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Lack of PDZD7 long isoform disrupts ankle-link complex and causes hearing loss in mice

Haibo Du¹, Linzhi Zou², Rui Ren¹, Nana Li¹, Jie Li², Yanfei Wang¹, Jinpeng Sun³, Jun Yang⁴, Wei Xiong², Zhigang Xu^{1,5}

¹Shandong Provincial Key Laboratory of Animal Cell and Developmental Biology, School of Life Sciences, Shandong University, Qingdao, China

²School of Life Sciences, IDG/McGovern Institute for Brain Research at Tsinghua, Tsinghua University, Beijing, China

³Key Laboratory Experimental Teratology of the Ministry of Education, Department of Biochemistry and Molecular Biology, Shandong University School of Medicine, Jinan, China

⁴Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah, Salt Lake City, Utah

⁵Shandong Provincial Collaborative Innovation Center of Cell Biology, Shandong Normal University, Jinan, China

Abstract

Usher syndrome (USH) is the most frequent form of combined hereditary deafness-blindness, characterized by hearing loss and retinitis pigmentosa, with or without vestibular dysfunction. PDZD7 is a PDZ domain-containing scaffold protein that was suggested to be a USH modifier and a contributor to digenic USH. In the inner ear hair cells, PDZD7 localizes at the ankle region of the stereocilia and constitutes the so-called ankle-link complex together with three other USH proteins Usherin, WHRN, and ADGRV1. *PDZD7* gene is subjected to alternative splicing, which gives rise to two types of PDZD7 isoforms, namely the long and short isoforms. At present, little is known which specific isoform is involved in ankle-link formation and stereocilia development. In this work, we showed that PDZD7 long isoform, but not short isoforms, localizes at the ankle region of the stereocilia. Moreover, we established *Pdzd7* mutant mice by introducing deletions into exon 14 of the *Pdzd7* gene, which causes potential premature translational stop in the long isoform but leaves short isoforms unaffected. We found that lack of PDZD7 long isoform affects the localization of other ankle-link complex components in the stereocilia. Consequently,

Correspondence: Wei Xiong, School of Life Sciences, IDG/McGovern Institute for Brain Research at Tsinghua, Tsinghua University, Beijing 100084, China, wei_xiong@tsinghua.edu.cn, Zhigang Xu, Shandong Provincial Key Laboratory of Animal Cell and Developmental Biology, School of Life Sciences, Shandong University, Qingdao, Shandong 266237, China. xuzg@sdu.edu.cn. Haibo Du and Linzhi Zou contributed equally.

AUTHOR CONTRIBUTIONS

Z. Xu performed conceptualization; H. Du, L. Zou, R. Ren, N. Li, J. Li, and Y. Wang conducted investigation; J. Sun, W. Xiong, and Z. Xu analyzed the data; J. Yang managed resources; Z. Xu, W. Xiong, and J. Yang wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Pdzd7 mutant mice showed stereocilia development deficits and hearing loss as well as reduced mechanotransduction (MET) currents, suggesting that PDZD7 long isoform is indispensable for hair cells. Furthermore, by performing yeast two-hybrid screening, we identified a PDZD7 long isoform-specific binding partner PIP5K1C, which has been shown to play important roles in hearing and might participate in the function and/or transportation of PDZD7.

Keywords

ankle links; hair cells; inner ear; PDZD7 long isoform; stereocilia

1 | INTRODUCTION

Usher syndrome (USH) is the most frequent form of inherited sensory deaf-blindness, characterized by hearing loss and vision defect with or without balancing deficit.¹⁻³ Clinically, USH is classified into three types, namely USH1, USH2, and USH3. As the most prevalent type, USH2 accounts for up to 60% of all USH cases.⁴ USH2 patients manifest congenital severe hearing impairment, retinitis pigmentosa, and little balance problem. Three genes have been identified so far as USH2 causative genes, namely *USH2A*, *ADGRV1*, and *WHRN*, which encodes an adhesion protein Usherin, a large G protein-coupled receptor ADGRV1 (also named as VLGR1), and a PDZ domain-containing scaffold protein WHRN (whirlin), respectively.⁴⁻⁶ Evidences suggest that the USH2 proteins localize at the ankle region of hair cell stereocilia, where they bind to each other and form the so-called ankle-link complex.⁷⁻¹⁰

PDZD7 is a paralog of whirlin and another Usher protein harmonin (USH1C), sharing 55% and 35% similarity with whirlin and harmonin, respectively. PDZD7 contains three PDZ domains, a harmonin-N like (HNL) domain as well as a proline-rich (PR) region. *PDZD7* gene mutations are associated with nonsyndromic hearing loss.¹¹⁻¹³ Furthermore, PDZD7 is suggested to be a USH modifier and a contributor to digenic USH.¹⁴ Consistently, PDZD7 interacts with all the three known USH2 proteins and forms a quaternary protein complex at the ankle region of the stereocilia, suggesting that PDZD7 is part of the ankle-link complex.¹⁴⁻¹⁷ PDZD7 also interacts with USH1 proteins harmonin, SANS (USH1G), and MYO7A (USH1B) in vitro.^{11,18,19} PDZD7 disruption in mice causes stereocilia disorganization and MET deficits, leading to congenital hearing loss.¹⁶ Noticeably, localization of all the three known USH2 proteins at the ankle region of the stereocilia is affected by PDZD7 disruption, suggesting that PDZD7 plays a pivotal role in organizing the ankle-link complex in the developing cochlear hair cells.¹⁶

Several alternative splicing variants of *PDZD7* have been detected, which encodes either a full-length long isoform or short isoforms mainly containing the first two PDZ domains.^{11,14,16} In the *Pdzd7* knockout mice mentioned above, exons 2-5 of *Pdzd7* gene are deleted, resulting in the disruption of all the PDZD7 isoforms.¹⁶ In the present work, we introduced deletions into exon 14 of mouse *Pdzd7* gene, which causes a premature translational stop before the third PDZ domain. This deletion would disrupt the expression of PDZD7 long isoform, while leaving the short isoforms unaffected. We show here that lack

of PDZD7 long isoform affects ankle-link complex formation and MET currents, and causes progressive, severe to profound hearing loss in mice.

2 | MATERIALS AND METHODS

2.1 | Mice

All animal experiments were approved by the Animal Ethics Committee of Shandong University School of Life Sciences (Permit Number: SYDWLL-2017-05) and performed accordingly. Mice of mixed genders were used in the present work. *Pdzd7*^{PDZ3/ PDZ3} mice were generated on a C57BL/6J background using CRISPR/Cas9 technology by Shanghai Biomodel Organism Science & Technology Development Co., Ltd. To generate *Pdzd7*^{PDZ3/ PDZ3} mice, genomic DNA sequences 5'-ACAGGAGGTGGCTGGGGAGG-3' and 5'-GAGGAGGTGCGCATGCGCCT-3' were chosen as the sgRNA targets. *Cas9* mRNA was synthesized by in vitro transcription using mMACHINE T7 Ultra Transcription Kit (Thermo Fisher, Cat. No. AM1345) according to the manufacturer's instructions. Guide RNAs were synthesized by in vitro transcription using MEGAshortscript T7 Transcription Kit (Thermo Fisher, Cat. No. AM1354) according to the manufacturer's instructions. The RNA mixture was then microinjected into one-cell embryos, and genomic DNA was collected from the offspring and screened using PCR and Sanger sequencing to determine mutations. The founder mice were then back-crossed with C57BL/6J mice. The following primers were used for genotyping: 5'-AGGATGAAGACGGAGAGATAA-3' and 5'-AGCTTTGGGCTGGGGTCTGGC-3'. The following primers were used for RT-PCR: 5'-ACCTGCTACCAGTGAACAGC-3' and 5'-CCAGGAGACTTGCCTTGACC-3'.

