Critical role of spectrin in hearing development and deafness

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Inner ear hair cells (HCs) detect sound through the deflection of mechanosensory stereocilia. Stereocilia are inserted into the cuticular plate of HCs by parallel actin rootlets, where they convert sound-induced mechanical vibrations into electrical signals. The molecules that support these rootlets and enable them to withstand constant mechanical stresses underpin our ability to hear. However, the structures of these molecules have remained unknown. We hypothesized that αI- and βII-spectrin subunits fulfill this role, and investigated their structural organization in rodent HCs. Using super-resolution fluorescence imaging, we found that spectrin formed ring-like structures around the base of stereocilia rootlets. These spectrin rings were associated with the hearing ability of mice. Further, HC-specific, βII-spectrin knockout mice displayed profound deafness. Overall, our work has identified and characterized structures of spectrin that play a crucial role in mammalian hearing development.

INTRODUCTION

The cuticular plate of hair cells (HCs) is thought to be critical in mammalian hearing by securing stereocilia rootlets in place (1–6) and providing them with the rigidity and support necessary for auditory transduction (6–9). Stereocilia rootlets are electron-dense structures penetrating into the cuticular plate and forming an anchoring complex. This anchoring complex is believed to be the key structural component for stereocilia to withstand constant mechanical stresses, and thus plays a critical role in hearing (10). However, the specific anchoring molecules that provide stereocilia rootlets with their necessary elasticity and flexibility remain elusive.

We hypothesized that spectrin is a likely candidate to fulfill this role. Spectrin is expressed in almost all cells (11), playing a variety of important roles (11–14). In particular, the spectrin α and β subunits form flexible fiber-like structures in many types of cells (11, 14, 15). They are assembled into antiparallel heterodimers, which, in turn, form end-to-end tetramers (11, 15). Recent studies have shown that spectrin can form different structural organizations in many types of cells, although the components are similar (16–18). It is known that spectrin isoforms are expressed in different regions of the inner ear HCs and that they are concentrated in the cuticular plate (19, 20). However, the spectrin ultrastructural organization and therefore its role in mammalian hearing are unclear.

RESULTS

Spectrin forms ring structures in the cuticular plate

To determine the extent to which spectrin structures underpin the function of stereocilia, we turned to super-resolution fluorescence imaging, which can provide molecular labeling specificity and live-cell imaging feasibility with nanoscale resolution (21–24). Immunostaining experiments investigated the structure of both αII- and βII-spectrin in the cuticular plates of HCs of mice (fig. S1A). Confocal imaging showed the enrichment of both αII- and βII-spectrin as individual dots in the cuticular plate, but no obvious organizational pattern was detected (fig. S1B). Conversely, stimulated emission depletion (STED) imaging revealed a remarkably regular organization, with both αII- and βII-spectrin forming two rows of ring-like structures with diameters ranging from 200 to 210 nm outlining the edges of the outer HC (OHC) and inner HC (IHC) stereocilia rootlets (Fig. 1A). αII- and βII-spectrin ring structures were observed in the apex, middle, and base regions of the cochlea, and their diameters were similar (fig. S1, C and D).

