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2 CONDITIONAL DELETION OF PEJVAKIN IN ADULT OUTER HAIR 3 CELLS CAUSES PROGRESSIVE HEARING LOSS IN MICE

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15 **Abstract—Mutations in the *Pejvakin* (*Pjvk*) gene cause autosomal recessive hearing loss DFNB59 with audiological features of auditory neuropathy spectrum disorder (ANSD) or cochlear dysfunction. The precise mechanisms underlying the variable clinical phenotypes of DFNB59 remain unclear. Here, we demonstrate that mice with conditional ablation of the *Pjvk* gene in all sensory hair cells or only in outer hair cells (OHCs) show similar auditory phenotypes with early-onset profound hearing loss. By contrast, loss of *Pjvk* in adult OHCs causes a slowly progressive hearing loss associated with OHC degeneration and delayed loss of inner hair cells (IHCs), indicating a primary role for pejvakin in regulating OHC function and survival. Consistent with this model, synaptic transmission at the IHC ribbon synapse is largely unaffected in *sirtaki* mice that carry a C-terminal deletion mutation in *Pjvk*. Using the C-terminal domain of pejvakin as bait, we identified in a cochlear cDNA library ROCK2, an effector for the small GTPase Rho, and the scaffold protein IQGAP1, involved in modulating actin dynamics. Both ROCK2 and IQGAP1 associate via their coiled-coil domains with pejvakin. We conclude that pejvakin is required to sustain OHC activity and survival in a cell-autonomous manner likely involving regulation of Rho signaling.** **© 2017 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: auditory neuropathy, inner ear, DFNB59, pejvakin, hearing loss, hair cell.

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Abbreviations: ABR, auditory brainstem response; ANSD, auditory neuropathy spectrum disorder; CHD, calponin homology domain; DIAPH3, Diaphanous homolog 3; DPOAEs, distortion product otoacoustic emissions; GSDM, gasdermin; IHCs, inner hair cells; KO, knockout; OAEs, otoacoustic emissions; OHCs, outer hair cells; OTOF, otoferlin.

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INTRODUCTION 17

Hair cells of the mammalian inner ear are highly specialized mechanosensors that convert mechanical stimuli into electrical impulses. There are two types of hair cells in the organ of Corti, the sensory epithelium of the cochlea, that have different morphological and physiological properties and subserve unique functions (Dallos, 1992). IHCs are the true sensory cells that transmit the electrical signals via specialized ribbon synapses to the auditory nerve (Wichmann and Moser, 2015), whereas outer hair cells (OHCs) act as mechanical amplifiers that enhance weak sounds in the cochlea and are regulated by efferent input from the brainstem (Warr and Guinan, 1979; Zheng et al., 2000; Liberman et al., 2002; Dallos et al., 2008). Much remains to be known about the molecular mechanisms that control the morphological and functional specialization of these two types of hair cells and maintain their integrity throughout life.

Sensorineural hearing loss is frequently caused by defects in OHCs that seem more susceptible to damage by noise and genetic perturbations than IHCs. By contrast, mutations in only a few genes disrupt IHC function, without directly compromising OHC activity, including otoferlin (OTOF), SLC17A8 (encoding the vesicular glutamate transporter-3 (VGLUT3)), and Diaphanous homolog 3 (DIAPH3) (Varga et al., 2003; Roux et al., 2006; Ruel et al., 2008; Schoen et al., 2010). Patients carrying mutations in these genes often present with an abnormal auditory brainstem response (ABR), indicative of IHC dysfunction, but preserved otoacoustic emissions (OAEs), reflecting normal OHC activity, and therefore meet the diagnostic criteria for auditory neuropathy spectrum disorder (ANSD) (Starr et al., 1996; Kemp, 2002; Moser and Starr, 2016). ANSD is caused by defects in the encoding and processing of sound at IHCs, the IHC synapse, or the auditory nerve itself. While mutations in the OTOF and SLC17A8 genes cause defects at the IHC synapse (also termed auditory synaptopathy) (Roux et al., 2006; Ruel et al., 2008), DIAPH3 is critical for the long-term maintenance of IHC stereocilia and possibly synapses (Schoen et al., 2010). Thus, while only few genetic forms of ANSD are known, they have mostly been attributed to defects at the IHC synapse. More complex mechanisms may contribute to DFNB59, an autosomal recessive form of hearing loss that can manifest as ANSD or cochlear deafness (Delmaghani et al., 2006; Collin et al., 2007; Schwander et al., 2007). Two missense mutations (p.T54I and p.R183W) in *Pjvk* were initially reported in DFNB59 patients

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with audiological hallmarks of ANSD including abnormal ABR and preserved OAEs (Delmaghani et al., 2006). Evidence suggests that these mutations affect the conduction of signals in the auditory nerve, while leaving OHC function unaffected (Delmaghani et al., 2006). However, subsequent studies identified *Pjvk* nonsense mutations in DFNB59 patients with progressive hearing loss and, in many cases, absent OAEs (Chaleshtori et al., 2007; Ebermann et al., 2007; Schwander et al., 2007; Collin et al., 2007; Zhang et al., 2015). Importantly, abnormal OAEs have also been reported in individuals carrying the ANSD-linked p.R183W mutation (Collin et al., 2007), raising the question of whether OHC defects may secondarily develop over time as the disease progresses and whether DFNB59 meets the diagnostic criteria of ANSD.