2.2 | ABR measurement

A RZ6 workstation and BioSig software were used for stimulus generation, presentation, ABR acquisition, and data management. Mice were placed on an isothermal pad to keep the body temperature at 37°C during the experiment. The mice were anesthetized by intraperitoneally injecting 5% chloral hydrate for 0.5 mL/100 g body weight, then electrodes were inserted subcutaneously at the vertex and pinna as well as near the tail. Acoustic stimuli (clicks or pure-tone bursts) of decreasing sound level from 90 dB SPL in 10 dB SPL steps were generated using high-frequency transducers. At each sound level, 512 responses were sampled and averaged. Hearing threshold of each mouse was determined as the lowest sound level at which all ABR waves were detectable.

2.3 | Immunostaining

All steps were performed at room temperature unless otherwise indicated. Dissected organ of Corti explants or cultured cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes, followed by permeabilization and blocking with PBT1 (0.1% Triton X-100, 1% BSA, and 5% heat-inactivated goat serum in PBS, pH 7.3) for 1 hour. For USH2A and WHRN staining, the samples were additionally incubated in 50 mM NH₄Cl for 15 minutes then in Tween tris buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 10 minutes before permeabilization. Samples were then incubated with primary antibody in PBT1 overnight at 4°C, followed by incubation with secondary antibody in PBT2 (0.1% Triton X-100 and 0.1% BSA in PBS) for 1 hour. After that, samples were incubated with

phalloidin in PBS for 30 minutes, then mounted in PBS/glycerol (1:1) and imaged with a confocal microscope (LSM 700, Zeiss, Germany). For nuclei staining, samples were incubated with DAPI in PBS for 15 minutes before mounting.

Primary antibodies were as follows: anti-PDZD7_N, anti-PDZD7_C, anti-ADGRV1, anti-WHRN, and anti-USH2A were described previously¹⁶; anti-PIP5K1C (Cell Signaling Technology, Cat. No. 3296, RID:AB_2164719); anti-HA (Cell Signaling Technology, Cat. No. 2367, RRID:AB_10691311). Secondary antibodies and additional reagents were as follows: Alexa Fluor 546-donkey anti-mouse IgG (Thermo Fisher Scientific, Cat. No. A10036, RRID:AB_2534012); Alexa Fluor 488-donkey anti-rabbit IgG (Thermo Fisher Scientific, Cat. No. A21202, RRID:AB_141607); TRITC-conjugated phalloidin (Sigma-Aldrich, Cat. No. P1951; RRID:AB_2315148); DAPI (Gen-View Scientific Inc, Cat. No. 28718-90-3).

2.4 | Scanning electron microscopy

Dissected mouse temporal bones were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C. Cochleae were dissected out of the temporal bone and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 2 hours. After dehydration in ethanol, samples were critically point dried using a Leica EM CPD300 (Leica, Germany). After that, samples were mounted and sputter coated with platinum (15 nm) using a Cressington 108 sputter coater (Cressington, United Kingdom), and imaged with a Quanta250 field-emission scanning electron microscope (FEI, The Netherlands).

2.5 | DNA constructions, immunoprecipitations, and western blots

The coding sequences of *Pdzd7* long isoform (GenBank [XM_006526538.1](#)), *Pdzd7* short isoform (GenBank [KF041446.1](#)), and *Pip5k1c* (GenBank [NM_001146687.2](#)) were amplified from mouse inner ear cDNA and cloned into pEGFP-C2 or pmCherry-N1 vector, or modified pEGFP-C2 vector in which the EGFP coding sequence was replaced by HA-tag or Myc-tag coding sequence. To express Myc-tagged PDZD7 long isoform, the coding sequence of *Pdzd7* was inserted into pcDNA3.1 and Myc-tag coding sequence was added to its 3'-end.

Cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Cat. No. 12100046) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Cat. No. 10438026) and 1% penicillin/streptomycin. All cells were grown at 37°C in a 5% CO₂ humidified atmosphere. After transfection with the expression vectors, the cultured cells were lysed in ice-cold lysis buffer consisting of 150 mM NaCl, 50 mM Tris at pH 7.5, 1% (vol/vol) Triton X-100, 1 mM PMSF, and 1 × protease inhibitor cocktail. The supernatant was then collected after centrifugation and incubated with immobilized anti-Myc antibody (Sigma-Aldrich, Cat. No. E6654, RRID:AB_10093201) at 4°C for 2 hours.

Immunoprecipitated proteins were separated by polyacrylamide gel electrophoresis (PAGE), and then transferred to PVDF membrane. After blocking in PBS containing 5% BSA and 0.1% Tween-20, the membrane was incubated with primary antibody at 4°C overnight, followed by incubation with HRP conjugate-goat anti-mouse antibody (Bio-Rad, Cat. No.

170-6516, RRID:AB_11125547) at room temperature for an hour. The signals were detected with the ECL system (Thermo Fisher Scientific). Primary antibodies were as follows: anti-Myc (Sigma-Aldrich, Cat. No. M4439, RRID:AB_439694); anti-HA (Cell Signaling Technology, Cat. No. 2367, RRID:AB_10691311); anti-GFP (Abmart, Cat. No. M20004, RRID:AB_2619674).

2.6 | Injectoporation

Cochlear culture and injectoporation were performed as previously described.²² Briefly, the organ of Corti was isolated from P3 mice and cut into three pieces in DMEM/F12 with 1.5 µg/mL ampicillin. For electroporation, a glass pipette (2 µm tip diameter) was used to deliver plasmids (0.2 µg/µL in 1 × Hanks' balanced salt solution) to hair cells in the sensory epithelium. EGFP was used as an indicator for the selection of transfected hair cells. A series of three pulses at 60 V lasting 15 ms at 1 second intervals were applied to cochlear tissues by an electroporator (ECM Gemini X2, BTX, CA). The cochlear tissues were cultured for 1 day in vitro and then used for immunostaining.

2.7 | Hair cell electrophysiology

Hair cells were recorded using whole-cell patch-clamp as previously described.²¹ The basilar membrane of neonatal mice was acutely dissected in the dissection solution containing (in mM) 141.7 NaCl, 5.36 KCl, 0.1 CaCl₂, 1 MgCl₂, 0.5 MgSO₄, 3.4 L-glutamine, 10 glucose, and 10 H-HEPES (pH 7.4). Then, the basilar membrane was transferred into a recording chamber with recording solution containing (in mM) 144 NaCl, 0.7 NaH₂PO₄, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 5.6 glucose, and 10 H-HEPES (pH 7.4). Patch pi-pettes were made from borosilicate glass capillaries (BF150-117-10, Sutter Instrument Co., Novato, CA) with a pipette puller (P-2000, Sutter) and polished on a microforge (MF-830, Narishige, Tokyo, Japan) to resistances of 4–6 MΩ. Intracellular solution contained (in mM) 140 CsCl, 1 MgCl₂, 0.1 EGTA, 2 Mg-ATP, 0.3 Na-GTP, and 10 H-HEPES, pH 7.2. A 40-Hz sinusoidal wave stimulus was delivered by a 27-mm-diameter piezoelectric disc driven by a home-made piezo amplifier pipette with a tip diameter of 3–5 µm positioned 5–10 µm from the hair bundle to evoke maximum MET currents. The evoked MET currents were recorded with a patch-clamp amplifier (EPC 10 USB and Patchmaster software, HEKA Elektronik, Lambrecht/Pfalz, Germany).

2.8 | FM 1-43FX uptake

FM 1-43FX was used to label functional hair cells as described.²⁰ Briefly, mouse basilar membrane was dissected and incubated with 3 µM FM 1-43FX (Thermo Fisher, Cat. No. F35355) in PBS for 30 seconds, then fixed with 4% PFA at room temperature for 20 minutes. The samples were mounted in PBS-glycerol (1:1) and imaged with a confocal microscope (LSM 700, Zeiss, Germany).

2.9 | Statistical analysis

The numbers of independent animals are indicated in the figure legends. Data were shown as means ± standard error of mean (SEM). Student's two-tailed unpaired *t* test was used to determine statistical significance, and *P* < .05 was considered statistically significant.