To investigate the spatial relation between individual spectrin rings and rootlets, we performed two-color STED imaging to characterize the structure of spectrin and F-actin bundles in the cuticular plate, which forms the central part of rootlets and was labeled with phallloidin-conjugated organic dyes. The STED results showed that each spectrin ring surrounded an F-actin dot (Fig. 1B), supporting the idea that the spectrin rings associate tightly with rootlets in the cuticular plate. Transmission electron microscopy of cochlear sections from mice found a mean stereocilia rootlet diameter of ~80 nm, which is comparable to previous findings (5, 6) but smaller than the mean diameter of the rings of spectrin (Fig. 1, C and D). We reasoned that spectrin forms cylinders that wrap around the rootlets acting as a mechanical sensor during stereocilia deflection because of its intrinsic extensibility and elasticity (25, 26). Given the highly conserved organization of spectrin...
Fig. 1. Structure of αII- and βII-spectrin in the cuticular plate. (A) Representative STED images of αII- and βII-spectrin from mouse OHCs and IHCs at P21, with magnification of yellow boxed regions on the right. Intensity profiles along the solid lines are shown. The rings are numerically labeled, and corresponding intensity curves are shown (n = 3 mice). Scale bars, 1 μm. (B) Representative two-color STED image of βII-spectrin (magenta) and F-actin (cyan) in the cuticular plate of OHC and the magnification region indicated by yellow boxes. Yellow triangles indicate that the rootlet is found inside the spectrin ring. Scale bar, 1 μm. (C) Representative transmission electron micrographs of the apical region of HCs from mice, with the sections parallel to the stereocilia staircase (left) and stereocilia row (right) (n = 3 mice). Scale bars, 200 nm. (D) Size comparison among spectrin rings and stereocilia rootlets. n = ring numbers or rootlet numbers from three to five mice for each group. ****P < 0.0001, Student’s t test. Error bars ± SD.
To directly analyze the effects of spectrin on hearing function, we crossed Sptbn1-flax (Sptbn1\textsuperscript{flax/flax}) mice with Atoh1\textsuperscript{+/+} mice, which express a Cre recombinase in the HC-specific Atoh1 locus to generate HC-specific βII-spectrin knockout (Atoh1-Sptbn1\textsuperscript{−/−}) mice (Fig. 3A). We performed immunostaining experiments to characterize the βII-spectrin expression in the apical, middle, and base regions of cuticular plate in Atoh1-Sptbn1\textsuperscript{−/−} mice. βII-spectrin was completely deleted in all regions of cuticular plate in Atoh1-Sptbn1\textsuperscript{−/−} mice (Fig. 3B). Because αII-spectrin exists in cuticular plate of HCs and may play a complementary role for βII-spectrin in HCs, we tested whether the expression of αII-spectrin was altered in Atoh1-Sptbn1\textsuperscript{−/−} mice. Our immunostaining experiments indicated a near-complete deletion of αII-spectrin in cuticular plates and the foniculus region of HCs in Atoh1-Sptbn1\textsuperscript{−/−} mice at 2 weeks after birth (Fig. 3C). αII-spectrin was previously found in the cortical lattice, which is likely associated with βV-spectrin in HCs (19). Notably, αII-spectrin was found specifically in the cortical lattice region of HCs in Atoh1-Sptbn1\textsuperscript{−/−} mice (Fig. 3C), suggesting that βII-spectrin may be required for the specific location of αII-spectrin in the cuticular plate of HCs. Next, we tested whether βII-spectrin affects the distribution of other proteins in the taper region of stereocilia. We immunostained taperin in HCs from Atoh1-Sptbn1\textsuperscript{−/−} mice. The amount of taperin expression was quite normal, but its spatial pattern was markedly changed according to the spatial pattern of stereocilia (Fig. 3D). Looking into details, taperin was located in the taper region of each stereocilium and also outside the taper region of stereocilia (Fig. 3D). FAM65B was found to be expressed in the base region of stereocilia and critical for hearing development (28). FAM65B expression level was not substantially decreased, but FAM65B spatial pattern was substantially changed in HCs of Atoh1-Sptbn1\textsuperscript{−/−} mice (Fig. S2). Our results suggested that βII-spectrin is important for the normal distribution of taperin and FAM65B in the base region of stereocilia.

