the OHC hair bundles. Instead, it means that Tub protein is essential for maintain of stereocilin at OHC hair bundles and formation of horizontal top connectors by stereocilin. This study firstly demonstrated that Tub protein is specifically located at OHC hair bundles of cochlea. Tub protein has long been suggested to be closely associated with primary cilia which is microtubule-based cellular organelle. However, the results of this study suggest that Tub protein may be closely associated with actin-based cellular organelles *in vivo*. The results of this study could also provide a new insight into the molecular mechanism of another phenotypes of tubby mice such as late onset obesity, and retinal degeneration. The remaining question is how Tub protein moves to the stereocilia and what is the molecular function of Tub protein on the stereocilia. Further study is needed to answer these questions.

REFERENCES

1. Coleman DL, Eicher EM. Fat (fat) and tubby (tub): two autosomal recessive mutations causing obesity syndromes in the mouse. J Hered 1990;81:424-7.
2. Ohlemiller KK, Hughes RM, Mosinger-Ogilvie J, Speck JD, Grosof DH, Silverman MS. Cochlear and retinal degeneration in the tubby mouse. Neuroreport 1995;6:845-9.
3. Ohlemiller KK, Hughes RM, Lett JM, Ogilvie JM, Speck JD, Wright JS, et al. Progression of cochlear and retinal degeneration in the tubby (rd5) mouse. Audiol Neurootol 1997;2:175-85.
4. Carroll K, Gomez C, Shapiro L. Tubby proteins: the plot thickens. Nat Rev Mol Cell Biol 2004;5:55-63.
5. Noben-Trauth K, Naggert JK, North MA, Nishina PM. A candidate gene for the mouse mutation tubby. Nature 1996;380:534-8.
6. Stubdal H, Lynch CA, Moriarty A, Fang Q, Chickering T, Deeds JD, et al. Targeted deletion of the tub mouse obesity gene reveals that tubby is a loss-of-function mutation. Mol Cell Biol 2000;20:878-82.
7. Boggon TJ, Shan WS, Santagata S, Myers SC, Shapiro L. Implication of tubby proteins as transcription factors by structure-based functional analysis. Science 1999;286:2119-25.
8. Santagata S, Boggon TJ, Baird CL, Gomez CA, Zhao J, Shan WS, et al. G-protein signaling through tubby proteins. Science 2001;292:2041-50.
9. Verpy E, Weil D, Leibovici M, Goodyear RJ, Hamard G, Houdon C, et al. Stereocilin-deficient mice reveal the origin of cochlear waveform distortions. Nature 2008;456:255-8.
10. Ikeda A, Zheng QY, Zuberi AR, Johnson KR, Naggert JK, Nishina PM. Microtubule-associated protein 1A is a modifier of tubby hearing (moth1). Nat Genet 2002;30:401-5.
11. Hildebrandt F, Benzing T, Katsanis N. Ciliopathies. N Engl J Med 2011;364:1533-43.
12. Girard D, Petrovsky N. Alstrom syndrome: insights into the pathogenesis of metabolic disorders. Nat Rev Endocrinol 2011;7:77-88.
13. Heckenlively JR, Chang B, Erway LC, Peng C, Hawes NL, Hageman GS, et al. Mouse model for Usher syndrome: linkage mapping suggests homology to Usher type I reported at human chromosome 11p15. Proc Natl Acad Sci U S A 1995;92:11100-4.
14. Kleyn PW, Fan W, Kovats SG, Lee JJ, Pulido JC, Wu Y, et al. Identification and characterization of the mouse obesity gene tubby: a member of a novel gene family. Cell 1996;85:281-90.
15. Ikeda S, Shiva N, Ikeda A, Smith RS, Nusinowitz S, Yan G, et al. Retinal degeneration but not obesity is observed in null mutants of the tubby-like protein 1 gene. Hum Mol Genet 2000;9:155-63.
16. Gu S, Lennon A, Li Y, Lorenz B, Fossarello M, North M, et al. Tubby-like protein-1 mutations in autosomal recessive retinitis pigmentosa. Lancet 1998;351:1103-4.
17. Hagstrom SA, North MA, Nishina PL, Berson EL, Dryja TP. Recessive mutations