3 | RESULTS

3.1 | PDZD7 long isoform, but not short isoform, localizes in the stereocilia of cochlear hair cells

To investigate the role of different PDZD7 isoforms in cochlear hair cells, we first established *Pdzd7* mutant mice that only express PDZD7 short isoforms but not long isoform. The mouse *Pdzd7* gene contains 16 exons, among which exons 1 to 13 are shared by the long and short isoforms, whereas exons 14 to 16 are unique to the long isoform (Figure 1A). We introduced two deletions (13 and 15 base pairs, respectively) into exon 14 using the CRISPR/Cas9 genome editing technique, which causes potential premature translational stop only in the long isoform but leaves short isoforms unaffected (Figure 1A and S1C). The deletions in *Pdzd7* exon 14 were confirmed by genotyping PCR, RT-PCR, and Sanger sequencing (Figure S1A–C). Given that the PDZ3 domain is the most significant difference between PDZD7 long and short isoforms, we designate the resultant mutant mice as *Pdzd7*^{PDZ3/ PDZ3} in the following text.

Expression of different PDZD7 isoforms was examined by performing whole-mount immunostaining using two PDZD7 antibodies. PDZD7_C antibody recognizes a C-terminal fragment unique to PDZD7 long isoform (Figure 1A). PDZD7_N antibody recognizes an N-terminal fragment of PDZD7, which is common for both the long and short isoforms (Figure 1A). PDZD7_C antibody detected immunoreactivity signals at the ankle region of cochlear hair cell stereocilia in the heterozygous control mice, but not in the homozygous mutant mice (Figure 1B), confirming that PDZD7 long isoform localizes in the stereocilia ankle region and that this localization is disrupted in the mutant mice. PDZD7_N antibody also detected immunoreactivity signals at the ankle region of cochlear hair cell stereocilia in the control mice, but not in the homozygous mutant mice (Figure 1C), suggesting that neither long nor short PDZD7 isoform is present in the stereocilia of *Pdzd7*^{PDZ3/ PDZ3} cochlear hair cells. Considering that only the long isoform is affected in *Pdzd7*^{PDZ3/ PDZ3} mice, this result suggested that unlike PDZD7 long isoform, PDZD7 short isoforms do not localize in the stereocilia.

To further confirm the localization of different PDZD7 isoforms in the stereocilia, we expressed HA-tagged PDZD7 in the hair cells of cultured mouse auditory sensory epithelia using injectoporation technique.^{21,22} Consistent with the immunostaining results, HA-tagged PDZD7 long isoform localized in the stereocilia in most transfected cells (94% of 378 cells, Figure 1D and D'). In contrast, HA-tagged PDZD7 short isoform was only weakly detected in the stereocilia in half of the transfected cells (51% of 247 cells), and completely localized in the cytoplasm in the other transfected cells (Figure 1E and E'). Taken together, our data suggested that PDZD7 long isoform, but not short isoforms, localizes in the hair cell stereocilia.

3.2 | Lack of PDZD7 long isoform causes progressive, severe to profound hearing loss in mice

The auditory function of *Pdzd7* mutant mice was examined by performing the auditory brainstem response (ABR) measurement, which detects the sound-evoked

electrophysiological potentials in the ascending auditory pathway. The results revealed that compared to wild-type or *Pdzd7*^{+/+} *PDZ3* mice, *Pdzd7*^{PDZ3/} *PDZ3* mice showed significant hearing threshold elevation both to click and pure-tone stimuli. At age of 2 weeks, *Pdzd7*^{PDZ3/} *PDZ3* mice had a nearly 30-dB threshold shift (Figure 2A). The threshold shift was around 40 dB at age of 4 weeks, and reached around 50 dB at age of 6 weeks (Figure 2B,C). Taken together, our present data showed that the threshold shift in *Pdzd7*^{PDZ3/} *PDZ3* mice increases when the mice get older, suggesting that lack of PDZD7 long isoform results in progressive, severe to profound hearing loss in mice (Figure 2D).

3.3 | Lack of PDZD7 long isoform affects the subcellular localization of other ankle-link complex components

Loss of PDZD7 immunoreactivity in the stereocilia of *Pdzd7*^{PDZ3/} *PDZ3* cochlear hair cells promoted us to examine the expression of the other ankle-link complex components in the stereocilia of these cells. Immunostaining showed that ADGRV1, WHRN, and USH2A all localized at the ankle region of the stereocilia in the control inner hair cells (IHCs) and outer hair cells (OHCs), and WHRN also localized at the tips of the stereocilia (Figure 3A, C and E). However, in both IHCs and OHCs of *Pdzd7*^{PDZ3/} *PDZ3* mice, ADGRV1 was distributed diffusely in the stereocilia (Figure 3A), whereas WHRN was mainly detected at the stereocilia tips (Figure 3C). Meanwhile, USH2A immunoreactivity was significantly decreased in the stereocilia of *Pdzd7*^{PDZ3/} *PDZ3* OHCs and IHCs (Figure 3E). Taken together, our data showed that localization of the other three ankle-link complex components at the stereocilia ankle region was significantly reduced in *Pdzd7*^{PDZ3/} *PDZ3* cochlear hair cells (Figure 3B, D, and F), suggesting that PDZD7 long isoform is required for the formation and/or maintenance of ankle-link complex.

3.4 | Lack of PDZD7 long isoform affects stereocilia development

It has been shown that the ankle links play important roles in stereocilia development.^{10,23} Stereocilia development in *Pdzd7*^{PDZ3/} *PDZ3* mice was first examined by performing phalloidin staining. The results showed that stereocilia disorganization could be detected at postnatal day 9 (P9) *Pdzd7*^{PDZ3/} *PDZ3* OHCs, whereas IHCs were largely unaffected (Figure S2A,B). In most *Pdzd7*^{PDZ3/} *PDZ3* OHCs, hair bundle lost its usual V-shaped symmetry. This situation was exacerbated when the mice got older, and eventually some OHCs completely lost their stereocilia when examined at age of P45 (Figure S2C–H).

Stereocilia development was examined furthermore by performing scanning electron microscopy (SEM). At P0, the stereocilia of both IHCs and OHCs in *Pdzd7*^{PDZ3/} *PDZ3* mice were largely normal compared with the control *Pdzd7*^{+/+} *PDZ3* mice (data not shown). *Pdzd7*^{PDZ3/} *PDZ3* OHCs started to show asymmetric hair bundles at P4, which was more obvious at P8, whereas stereocilia in IHCs still remained normal (Figure 4A–D’). At P8, around 9% of *Pdzd7*^{+/+} *PDZ3* OHCs showed asymmetric hair bundles, whereas 78% of *Pdzd7*^{PDZ3/} *PDZ3* OHCs had asymmetric hair bundles. By age of P42, the stereocilia in *Pdzd7*^{PDZ3/} *PDZ3* OHCs became more disorganized, and some OHCs completely lost their stereocilia (Figure 4E–F’). Even at this stage, IHCs in *Pdzd7*^{PDZ3/} *PDZ3* mice still looked normal (Figure 4F’). Taken together, both phalloidin staining and SEM results suggested that PDZD7 long isoform is important for OHC stereocilia development.

Stereocilia disorganization could lead to hair cell loss. DAPI staining was then performed to examine hair cell loss at different developmental stages. The results revealed robust OHC loss in 1-month-old *Pdzd7*^{PDZ3/ PDZ3} mice compared to *Pdzd7*^{+/+} *PDZ3* mice, especially at the basal turn (Figure 5A–C). By age of 3 months, OHC loss at the basal turn in *Pdzd7*^{PDZ3/ PDZ3} mice reached around 50% (Figure 5D–F). Eventually, when examined at 7 months, OHC loss at the middle turn in *Pdzd7*^{PDZ3/ PDZ3} mice also reached around 60% (Figure 5G–I). Meanwhile, IHC numbers remained unchanged through development in *Pdzd7*^{PDZ3/ PDZ3} mice (Figure 5 and data not shown). Taken together, our data suggested that lack of PDZD7 long isoform results in robust OHC loss in mice.