Next, we imaged the F-actin structure at different stages of HC development in Atoh1-sptbn1\textsuperscript{−/−} mice. Typically, HCs displayed a V-shape organization of stereocilia even at early development, which can be detected by the F-actin structure of HCs (Fig. 4A). The stereocilia started to lose their polarity and developed dysregulated spacing as early as postnatal day 4 (P4) in Atoh1-sptbn1\textsuperscript{−/−} mice (Fig. 4B). The extent of the disruption was larger at later developmental stages (Fig. 4, A and B). Further, we characterized the HC morphology by immunostaining myosin7a and found that HCs were gradually degraded during development in Atoh1-sptbn1\textsuperscript{−/−} mice (Fig. S3). HCs started to degenerate at around 2 weeks after birth, and this supports the idea that spectrin in the HC is essential to HC development and that it might affect its polarity and the disruption of protein distribution, specifically in the cuticular plate region (Fig. 3S). We further examined the expression of spectrin and F-actin in HCs in the Atoh1-sptbn1\textsuperscript{−/−} mice. βII-spectrin was completely deleted in HCs of Atoh1-sptbn1\textsuperscript{−/−} mice (Fig. S4A). The morphology of HCs was assessed by fluorescence-labeled phalloidin, and no obvious defects were visualized in HCs of Atoh1-sptbn1\textsuperscript{−/−} mice (Fig. S4B). Notably, no circling or head tilt behaviors were observed from Atoh1-sptbn1\textsuperscript{−/−} mice. Furthermore, vestibular ocular reflex (VOR) has been measured for assessment of vestibular function, and no significant difference was observed between Atoh1-sptbn1\textsuperscript{−/−} mice and control mice (Fig. S4C). These results suggest that βII-spectrin deletion did not substantially affect the function of HCs. Next, to study the role of βII-spectrin in HCs in hearing development, we performed auditory brainstem response (ABR) tests to assess the hearing ability of Atoh1-Sptbn1\textsuperscript{−/−} mice. We found that the Atoh1-Sptbn1\textsuperscript{−/−} mice displayed severe hearing loss (Fig. 4, C and D). Our results demonstrate a crucial role of spectrin in HC function and hearing development.

Spectrin rings correlate with hearing ability
Mice acquire the ability to hear at around 2 weeks after birth (29), which corresponds to our observation of spectrin forming organized structures in the cuticular plate and playing an essential role in hearing development. Additionally, we wished to investigate whether spectrin is necessary for the maintenance of hearing in adults. As follows, we investigated the structure of spectrin rings in mice with hearing disability due to aging or noise exposure. We hypothesized that spectrin’s regular ring-like organization would be perturbed under both HC damage conditions.

In aging mice, hearing function was gradually lost over time (Fig. 5A), as measured by ABR tests. βII-spectrin rings were also gradually disrupted in the OHCs of aging mice (Fig. 5B) and were...
Fig. 2. Spectrin structure in HCs during postnatal development. (A) Representative STED images of βII-spectrin staining in the cuticular plates of OHCs at different developmental stages. \( n = 3 \) to 8 mice from each stage. Scale bar, 1 \( \mu \)m. (B) Representative confocal images of stereocilia in OHCs at different developmental stages (\( n = 3 \) to 5 mice from each stage). Scale bar, 1 \( \mu \)m. (C and D) Same as (A) and (B), but for IHCs at different developmental stages. (C) \( n = 4 \) to 9 mice from each stage; (D) \( n = 3 \) to 5 mice from each stage. Scale bars, 1 \( \mu \)m. (E) Spectrin ring diameter comparison among different developmental stages in OHCs and IHCs, respectively. \( n = \) ring numbers from three to five mice for each group. No significances. Two-way analysis of variance (ANOVA). Error bars ± SD. (F) Representative confocal images of βII-spectrin (magenta) and myosin7a (cyan) in utricle from P14 mice (\( n = 3 \) mice). S, striolar region; ES, extrastriolar region. Scale bar, 15 \( \mu \)m. (G) Representative confocal and STED images of βII-spectrin in VHCs (\( n = 3 \) mice). Scale bar, 1 \( \mu \)m.
more disrupted in the base region of their OHCs in line with the degree of hearing loss at high frequencies (Fig. 5C). In the noise-induced permanent threshold shift (PTS) mouse model, we tested the structure of βIII-spectrin rings (fig. S4A). Notably, we examined the spectrin structure 1 day after noise exposure and observed that spectrin rings were severely disrupted in the cuticular plate of OHCs in these mice (fig. S4, A to C). Our results indicate that the nanoscale structure of some proteins in these seemingly normal HCs was already disrupted and that the level of disruption of spectrin rings correlates with the degree of hearing disability in both aging and PTS mice. Further, we used a finite element model of the organ of Corti to test whether the spectrin rings play a role in the cochlear amplifier (30). Micromechanics modeling showed that the loss of spectrin rings had a direct depressive effect on the basilar membrane (BM) displacement amplitude (Fig. 5D) and thus played a crucial role in hearing. Without spectrin rings, the modeling result