Pejvakin is a distantly related member of the gasdermin family of genes (Saeki et al., 2000). All gasdermins share a common N-terminal gasdermin (GSDM) domain. The GSDM N-domain of some gasdermins bears intrinsic cytotoxic activity (Op de Beeck et al., 2011; Shi et al., 2015), although no such function has been reported for the GSDM N-domain of pejvakin. The C-terminal domain of pejvakin bears homology with Zinc binding proteins, and its deletion causes progressive hearing loss and abnormal OAEs in the ENU-induced *sirtaki* mouse line (Schwander et al., 2007), suggesting a critical role for the C-terminal domain in pejvakin function. A recent study suggested a possible role for pejvakin in regulating peroxisome proliferation in sensory hair cells and auditory neurons in response to oxidative stress (Delmaghani et al., 2015), although no peroxisomal targeting sequence has been detected in its primary sequence. Thus, clarification of the mechanisms underlying the phenotypic variability associated with mutations in the *Pjvk* gene awaits identification of its molecular and cell-type-specific functions.

To determine the extent to which pejvakin regulates the development and maintenance of IHCs and OHCs, we have carried out targeted disruption of the *Pjvk* gene in the early postnatal and adult cochlea. Here, we report that genetic ablation of pejvakin in all cochlear hair cells or only in OHCs leads to an early-onset profound hearing loss. Pejvakin is also required to sustain the activity and survival of OHCs in the adult cochlea but is largely dispensable for synaptic transmission at the IHC ribbon synapse. Using yeast two-hybrid screens of a cochlear cDNA library, we identified ROCK2 and IQGAP1, well-known regulators of actin dynamics, as binding proteins for pejvakin (Mateer et al., 2002; Shimizu et al., 2003; Brown and Sacks, 2006; Truebestein et al., 2015). Our findings show that loss of function mutations in *Pjvk* affect OHC function in an age-dependent manner, possibly by compromising the integrity of the hair cell cytoskeleton.

EXPERIMENTAL PROCEDURES

Mouse strains, ABR and DPOAE measurements

All procedures were performed in accordance with research guidelines of the institutional animal care and use committee of Rutgers University. Mice of either sex were studied. The measurement of ABRs and distortion product otoacoustic emissions (DPOAEs) was carried out as described (Schwander et al., 2007). tdTomato reporter mice (B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J) and wild-type C57BL6 mice were obtained from The Jackson Laboratory. *Atoh1-CreER*^{TM} (Chow et al., 2006) and *Prestin-CreER*^{T2} (Fang et al., 2012) mice were a kind gift from S. Baker and J. Zuo (St. Jude Children's Research Hospital, Memphis, TN), respectively. Generation of pejvakin floxed mice (*Pjvk*$^{fl/fl}$) mice will be described in detail elsewhere (M. Kazmierczak, P. Kazmierczak, A.W. Peng, S.L. Harris, P. Shah, J.-L. Puel, M. Lenoir, S.J. Franco, and M. Schwander, unpublished observations). *Pjvk*$^{fl/fl}$ mice were crossed with *Atoh1-CreER*^{TM} and Prestin-*CreER*^{T2} mice and genotyped as previously described (Graus-Porta et al., 2001). Double heterozygous *Pjvk*$^{fl/+}$*; Atoh1-CreER*^{TM} and *Pjvk*$^{fl/+}$*; Prestin-CreER*^{T2} mice were crossed with homozygous *Pjvk*$^{fl/fl}$ mice to obtain animals used in experiments. To induce Cre activity in crosses with *Atoh1-CreER*^{TM} mice, pups were intraperitoneally (IP) injected once daily with tamoxifen (T5648, Sigma) dissolved in corn oil (C8267, Sigma) at a dose of 3 mg/40 g body weight at P0 and P1. To induce Cre activity in crosses with *Prestin-CreER*^{T2} mice, pups were injected IP once daily either at P2-P4 with 3 mg/40 g or at P21 and P22 with 9 mg/40 g body weight. *Pjvk* conditional knockout (KO) mice were genotyped for the presence of Cre recombinase and the pejvakin floxed allele. Detection of Cre allele: Cre_fw GACATGTTCAGGGATCGCCAGGCG, Cre_rv1 GACGGAAATCCATCGCTCGACCAG; Detection of Flox allele: FloxLongfw GAATTCCTCTTGGATGATGGC CACTGCAGA, FloxLongrv AACGAAGCTCTTGGTAG CAGCAGCAAACAT. *Sirtaki* mice were genotyped as previously described (Schwander et al., 2007).

Histology and immunohistochemistry

Inner ear sections were stained with hematoxylin and eosin as described (Schwander et al., 2007). Whole-mount staining of cochlear sensory epithelia with anti-myosin VIIa (rabbit; Proteus Biosciences) and 488-phalloidin (Life Technologies) were carried out as described (Senften et al., 2006; Schwander et al., 2007). The whole-mount preparations were imaged with a BX63 fluorescence microscope (Olympus). Hair cells were counted as present if myosin VIIa-positive cell bodies and V-shaped hair bundles were intact. CellSense software (Olympus) was used to measure the total length of cochlear whole mounts and the length of individual counted segments. The total number of IHCs and OHCs was counted in each of three cochlear segments (apical, medial and basal) of 600–1600 μm. Density (cells per 100 μm) of hair cells was then calculated for each segment.

Immunohistochemistry for CtBP2 and GluR2/3 was performed as described previously (Khimich et al., 2005). In brief, the organs were fixed with 4% formaldehyde for 10 min on ice, immunolabeled by mouse IgG1 anti-CtBP2 (BD Biosciences, 1:200) and rabbit anti-GluR2/3 (Chemicon, 1:200) primary antibodies and secondary AlexaFluor488- and AlexaFluor568-labeled antibodies (Molecular Probes, 1:200). Confocal images

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