3.5 | Lack of PDZD7 long isoform affects MET currents

Saturating MET currents of cochlear hair cells were recorded by applying fluid jet stimulation that generally deflects hair bundle in a physiological way. In P6 control OHCs, a large MET current around 800 pA was recorded (Figure 6A). In contrast, the MET current in *Pdzd7*^{PDZ3/ PDZ3} OHCs was reduced to about half of that in the control OHCs (Figure 6A). Similar results were also observed in P6 *Pdzd7*^{PDZ3/ PDZ3} IHCs (Figure 6B). MET function of cochlear hair cells at older ages were examined by performing FM1-43FX uptake experiment. When applied briefly, fluorescent dye FM1-43 or its analog FM1-43FX enters hair cells through the MET channels, hence provides an indicator of the functional integrity of hair cells.^{24,25} Our data showed that compared to the control OHCs and IHCs, FM1-43FX uptake was significantly reduced in *Pdzd7*^{PDZ3/ PDZ3} hair cells when examined at P9 and P14 (Figure 6C). FM1-43FX uptake was barely detectable in *Pdzd7*^{PDZ3/ PDZ3} cochlear hair cells at P28, and completely undetected at P42 (Figure 6C). Taken together, our data suggested that lack of PDZD7 long isoform significantly affects MET function in both OHCs and IHCs.

3.6 | PIP5K1C binds PDZD7 long isoform and localizes in the stereocilia of cochlear hair cells

To further investigate the mechanism how PDZD7 long isoform regulates hair cell function, we performed yeast two-hybrid screen to identify proteins that specifically bind PDZD7 long isoform but not short isoforms. The C-terminal part of PDZD7 long isoform was used as bait to screen an inner ear cDNA library. Among the candidate PDZD7 long isoform-binding partners identified from the screen, phosphatidylinositol-4-phosphate 5-kinase type 1 gamma (PIP5K1C) caught our attention. PIP5K1C belongs to type I PI(4)P 5-kinase (PIPKI) family, which catalyzes the generation of PIP₂ by phosphorylation of PI(4)P at the D-5 position of the inositol ring.²⁶ PIP5K1C was suggested to be primarily responsible for the synthesis of PIP₂ in the cochlea, and *Pip5k1c* heterozygous mice suffered from high-frequency hearing loss.²⁷

We first confirmed the interaction between PDZD7 and PIP5K1C by performing co-immunoprecipitation (co-IP) experiments. Expression vectors were transfected into HEK293T cells, and cell lysates were subjected to IP with immobilized anti-Myc agarose. The results showed that EGFP-tagged PDZD7 C-terminal fragment could be co-IPed with Myc-tagged PIP5K1C, and HA-tagged PIP5K1C could be co-IPed with Myc-tagged PDZD7 C-terminal fragment (Figure 7A,B). Additionally, our data confirmed that only PDZD7

long isoform, but not short isoform, could be co-IPed with PIP5K1C, suggesting that the interaction with PIP5K1C is PDZD7 long isoform-specific (Figure 7B). The interaction was also supported by the subcellular localization of these two proteins. When over-expressed in HeLa cells, PDZD7-mCherry mainly localized in the cytoplasm. On the other hand, Myc-PIP5K1C were almost exclusively associated with the plasma membrane (Figure 7C). When expressed together, PDZD7-mCherry colocalized with Myc-PIP5K1C on the plasma membrane, supporting the interaction between these two proteins (Figure 7C).

Expression of PIP5K1C in the mouse inner ear was examined by performing western blot using a specific PIP5K1C antibody. The results showed that PIP5K1C was abundantly expressed in the brain, and weakly expressed in other tissues including the inner ear (Figure S3). This antibody failed in whole-mount immunostaining experiment; hence, we performed injectoporation experiment to examine the localization of PIP5K1C in cochlear hair cells. The results showed that HA-tagged PIP5K1C localized in the stereocilia in injectopored hair cells in a PDZD7-independent way, suggesting that PIP5K1C is a novel stereociliary protein and its localization in the stereocilia does not require PDZD7 (Figure 7D).

4 | DISCUSSION

PDZD7 gene is subjected to alternative splicing, which gives rise to several *PDZD7* isoforms. *PDZD7* long isoform contains three PDZ domains, an HNL domain and a proline-rich domain. *PDZD7* short isoforms mainly consist of the first two PDZ domains.^{11,14,16} Different *PDZD7* isoforms might play different roles in the inner ear, just as another ankle-link complex component *WHRN* does. As a paralog of *PDZD7*, *WHRN* also has long and short isoforms. *WHRN* short isoform localizes at the tips of the stereocilia, whereas the long isoform localizes at the ankle region of the stereocilia.^{28–30} *WHRN* short isoform interacts with *MYO15A* and *EPS8*, and plays pivotal roles in stereocilia length regulation.^{28,31,32} On the other hand, *WHRN* long isoform interacts with *ADGRV1*, *Usherin*, and *PDZD7* and is an important component of the ankle-link complex.^{33,34}

In the present work, we showed that *PDZD7* long isoform localizes at the ankle region of the stereocilia in cochlear hair cells. Furthermore, lack of *PDZD7* long isoform affects the organization of ankle-link complex as well as stereocilia development, and eventually results in progressive, severe to profound hearing loss. In contrast, we could not detect *PDZD7* short isoforms in the stereocilia through immunostaining. Our *PDZD7* antibodies do not behave well in western blot, precluding further examination of *PDZD7* short isoform at the protein level. In fact, native *PDZD7* isoforms have not been detected at the protein level by any report. Nevertheless, we showed here that injectopored *PDZD7* short isoform is not targeted to the stereocilia. Taken together, our present data suggested that *PDZD7* long isoform localizes at the ankle region of the stereocilia and is indispensable for stereocilia development and hair cell function, whereas *PDZD7* short isoform, if exists at all, is not localized in the stereocilia and perhaps not required for stereocilia development and/or function.

Among the three PDZ domains of *PDZD7*, the first two PDZ domains mediate interaction with the other ankle-link complex components *ADGRV1*, *WHRN*, and *USH2A*, whereas

the third PDZ domain is only involved in the interaction between PDZD7 and WHRN.¹⁷ In the previous study, we identified several proteins that bind PDZD7 short isoform.³⁵ We showed here that PDZD7 short isoform does not localize in the stereocilia, suggesting that the long isoform-specific C-terminal fragment (including PDZ3 and proline-rich domain) might be important for transporting PDZD7 to the stereocilia. Identification of proteins that specifically interact with the C-terminal fragment of PDZD7 will shed light on the mechanism how this process is regulated. Our present data suggested that PIP5K1C is a promising candidate for transporting PDZD7 to the plasma membrane. PIP5K1C binds PDZD7 long isoform, but not short isoform. In transfected cells, plasma membrane localization of PDZD7 long isoform was greatly increased in the presence of PIP5K1C, consistent with the hypothesis that it might participate in the transportation of PDZD7.