**Fig. 3. Spectrin is required for the right distribution of taperin in HCs.** (A) Schematic methodology of generating βIII-spectrin HC-specific KO mice. (B) Representative confocal images of βIII-spectrin signals in the HCs in apical, middle, and basal turns from control mice (n = 3 mice) and Atoh1-Sptbn1−/− mice at P30 (n = 3 mice). Yellow numbers indicate the location of HCs. Scale bars, 4 μm. (C) Same as (B), but for αII-spectrin (n = 3 mice). Scale bars, 10 μm (left) and 5 μm (right). (D) Representative confocal and STED images of taperin (magenta) and F-actin (cyan) from control mice (n = 3 mice) and Atoh1-Sptbn1−/− mice (n = 3 mice) at P17. Scale bar, 1 μm.
indicated a severe effect on the displacement amplitude of BM (Fig. 5D). Thus, we hypothesized that mice without spectrin rings display severe hearing loss or even deafness.

**DISCUSSION**

Together, using super-resolution fluorescence microscope, we have identified a previously unknown structure of spectrin in the cuticular plate of HCs: αII- and βII-spectrin form a cylindrical structure corresponding to each stereocilium in the rootlet region. Furthermore, we have provided evidence that spectrin in HCs plays a critical role in hearing development and deafness.

**Spectrin as a mechanical support for rootlets and shear mechanical stress**

Our previous studies support the idea that spectrin tends to form a periodic structure under the plasma membrane in a concentration-dependent manner in either neurons or glial cells (17, 27). Spectrin concentrates in the rootlet region of HCs, which could be self-assembled into higher-order structures like those found in neurons or glial cells. Spectrin likely forms heterotetramers, which, in turn, form cylindrical structures perpendicular to the cellular submembrane cortex, and this organization is in marked contrast to those structures in neurons or red blood cells (17, 18, 27). These intracellular spectrin structures corresponding to each stereocilium may help to maintain its function because of the intrinsic extensibility and flexibility of spectrin. The cylindrical spectrin structure may help rootlets shear mechanical stress during deflection. Previous studies have supported an elastic function for spectrin (14). β-Spectrin is
required for membrane elasticity, which is essential for red blood cells to withstand deformation when passing through arterioles (26). Neurons can be stretched up to 65% of their length without breaking, and spectrin has been proposed to be involved in maintaining membrane elasticity (31). Thus, spectrin forms an isolated and specific nanodomain in the cuticular plate corresponding to each rootlet, helping to maintain stereocilium function.

**Spectrin as an intracellular trafficking protein**

We propose that this cylindrical structure perpendicular to the cell membrane maintains stereocilia by allowing molecules into and out of the stereocilium. Some inherited neuronal diseases are the direct result of mutations in β-spectrin (32) such as an inherited progressive spinocerebellar ataxia (SCA5) (33, 34). Mutations in βIII-spectrin are related to the dislocation of membrane proteins (33); this association suggests that spectrin could act as a trafficking protein for each stereocilium and thus could be involved in developing and maintaining stereocilium identity and function.

**Spectrin is critical to HC polarity and survival**

In early developmental stages, HC polarity is disrupted in the absence of βII-spectrin, and the extent of the disruption becomes largest in later developmental stages such that eventually the HC starts to degenerate around 2 weeks after birth. Our data suggest that βII-spectrin is required for the normal expression pattern of proteins, such as αII-spectrin and FAM65B. In the cuticular plate, it is likely that spectrin and other components form a functional domain, which is required for HC polarity and function. The spectrin rings are formed during the maturation of membrane properties of HCs, likely by dictating the trafficking of molecules into and out of the stereocilium. Further, spectrin rings were associated with hearing ability in different animal models of hearing loss, supporting the idea that spectrin rings may be required for maturation of stereocilia and HCs. These results support a crucial role of spectrin in the cuticular plate for hearing development. Our study with multiple approaches provides a structural basis for understanding the function of spectrin in the auditory system.