in the gene encoding the tubby-like protein TULP1 in patients with retinitis pigmentosa. Nat Genet 1998;18:174-6.
18. Hagstrom SA, Adamian M, Scimeca M, Pawlyk BS, Yue G, Li T. A role for the Tubby-like protein 1 in rhodopsin transport. Invest Ophthalmol Vis Sci 2001;42:1955-62.
19. North MA, Naggert JK, Yan Y, Noben-Trauth K, Nishina PM. Molecular characterization of TUB, TULP1, and TULP2, members of the novel tubby gene family and their possible relation to ocular diseases. Proc Natl Acad Sci U S A 1997;94:3128-33.
20. Ikeda A, Ikeda S, Gridley T, Nishina PM, Naggert JK. Neural tube defects and neuroepithelial cell death in Tulp3 knockout mice. Hum Mol Genet 2001;10:1325-34.
21. Mukhopadhyay S, Wen X, Chih B, Nelson CD, Lane WS, Scales SJ, et al. TULP3 bridges the IFT-A complex and membrane phosphoinositides to promote trafficking of G protein-coupled receptors into primary cilia. Genes Dev 2010;24:2180-93.
22. Norman RX, Ko HW, Huang V, Eun CM, Abler LL, Zhang Z, et al. Tubby-like protein 3 (TULP3) regulates patterning in the mouse embryo through inhibition of Hedgehog signaling. Hum Mol Genet 2009;18:1740-54.
23. Cameron DA, Pennimpede T, Petkovich M. Tulp3 is a critical repressor of mouse hedgehog signaling. Dev Dyn 2009;238:1140-9.
24. Mukhopadhyay S, Jackson PK. The tubby family proteins. Genome Biol 2011;12:225.
25. Li QZ, Wang CY, Shi JD, Ruan QG, Eckenrode S, Davoodi-Semiromi A, et al. Molecular cloning and characterization of the mouse and human TUSP gene, a novel member of the tubby superfamily. Gene 2001;273:275-84.
26. van Duyvenvoorde HA, Lui JC, Kant SG, Oostdijk W, Gijsbers AC, Hoffer MJ, et al. Copy number variants in patients with short stature. Eur J Hum Genet 2014;22:602-9.
27. Vieira AR, de Carvalho FM, Johnson L, DeVos L, Swailes AL, Weber ML, et al. Fine Mapping of 6q23.1 Identifies TULP4 as Contributing to Clefts. Cleft Palate Craniofac J 2015;52:128-34.
28. Coyle CA, Strand SC, Good DJ. Reduced activity without hyperphagia contributes to obesity in Tubby mutant mice. Physiol Behav 2008;95:168-75.
29. Backberg M, Madjid N, Ogren SO, Meister B. Down-regulated expression of agouti-related protein (AGRP) mRNA in the hypothalamic arcuate nucleus of hyperphagic and obese tub/tub mice. Brain Res Mol Brain Res 2004;125:129-39.
30. Wang Y, Seburn K, Bechtel L, Lee BY, Szatkiewicz JP, Nishina PM, et al. Defective carbohydrate metabolism in mice homozygous for the tubby mutation. Physiol Genomics 2006;27:131-40.
31. Guan XM, Yu H, Van der Ploeg LH. Evidence of altered hypothalamic pro-opiomelanocortin/ neuropeptide Y mRNA expression in tubby mice. Brain Res Mol Brain Res 1998;59:273-9.
32. Ikeda S, He W, Ikeda A, Naggert JK, North MA, Nishina PM. Cell-specific expression of tubby gene family members (tub, Tulp1,2, and 3) in the retina. Invest Ophthalmol Vis Sci 1999;40:2706-12.
33. Ikeda A, Zheng QY, Rosenstiel P, Maddatu T, Zuberi AR, Roopenian DC, et al. Genetic modification of hearing in tubby mice: evidence for the existence of a major gene (moth1) which protects tubby mice from hearing loss. Hum Mol Genet 1999;8:1761-7.