Detailed expression pattern of PIP5K1C in the inner ear has not been reported. Nevertheless, *Pip5k1c* mRNA was detected in cochlear hair cells as well as supporting cells by RNA transcriptome sequencing.³⁶ We found that PIP5K1C protein was expressed in the inner ear by western blot analysis using a specific anti-PIP5K1C antibody. The antibody failed in whole-mount immunostaining experiment, precluding further examination of PIP5K1C expression in the hair cells. Nevertheless, by performing injectoporation, we showed that HA-tagged PIP5K1C protein could localize to the stereocilia, consistent with a potential role of PIP5K1C in stereocilia structure and/or function. *Pip5k1c* homozygous knockout mice die embryonically or postnatally, which hinders the investigation of its physiological function.^{37,38} However, *Pip5k1c* heterozygous mice were viable and showed high-frequency hearing loss, suggesting that PIP5K1C plays a pivotal role in hearing.²⁷

Besides regulating the transportation and/or function of PDZD7, PIP5K1C might affect hearing transduction through regulating PIP₂ level in the stereocilia. PIP₂ is localized along the stereocilia but absent from the taper region.^{39–41} PIP₂ was shown to be an important regulator of the plasma membrane Ca²⁺-ATPase isoform 2 (PMCA2), a calcium pump that is highly concentrated in hair cell stereocilia.^{42–44} Consistently, it has been shown that PIP₂ affects transduction and adaptation in the hair cells.^{39,41} Together with phosphatidylinositol phosphatases such as protein tyrosine phosphatase receptor type Q (PTPRQ),³⁹ PIP5K1C might regulate the level of PIP₂ in the stereocilia, hence play important roles in hearing transduction. In the present work, we could not determine the precise localization of PIP5K1C in the stereocilia. It will be very interesting if PIP5K1C is localized in the ankle links together with PDZD7, since the localization of ankle links is immediately adjacent to the PIP₂-free zone in the stereocilia tapers.⁴⁰ Further investigation is required to explore the potential role of PIP5K1C as well as PDZD7 in controlling PIP₂ level in more details.

Ankle links are very important for stereocilia development. Interestingly, despite that PDZD7 long isoform is localized at the ankle-link region in both OHCs and IHCs, lack of PDZD7 long isoform mainly affected stereocilia morphology in OHCs, but not IHCs. Consistently, severe stereocilia disorganization was only observed in OHCs that lack both PDZD7 isoforms.¹⁶ Similar phenotype was also observed in mice lacking other ankle-link complex components.^{28,34,45} The different consequences of ankle-link complex disruption in the stereocilia of OHCs and IHCs might result from inherent differences in stereocilia development or organization between OHCs and IHCs, which needs further investigation.

Although lack of PDZD7 long isoform only affected OHC stereocilia development, it caused decreased MET currents in both IHCs and OHCs. This is in sharp contrast with *Pdzd7* knockout mice that lack both isoforms, in which MET currents in OHCs were significantly reduced, whereas MET currents in IHCs remained unaffected.¹⁶ One possible explanation for this discrepancy lies in the different stimulation methods used in these two works. In the previous work, a rigid piezo-driven stiff glass probe was used to deflect the hair bundle to a predetermined angle independent of its stiffness,¹⁶ hence could not provide insight into potential changes of stereocilia stiffness. In contrast, calibrated fluid-jet stimuli were used in the present work to deflect the hair bundle in a physiological way. Using this method, changes of stereocilia stiffness will cause stereocilia deflection to different extent, which then will result in different maximum MET currents. Our electrophysiology results were further supported by the FM1-43FX dye uptake experiment, which showed that FM1-43FX uptake was affected in both IHCs and OHCs. Taken together, our present data suggest that although lack of PDZD7 long isoform does not cause obvious morphological deficits in IHCs, it does affect MET currents in IHCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ABR	auditory brainstem response
co-IP	co-immunoprecipitation
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HNL	harmonin-N like
IHC	inner hair cell
MET	mechano-electrical transduction
OHC	outer hair cell
PAGE	polyacrylamide gel electrophoresis
PFA	paraformaldehyde
PIP5K1C	phosphatidylinositol-4-phosphate 5-kinase type 1 gamma

PR	proline-rich
PTPRQ	protein tyrosine phosphatase receptor type Q
SEM	scanning electron microscopy
USH	Usher syndrome

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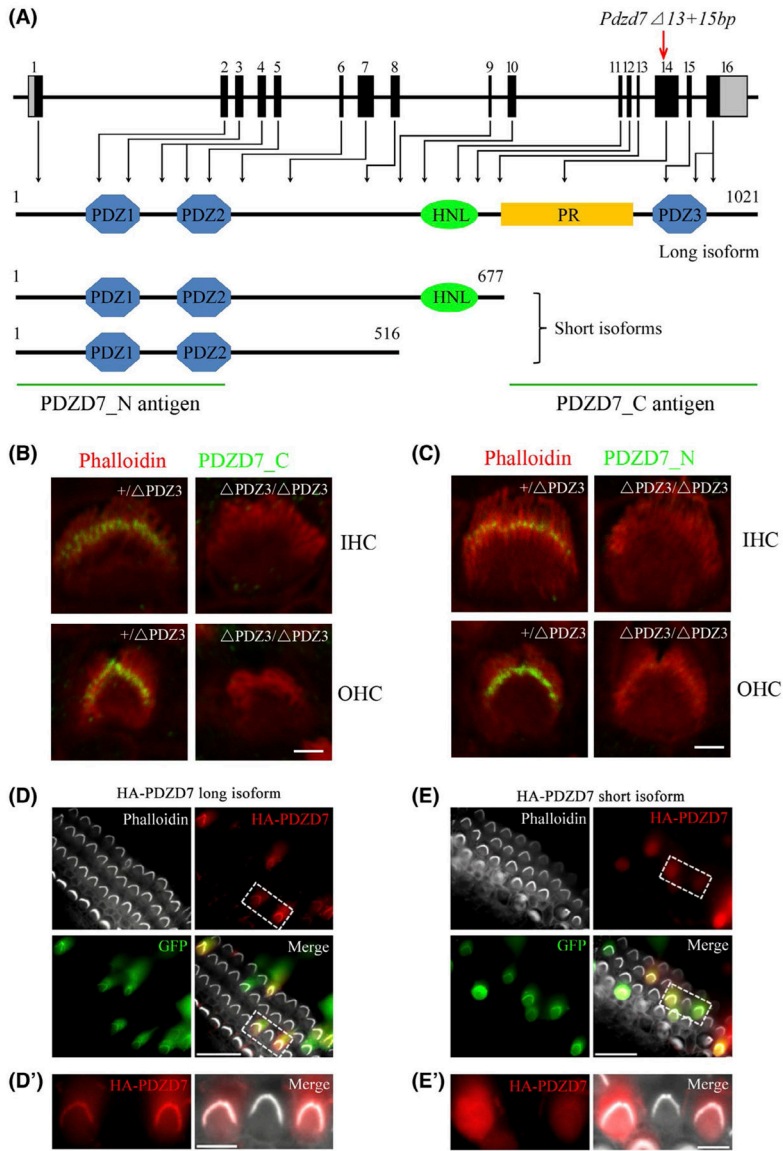


FIGURE 1. PDZD7 long isoform, but not short isoform, localizes in the stereocilia of auditory hair cells. A, Schematic drawing of *Pdzd7* genomic structure and the domain architecture of PDZD7 protein. Mouse *Pdzd7* gene contains 16 exons, and deletions were introduced into exon 14 that is unique for PDZD7 long isoform. The coding sequences were indicated by black boxes, whereas the uncoding sequences were indicated by grey boxes. The various domains and the antigens recognized by different antibodies were indicated. B and C, Cochlear whole mounts from *Pdzd7*^{+/+} *PDZ3* and *Pdzd7*^{ΔPDZ3/ΔPDZ3} mice at P4 were stained with PDZD7_C antibody (B) or PDZD7_N antibody (C). Phalloidin staining was also performed to indicate the stereocilia. Images were taken from the middle turn of the cochlea. D-E', Cochlear explants from wild-type mice at P3 were injectoparated with expression vectors to express HA-tagged PDZD7 long isoform (D and D') or short isoform (E and E') in hair cells. GFP fluorescence indicated successfully injectoparated hair cells. Immunostaining with anti-HA