**MATERIALS AND METHODS**

**Experimental design**

The objective of this study was to analyze the subdiffraction-limited structure of spectrin in HCs, followed by functional investigations of spectrin in HCs with regard to hearing ability. STED microscopy improves the resolution of imaging system up to nanometer level. Thus, the fine spectrin structure in HCs was revealed using this technology. The phenotype in spectrin-specific knockout mice with hearing disability demonstrated the critical role of spectrin in HC and proper hearing function.

**Animals**

C57BL/6 mice, CD-1 mice, Sprague-Dawley rats, Spbtn1^flx/flx^ (Jax mice, stock no. 020288), and Atoh1-Cre (35) mice of both sexes were used in this study. Spbtn1^flx/flx^ mice were mated with Atoh1Cre mice to spatially eliminate βII-spectrin in inner ear HCs. The day of birth was counted from P0. Genotyping primers for Spbtn1^flx/flx^ and Atoh1-Cre mice were as follows: Spbtn1^flx/flx^: 5′-GTAGCCTCCTTCTCTGGGATG-3′ (forward) and 5′-TAGAGCCCTCCTCCATGGTCT-3′ (reverse); Atoh1WT, 5′-TGACGCGACGCGACCGACTCGTA-3′ (forward) and 5′-GGACAGCTTTGTGCTGG-3′ (reverse); and Atoh1-Cre, 5′-GGCGACGCCCTTACAGCAAC-3′ (forward) and 5′-GGCCAAAATGTCTGCTGATGT-3′ (reverse). Animals were housed under a 12-hour light/dark cycle at a room temperature of 22° ± 1°C with food and water available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committees of ShanghaiTech University and Southeastern University, China.

**Immunohistochemistry**

Mice were sacrificed with an overdose of pentobarbital sodium (100 mg/kg body weight, intraperitoneally), and the temporal bone was rapidly dissected out under the stereoscope in cold phosphate-buffered saline (PBS) (pH 7.2). To obtain whole-mount preparations of the organ of Corti from mice before P7, the cochlear spiral was microdissected from the temporal bone and adhered to a microscope slide cover glass (thickness, 0.17 mm; diameter, 10 mm) coated with Cell-Tak (BD Biosciences). Cochleae were fixed for 1 hour in 4% paraformaldehyde (in PBS, pH 7.2) at room temperature. For cochleae from mice older than P7, the temporal bone was fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 2 hours at room temperature before being cut into pieces after 0.5- to 6-hour treatment of 0.5 mM EDTA (pH 8.0) until the temporal bone became soft. After complete washing with 0.01 M PBS, samples were immersed in blocking solution containing 10% donkey serum, 0.3% Triton X-100, 1% bovine serum albumin, and 0.02% sodium azide (NaN3) in PBS (pH 7.2) for 1 to 2 hours at room temperature. We used a mouse monoclonal antibody that specifically targets the C terminus of βII-spectrin (amino acids 2101 to 2189; Santa Cruz Biotechnology, catalog no. SC-136074) or a mouse monoclonal antibody that specifically targets αII-spectrin (clone D8B17, BioLegend, catalog no. 803201) to label different isoforms of spectrin (11, 12, 17, 27). These cochlear tissues were subsequently incubated with the primary antibody at 4°C overnight. After complete washing with PBS, the samples were incubated with the secondary antibody Alexa Fluor 555–conjugated donkey anti-mouse IgG (H+L) (Thermo Fisher Scientific, catalog no. A-31570) for 1 hour at room temperature. Cochlear samples were mounted with ProLong Gold (Life Technologies) or Vectashield (Vector Labs) mounting medium. To label F-actin in whole-mount rodent cochlear preparations, phalloidin conjugated with fluorescent dyes (Alexa Fluor 488, Thermo Fisher Scientific, catalog no. A12379; ATTO 488, ATTO-TEC, catalog no. AD488-82) was used. After the staining procedures, all samples were carefully examined under a conventional confocal microscope (Zeiss LSM700), and only those samples with high specificity and preferable signal-to-noise ratio were selected for further super-resolution imaging.