34. Kong L, Chen GD, Zhou X, McGinnis JF, Li F, Cao W. Molecular mechanisms underlying cochlear degeneration in the tubby mouse and the therapeutic effect of sulforaphane. Neurochem Int 2009;54:172-9.
35. Kotlarz JP, Eby TL, Borton TE. Analysis of the efficiency of retrocochlear screening. Laryngoscope 1992;102:1108-12.
36. Melcher JR, Kiang NY. Generators of the brainstem auditory evoked potential in cat. III: Identified cell populations. Hear Res 1996;93:52-71.
37. Mehrparvar AH, Mirmohammadi SJ, Davari MH, Mostaghaci M, Mollasadeghi A, Bahaloo M, et al. Conventional Audiometry, Extended High-Frequency Audiometry, and DPOAE for Early Diagnosis of NIHL. Iran Red Crescent Med J 2014;16:e9628.
38. Avan P, Buki B, Petit C. Auditory distortions: origins and functions. Physiol Rev 2013;93:1563-619.
39. Goodyear RJ, Marcotti W, Kros CJ, Richardson GP. Development and properties of stereociliary link types in hair cells of the mouse cochlea. J Comp Neurol 2005;485:75-85.
40. Betz WJ, Bewick GS. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. Science 1992;255:200-3.
41. Betz WJ, Mao F, Bewick GS. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J Neurosci 1992;12:363-75.
42. Betz WJ, Mao F, Smith CB. Imaging exocytosis and endocytosis. Curr Opin Neurobiol 1996;6:365-71.
43. Gale JE, Marcotti W, Kennedy HJ, Kros CJ, Richardson GP. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. J Neurosci 2001;21:7013-25.
44. Meyers JR, MacDonald RB, Duggan A, Lenzi D, Standaert DG, Corwin JT, et al. Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 2003;23:4054-65.
45. Gillespie PG, Muller U. Mechanotransduction by hair cells: models, molecules, and mechanisms. Cell 2009;139:33-44.
46. Verpy E, Leibovici M, Michalski N, Goodyear RJ, Houdon C, Weil D, et al. Stereocilin connects outer hair cell stereocilia to one another and to the tectorial membrane. J Comp Neurol 2011;519:194-210.
47. Fettiplace R, Hackney CM. The sensory and motor roles of auditory hair cells. Nat Rev Neurosci 2006;7:19-29.
48. Schwander M, Kachar B, Muller U. Review series: The cell biology of hearing. J Cell Biol 2010;190:9-20.
49. Sun X, Haley J, Bulgakov OV, Cai X, McGinnis J, Li T. Tubby is required for trafficking G protein-coupled receptors to neuronal cilia. Cilia 2012;1:21.
50. Mukhopadhyay S, Jackson PK. Cilia, tubby mice, and obesity. Cilia 2013;2:1.
51. Verpy E, Masmoudi S, Zwaenepoel I, Leibovici M, Hutchin TP, Del Castillo I, et al. Mutations in a new gene encoding a protein of the hair bundle cause non-syndromic deafness at the DFNB16 locus. Nat Genet 2001;29:345-9.
52. Peng AW, Salles FT, Pan B, Ricci AJ. Integrating the biophysical and molecular mechanisms of auditory hair cell mechanotransduction. Nat Commun 2011;2:523.
53. Belyantseva IA, Boger ET, Friedman TB. Myosin XVa localizes to the tips of inner ear sensory cell stereocilia and is essential for staircase formation of the hair bundle. Proc Natl Acad Sci U S A 2003;100:13958-63.
54. Belyantseva IA, Boger ET, Naz S, Frolenkov GI, Sellers JR, Ahmed ZM, et al. Myosin-XVa is required for tip localization of whirlin and differential elongation of