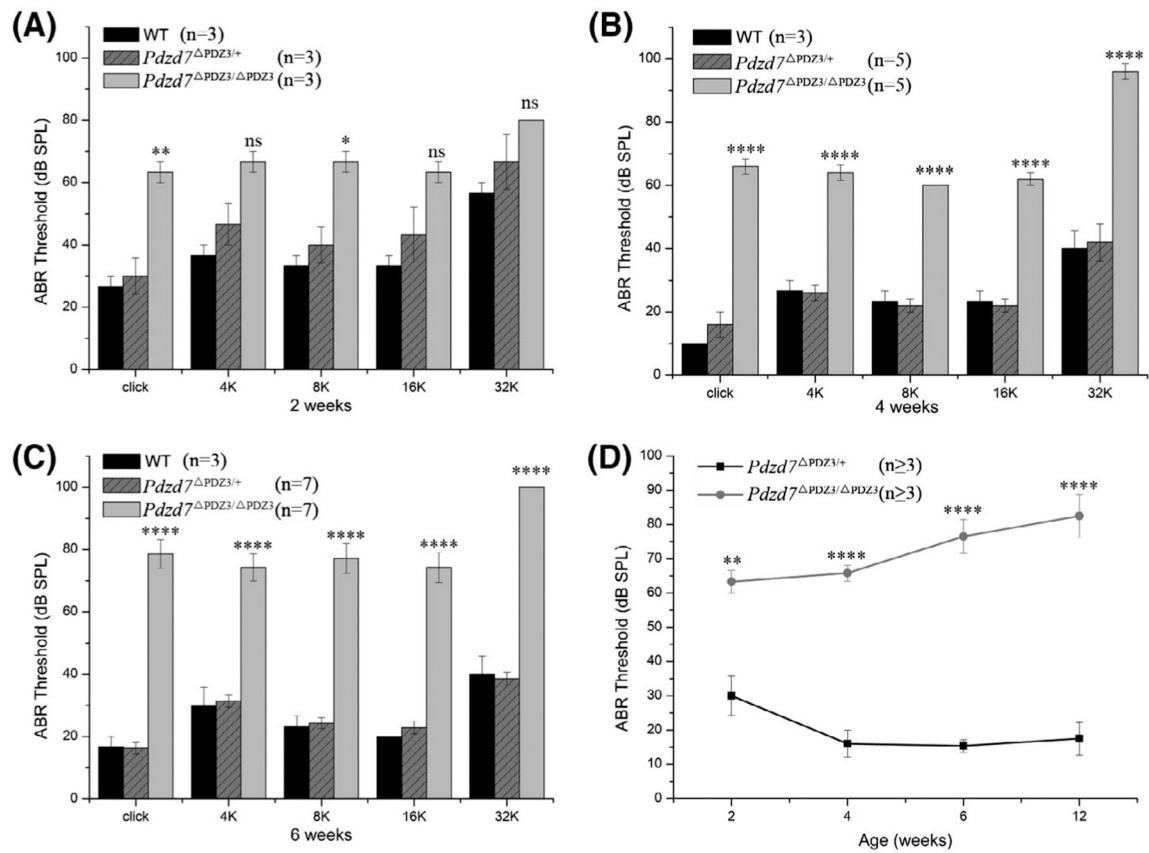
antibody was performed to locate HA-PDZD7. Phalloidin staining was performed to indicate the stereocilia. Scale bars: 2 μm in (B) and (C), 20 μm in (D) and (E), and 5 μm in (D') and (E')

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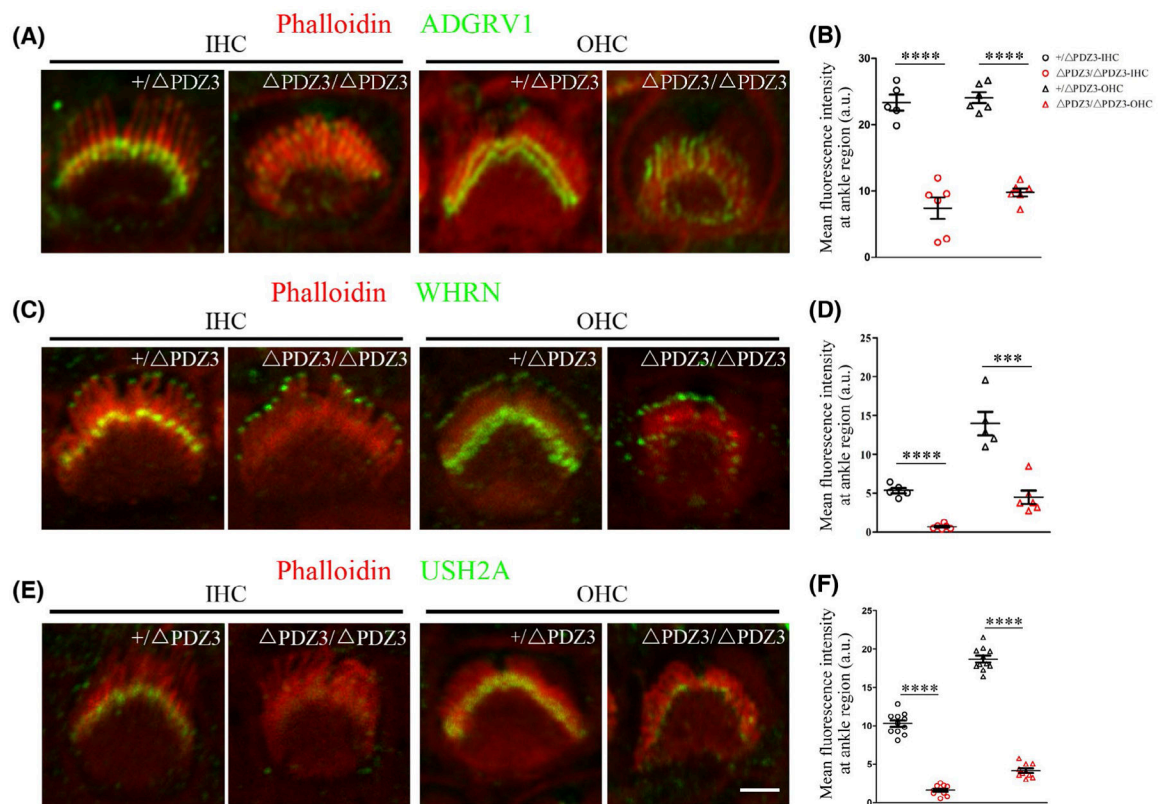
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**FIGURE 2.**

Lack of PDZD7 long isoform causes progressive, severe to profound hearing loss in mice.

A, ABR thresholds for click stimuli and pure tones at 2 weeks of age. B, ABR thresholds for click stimuli and pure tones at 4 weeks of age. C, ABR thresholds for click stimuli and pure tones at 6 weeks of age. D, Summary of ABR thresholds for click stimuli at different ages.

The differences between homozygotes and heterozygotes were evaluated by Student's *t* test (ns, not significant; **P* < .05; ***P* < .01; *****P* < .0001)

**FIGURE 3.**

Lack of PDZD7 long isoform affects the subcellular localization of other ankle-link complex components. Cochlear whole mounts from *Pdzd7*^{+/+} *PDZ3* and *Pdzd7*^{PDZ3/} *PDZ3* mice at P4 were stained with anti-ADGRV1 (A), anti-WHRN (C), or anti-USH2A (E) antibody. Phalloidin staining was also performed to indicate the stereocilia. Images were taken from the middle turn of the cochlea. Scale bar: 2 μ m. The fluorescence intensity of corresponding antibodies at ankle regions was measured and analyzed in (B), (D), and (F). The differences were evaluated by Student's *t* test (****P* < .001; *****P* < .0001)