**STED imaging**

STED images were obtained with a Leica TCS SP8 STED 3X microscope equipped with a white light pulse laser (WLL2), STED laser (592 nm), an oil immersion 100x/numerical aperture 1.4 objective lens (HC PL APO CS2, Leica), and a TCS SP8 time-gated system. The STED depletion laser was co-aligned with the excitation laser and used to selectively deactivate the excited fluorophores surrounding the focal point, which allows an increased resolution of 30 to 40 nm by shrinking the point-spread function of the microscope. Images (1024 × 1024 pixels) were acquired in both confocal and STED modes. Acquisition settings such as laser power, image size, pixel dwell times, line average, frame accumulation, and time-gating...
interval (1- to 6-ns post-pulse time window) were optimized to achieve the best imaging quality. Deconvolution of STED images was performed using Huygens software (Scientific Volume Imaging) with the Huygens classical maximum likelihood estimation deconvolution algorithm.

**Image processing and analysis**

All images were exported from LAS X (Leica Microsystems) and further processed using Fiji software (National Institutes of Health). The brightness and contrast of all images were linearly adjusted across the entire image. To quantitatively analyze the diameter and distribution pattern of spectrin rings, lines across the structures were drawn and the intensity profiles were measured using Fiji or LAS X, and these were further analyzed using MATLAB (MathWorks Inc.). For diameter measurement, the raw intensity data from one single ring structure (characterized by twin peaks) were normalized, the positions of each peak were found, the corresponding x values were taken as boundaries of the ring structure, and the diameters of the ring structures were calculated. For distribution pattern analysis, a fast Fourier transform (FFT) algorithm was applied to the normalized intensity profiles, and the fundamental frequency was determined. To quantitatively analyze the disruption index of the spectrin rings in the hearing loss models, spectrin ring numbers were counted using Fiji software from each HC in the apical/middle/basal regions and normalized by the highest ring numbers from single HC at the corresponding region and developmental stage. The ring disruption index ranged from 0 to 1 (0 representing severe disruption and 1 representing no disruption). All the intensity, diameter, FFT, and ring disruption index data were plotted using GraphPad Prism (GraphPad Software Inc.), and all the figure layouts were prepared in Illustrator (Adobe Systems Inc.).

**ABR audiometry**

ABR is the method to assess hearing by measuring the hearing threshold. In this test, on the basis of Tucker–Davis Technology System III [Tucker–Davis Technologies (TDT), Gainesville, FL, USA], ABR was performed in a soundproof room and changes in the electrical activity of the brain in response to sound were recorded via electrodes that were placed on the scalp of the mouse (36). In detail, mice were placed on a 37°C heating pad in the sound-attenuated space to maintain their homeostasis after being anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg). ABRs were recorded by using three needle electrodes placed over the vertex and bilaterally behind the pinnae. An MF1 magnetic speaker, a high-fidelity speaker for free-field presentations (4, 8, 16, 24, and 32 kHz). The acquired ABR response signal was amplified, averaged, and presented in a computer-based data acquisition system (BioSigRZ software, TDT, Gainesville, FL, USA). The sound pressure level for each frequency was performed between 15 and 90 dB (decibels SPL) in 5- or 10-dB steps. At each sound level, 1024 responses were averaged to get a reliable result. The ABR threshold was defined as the lowest SPL that could elicit a detectable response. All ABR tests were performed on mice older than P21.

**Hearing damage model**

For the noise-induced hearing damage model, awake adult mice in a wire mesh box were exposed to white noise (2 to 20 kHz) of 110 dB for 2 hours in a soundproof space. The white noise was generated and amplified in a TDT system and produced using four high-fidelity speakers placed above the box (37). After noise exposure, the mice were transferred to the standard animal facility for 1 day of recovery before the ABR test was performed. Immunofluorescence experiments of the cochlear specimens were then performed as described above.