hair-cell stereocilia. Nat Cell Biol 2005;7:148-56.
55. Salles FT, Merritt RC, Jr., Manor U, Dougherty GW, Sousa AD, Moore JE, et al. Myosin IIIa boosts elongation of stereocilia by transporting espin 1 to the plus ends of actin filaments. Nat Cell Biol 2009;11:443-50.
56. Grant SG. Putting tubby on the MAP. Nat Genet 2002;30:347-8.
57. Borman AD, Pearce LR, Mackay DS, Nagel-Wolfrum K, Davidson AE, Henderson R, et al. A homozygous mutation in the TUB gene associated with retinal dystrophy and obesity. Hum Mutat 2014;35:289-93.
58. Szentpetery Z, Balla A, Kim YJ, Lemmon MA, Balla T. Live cell imaging with protein domains capable of recognizing phosphatidylinositol 4,5-bisphosphate; a comparative study. BMC Cell Biol 2009;10:67.
59. Hirono M, Denis CS, Richardson GP, Gillespie PG. Hair cells require phosphatidylinositol 4,5-bisphosphate for mechanical transduction and adaptation. Neuron 2004;44:309-20.

ABSTRACT (IN KOREAN)

Tubby 마우스의 청력 손실에 대한 분자적 기전

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Tubby 마우스는 비만, 망막 퇴화, 청력 손실과 같은 세 가지 표현형을 나타내는 자연발생적으로 생겨난 돌연변이 마우스이다. *Tub* 유전자의 splice site 에 생긴 point mutation 에 의해 Tub 단백질의 C-terminal 부분의 44개 아미노산이 intron 에 의해 코딩 된 전혀 다른 24개의 아미노산으로 치환되면서 Tub 단백질은 기능을 완전히 상실하게 된다. Tub 단백질은 nuclear localization sequence, DNA binding domain, 그리고 *in vitro* 전사 활성과 같은 특징들로 인해 전사 조절자로서 작용할 가능성이 오랫동안 제기되어 왔다. 하지만 Tub 단백질이 실제로 생체내에서

어떠한 특정 유전자의 발현을 조절 하는지, 그리고 *tubby* 마우스 표현형의 분자적 기전이 무엇인지에 대해서는 잘 알려져 있지 않다. 본 연구에서 나는 *tubby* 마우스에서 나타나는 청력 손실의 분자적 기전에 대해 조사했다. 청력 측정 결과와 전자현미경 분석 결과는 *tubby* 마우스의 청력 손실이 달팽이관 외유모세포 (outer hair cell) 의 기능 이상으로부터 유래한다는 것을 보여주었다. 면역염색 결과 Tub 단백질은 달팽이관 외유모세포의 섬모 (hair bundle) 상에 존재한다는 사실을 발견했다. 게다가 Tub 단백질이 달팽이관의 외유모세포 상에서 섬모 연결 고리 (stereociliary link), 특히 horizontal top connector, 의 형성에 필수적이라는 사실도 발견했다. Stereocilin 또한 horizontal top connector 의 형성에 중요하다고 알려져 있는 단백질이다. Stereocilin 은 달팽이관의 외유모세포에 특이적으로 발현하며, 외유모세포의 섬모 상에 위치하고 있다. 특히 나는 *tubby* 마우스의 외유모세포 섬모 상에서 stereocilin 이 사라진 것을 발견했다. 이것은 외유모세포의 섬모상에 있는 Tub 단백질이 stereocilin 을 통한 horizontal top connector 의 형성에 필수적이라는 사실을 암시한다. 이상의 결과들은, 적어도 *tubby* 마우스의 청력손실 표현형에 있어서 만큼은, Tub 단백질이 이전 연구에서 제시되었던 바와 같이 전사조절자가 아니라, 달팽이관 외유모세포의 섬모 연결 고리를 형성하는데 직접적인 역할을 한다는 사실을 암시한다. 또한 본 연구의 결과들은 *tubby* 마우스의 청력 손실이 이전 연구에서 제

시되었던 달팽이관 신경 접합부의 (synapse) 기능 보다는 달팽이관 감각 유모 세포의 기능 장애 때문이라는 사실을 암시한다.

핵심되는 말: *tubby* 마우스, Tub 단백질, 청력 손실, 달팽이관, 외유모 세포, horizontal top connector, stereocilin