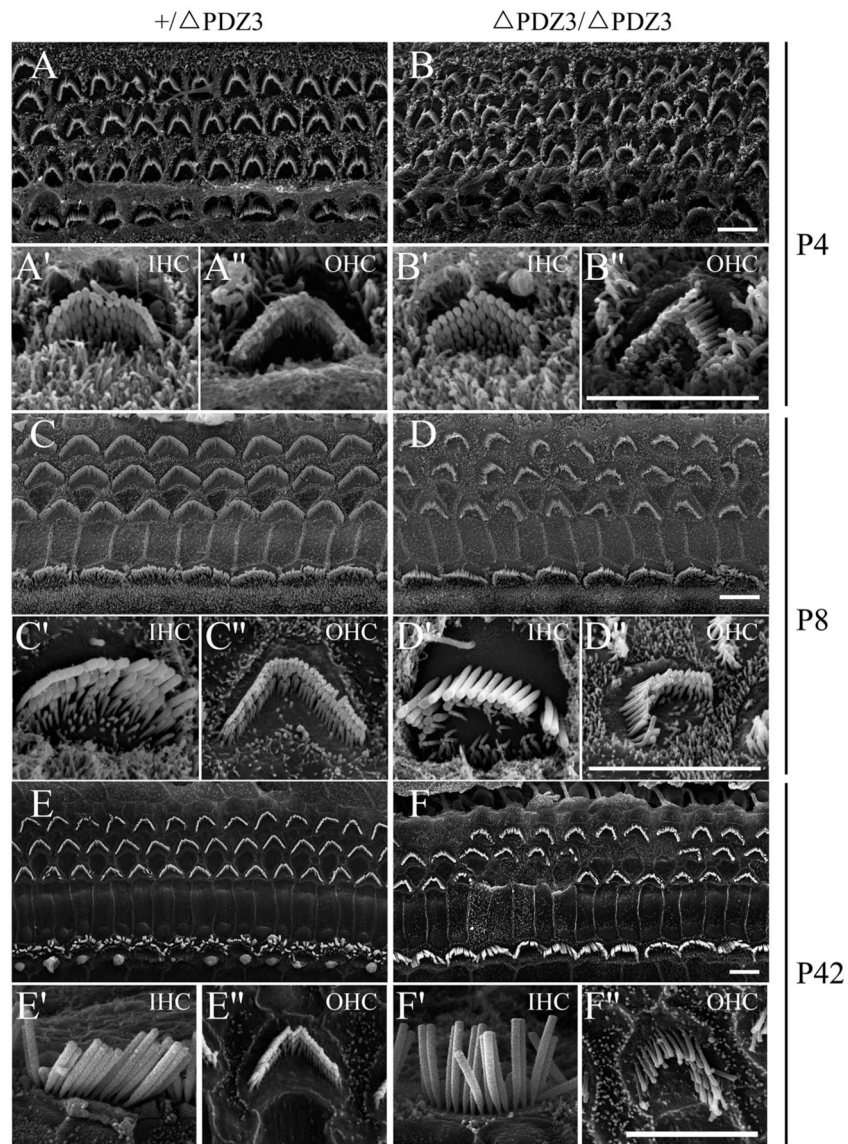


FIGURE 4.

Lack of PDZD7 long isoform affects stereocilia development. Scanning electron microscope images showing auditory sensory epithelia from *Pdzd7*^{+/ΔPDZ3} and *Pdzd7*^{ΔPDZ3/ΔPDZ3} mice at P4 (A-B''), P8 (C-D''), and P42 (E-F''). Images were taken from the middle turn of the cochlea. Bundle disorganization in *Pdzd7*^{ΔPDZ3/ΔPDZ3} mice could be observed as early as P4, which became more obvious at P8. By P42, stereocilia were completely lost in some *Pdzd7*^{ΔPDZ3/ΔPDZ3} OHCs. Scale bars, 5 μm

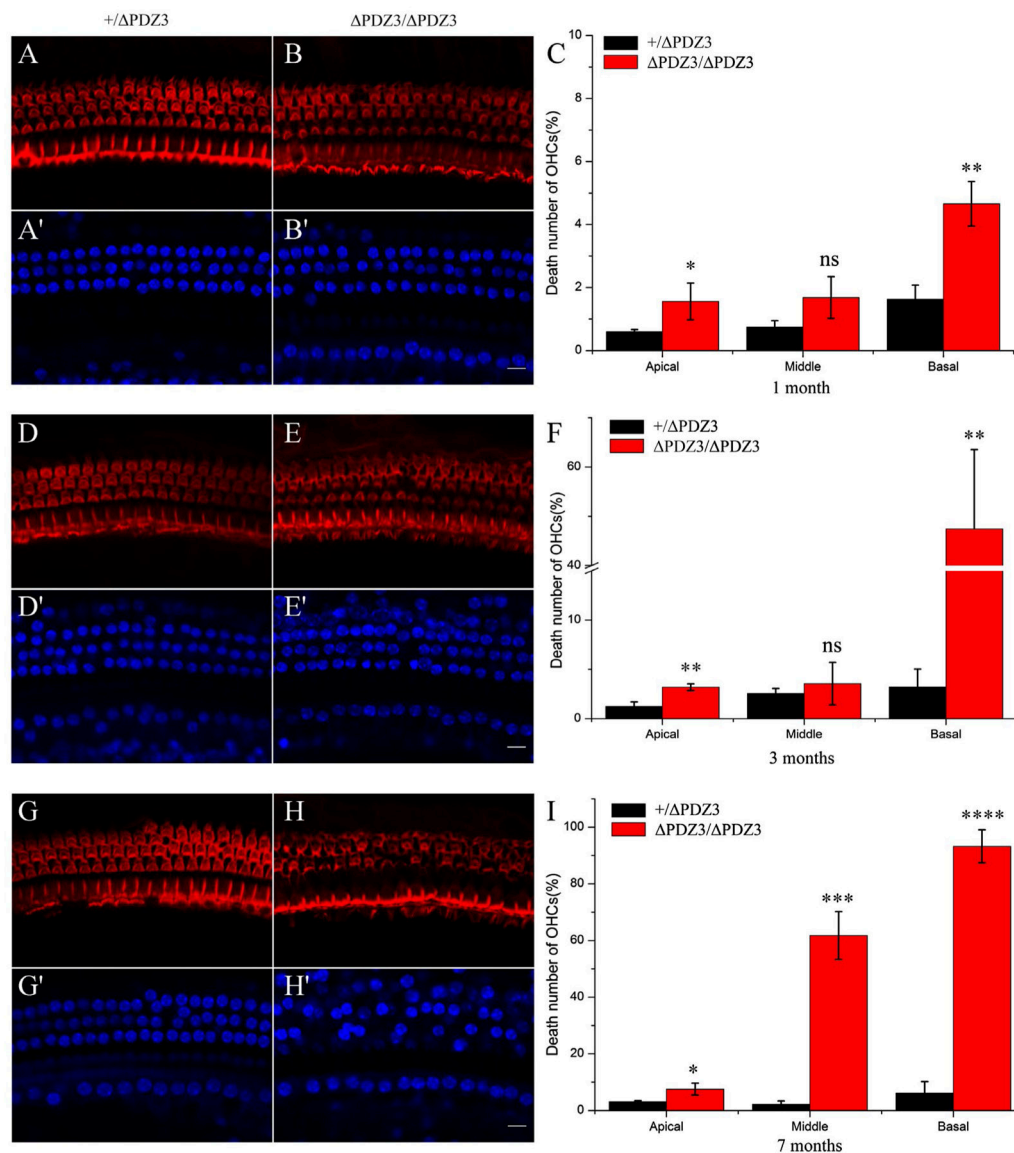


FIGURE 5. Lack of PDZD7 long isoform causes OHC loss. Cochlear whole mounts from *Pdzd7^{+/+} PDZ3* and *Pdzd7^{PDZ3/} PDZ3* mice at ages of 1 month (A-C), 3 months (D-F), and 7 months (G-I) were stained with Phalloidin and DAPI. Images were taken from the middle turn of the cochlea. Scale bar: 10 μ m. Percentages of OHC loss at different positions along the cochlea were shown in (C), (F), and (I). The differences were evaluated by Student's *t* test (ns, not significant; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001)