**Transmission electron microscopy**

Cochlear specimens from P30 mice were fixed in 2.5% glutaraldehyde (in PBS, pH 7.2) at 4°C overnight. After dehydration in a graded series of ethanol, the specimens were embedded in araldite CY 212 (TAAB, Aldermaston, UK). Ultrathin sections were cut using a diamond knife in a direction parallel to the stereocilia and then stained with alcoholic uranyl acetate (Polysciences, Warrington, USA) and alkaline lead citrate (Sigma-Aldrich). After washing gently with distilled water, the sections were randomly examined with a JEOL 1230 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

**Two-dimensional micromechanical model**

A two-dimensional model of the cochlea was modified from a previous study (30). This model predicts the BM motion and the shelling motion between the tectorial membrane and the reticular lamina within an active organ of Corti in response to both acoustic and electrical excitations. The spectrin rings were introduced between the stereocilia bottom and the cuticular plate of the OHCs. The transverse BM motions were compared under the following conditions: normal spectrin rings, reduced spectrin rings, and no spectrin rings. The BM displacement was shown as $|W_{BM}|$ (nm). Normalized frequency was calculated as $F \times CF(x)$, i.e., excitation frequencies were divided by the characteristic frequency at $x_0$ where the model geometry was taken from.

**Quantification and statistical analysis**

Statistical analyses were performed using Excel (Microsoft) and GraphPad Prism 6.0 software. Student’s t test and one-way analysis of variance (ANOVA) were used to determine the statistical significance. $P < 0.05$ was considered significant. All replicate numbers (number of mice, number of spectrin rings, or number of OHCs and IHCs analyzed) are specifically indicated in the figure legends. When possible, all analyses were undertaken blind to genotype and/or development stage.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/4/eaav7803/DC1

Fig. S1. The spectrin structure in the cuticular plate of HCs.

Fig. S2. Localization and irregular distribution of FAM65B in Atoh1−/− mice.

Fig. S3. HC maintenance.

Fig. S5. Spectrin rings are disrupted in OHCs of noise-induced hearing loss mice.

Fig. S4. Normal morphology and function of VHC in 22.

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Acknowledgments: We thank the Shanghai Municipal Government and ShanghaiTech University for financial support, J. Gao (Shandong University) for providing Atoh1-Cre mice, and the Bioimaging Core Facilities of the iHuman Institute and the animal facility of National Center for Protein Science Shanghai for their support. We also thank K. Zhang, J. Wang, Y. Li, F. Hao, and H. Liu for helpful discussions. Funding: This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA16010303), the National Key Research and Development Program of China (2016YFC0905900 to G.Z. and 2017YFC0103100 to G.Z. and S.H.), the National Natural Science Foundation of China (31771310 to G.Z.), the National Key Research and Development Program of China (2015CB965000 and 2017YFA0103900 to R.C.), and the 2015 Young Scholars Talent Plans of China (to G.Z. and R.C.), the National Natural Science Foundation of China (31200743 to Y.L, 31671063 to S.H., and 81622013, 81470692, and 31500852 to R.C.), Boehringer Ingelheim Pharma GmbH, and the Hoo Yingdong Education Foundation. Author contributions: G.Z., R.C., S.H., and M.T. conceived and designed the experiments. Y.L., J.Q., and X.C. performed most of the experiments and data analyses. Y.L., J.Q., C.C., H.L., and C.T. acquired the super-resolution fluorescence image data. J.Q. and W.Z. acquired the ABRA data. J.Q. acquired the electron microscopy data. G.Z., Y.L., J.Q., C.C., H.L., X.C., G.N., Y.Z., and C.T. contributed to data analysis, G.Y., M.T., and C.Z. discussed data analysis, interpretation, and presentation. G.Z., Y.L., and J.Q. wrote the manuscript with contributions from all authors. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 18 October 2018
Accepted 1 March 2019
Published 17 April 2019
10.1126/sciadv.aav7803
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DOI: 10.1126/sciadv.aav7803