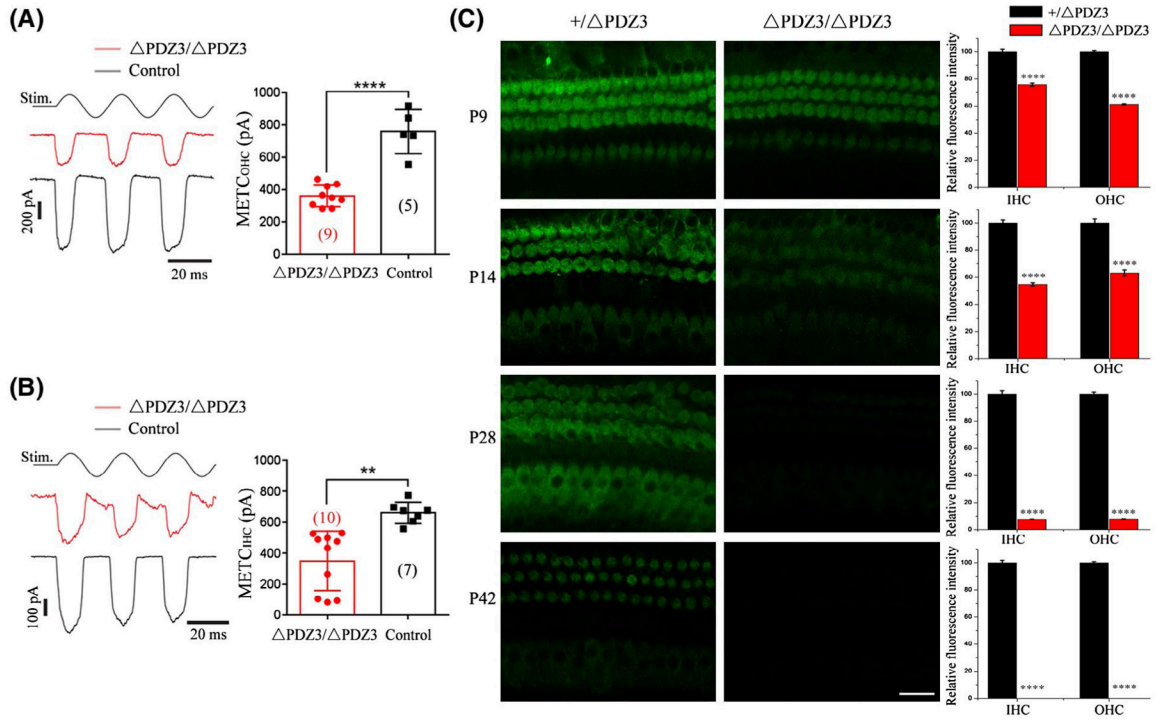


FIGURE 6.

Lack of PDZD7 long isoform affects MET currents in both OHCs and IHCs. A and B, A fluid jet system that drives a sinusoidal deflection of hair bundles was used to evaluate the saturating MET current from OHCs (A) and IHCs (B) at P6. C, Auditory sensory epithelia from *Pdzd7^{+/+} PDZ3* and *Pdzd7^{PDZ3/} PDZ3* mice at P9, P14, P28, and P42 were treated with FM 1-43FX. Images were taken from the middle turn of cochlea using a confocal microscope. Scale bar, 20 μ m. Relative fluorescence intensity in IHCs and OHCs of different genotypes at different developmental stages was measured and analyzed. The differences were evaluated by Student's *t* test (**** $P < .0001$)

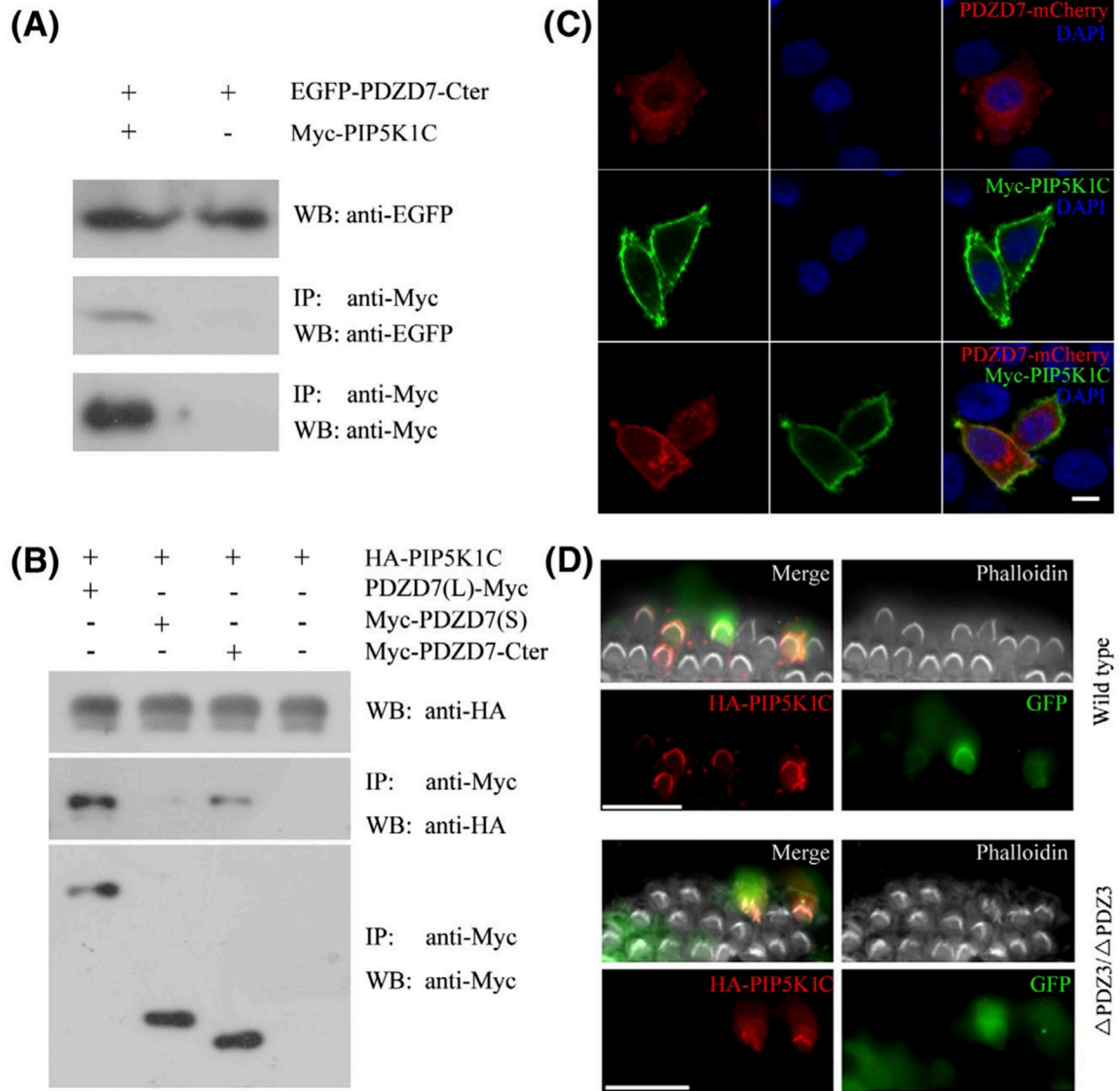


FIGURE 7.

PIP5K1C binds PDZD7 long isoform and localizes in the stereocilia. A and B, HEK293T cells were transfected with the constructs indicated on the top of each panel. Immunoprecipitations were performed with immobilized Myc-antibody followed by western blotting to detect corresponding proteins. C, HeLa cells were transfected to express Myc-tagged PIP5K1C and mCherry-tagged PDZD7 long isoform. Immunostaining with anti-PIP5K1C antibody was performed to locate PIP5K1C. DAPI staining was performed to indicate nuclei. D, Cochlear explants from wild-type or *Pdzd7*^{PDZ3/ PDZ3} mice at P3 were injectoprated with expression vectors to express HA-tagged PIP5K1C in hair cells. GFP fluorescence indicated successfully injectoprated hair cells. Immunostaining with anti-HA antibody was performed to locate HA-PIP5K1C. Phalloidin staining was performed to indicate the stereocilia. Scale bars: 5 μm in (C), 20 μm in (D)