Outer Hair Cell Function is Normal in β V Spectrin Knockout Mice.

Michael C. Stankewich^{1*}, Jun-Ping Bai^{2*}, Paul R. Stabach¹, Saaim Khan³, Lei Song⁴, Jon S. Morrow^{1,5}, Joseph Santos-Sacchi^{6,7,8}, Dhasakumar S. Navaratnam^{2,6,7 ^}

1 Department of Pathology, Yale School of Medicine, New Haven, CT, U.S.A

2 Department of Neurology, Yale School of Medicine, New Haven, CT, U.S.A

3 Cranbrook Kingswood Upper School, MI, USA

4 Department of Otolaryngology-Head and Neck Surgery, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

5 Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, U.S.A

6 Department of Surgery, Yale School of Medicine, New Haven, CT, U.S.A

7 Department of Neurobiology, Yale School of Medicine, New Haven, CT, U.S.A

8 Department of Cellular and Molecular Physiology, Yale University, New Haven,

CT, U.S.A

* Co-first authors

^ Corresponding Author

Address correspondence to Dr. Dhasakumar Navaratnam
Yale School of Medicine, Department of Neurology, PO Box 208018,
New Haven, CT., 06519
email: dhasakumar.navaratnam@yale.edu

Keywords: cochlea, auditory brainstem response, electromotility, distortion

product otoacoustic emissions, Spnb5, ribbon synapse, cochlear nerve

ABSTRACT

Reports have proposed a putative role for β V spectrin in outer hair cells (OHCs) of the cochlea. In an ongoing investigation of the role of the cytoskeleton in electromotility, we tested mice with a targeted exon deletion of β V spectrin (Spnb5), and unexpectedly find that Spnb5^(-/-) animals' auditory thresholds are unaffected. Similarly, these mice have normal OHC electromechanical activity (otoacoustic emissions) and non-linear capacitance. Moreover, Spnb5 mRNA is undetectable in the organ of Corti or OHCs. In contrast, magnitudes of auditory brainstem response (ABR) peak 1-amplitudes are significantly reduced. Evidence of a synaptopathy was absent with normal hair cell CtBP-2 counts. In Spnb5^(-/-) mice, the number of afferent and efferent nerve fibers is decreased. Taken together, these data establish that β V spectrin is important for hearing, affecting neuronal structure and function. Significantly, these data exclude β V spectrin as functionally important to OHCs as has been previously suggested.

INTRODUCTION

In the early 1990s, several groups made key seminal observations of a spectrin/actin lattice in the cochlear outer hair cells (OHCs) (Holley & Ashmore, 1990) (Holley, Kalinec, & Kachar, 1992). Their electron micrographs propelled an exquisite model of the spectrin/actin lattice assembled just beneath the OHC lateral membrane with longitudinal spectrin molecules linked to circumferential actin filaments that, in turn, were linked to the plasma membrane by electron-dense pillars. Their work also identified a pool of spectrin in the actin-rich cuticular plate. Another study evaluated spectrin proteins in cochlear innervation during rodent development (Hafidi, Despres, & Romand, 1990). They identified differences in developmental expression of the erythroid beta spectrin (Spnb1 spectrin) compared to the most ubiquitous nonerythroid form α II/ β II spectrin, similar to what is observed in the brain. What is not certain from these earlier studies is the specific spectrins involved, a family now known to consist of two alpha spectrins (Spna1 and 2) and five beta spectrins (Spnb1-5). More recently, it has been shown that Spnb4 is restricted to the nodes of Ranvier in myelinated axons and is also a deafness-related gene (Parkinson et al., 2001) (Lewis et al., 2018). It appears from these studies that the deafness in the quivering mouse is likely not originating in the cochlea but rather is central in origin. Furthermore, Spnb2 spectrin, when selectively deleted from the mouse cochlea sensory epithelia by crossing Spnb2 floxed mice with Atoh-1 Cre-recombinase, causes profound deafness due to a disruption of the stereocilia rootlets in the cuticular plate in hair cells (Liu et al., 2019). Thus, the function of specific spectrins,

particularly as it relates to the OHC lateral membrane and cochlear innervation, remains unclear. In humans, the molecule β V spectrin (SPTBN5) that was first discovered in the retina, is involved with the retinal disorder, Usher syndrome (Stabach & Morrow, 2000) (Papal et al., 2013). Further reports have proposed a putative role for β V spectrin in a second sensorineural function, hearing. One group's observations demonstrated that antisera raised against the C-terminal peptide of SPTBN5, the largest form of spectrin, and an orthologue of beta(Heavy) spectrin in *Drosophila*, labeled structures along the lateral membrane of the OHC (Legendre, Safieddine, Kussel-Andermann, Petit, & El-Amraoui, 2008) (Cortese et al., 2017). This observation drew attention because detection of this giant spectrin in tissue other than retina and testis has been elusive. This finding raised the possibility that β V spectrin was involved in the electromotile properties of OHCs.

Continuing an ongoing effort in our lab to define the cytoskeleton's role in electromotility, we sought to determine the role of β V spectrin in OHC function (Bai et al., 2010). In this study, we analyzed β V spectrin expression in the cochlea and addressed its function in mice with a targeted deletion of *Spnb5*. We tested hearing by auditory brainstem response (ABR), measured OHC-mediated otoacoustic emissions and OHC nonlinear capacitance (NLC), and evaluated the number of inner hair cell (IHC) ribbon synapses and nerve fibers that innervate the cochlear sensory epithelium. We find that while OHC function is unaffected in the absence of β V spectrin, nerve counts are reduced at the habenular, in all three turns of the cochlea, and in efferent fibers in the mid and basal turns, which collectively account for the reduced wave I amplitudes in ABRs. We conclude that β V spectrin is

100 necessary to maintain the integrity or function of afferent and efferent nerves of the
101 peripheral auditory system, but plays no role in the OHC itself.

102

103

RESULTS

Mouse β V spectrin amino acid sequence is divergent from the human protein.

Surprisingly, a sequence alignment comparing the mouse amino acid sequence to the human amino acid sequence reveals a significant divergence. In contrast, other beta spectrins, including beta I-IV, have ~ 90% amino acid conservation between human and mouse sequences. By aligning human and mouse β V spectrin, we note a significant divergence at the N- and C-termini of the proteins and an internal stretch of amino acids from position 817-922 (**Figure 1A**). Of note is that the initiator methionine residue in the human form is conserved between mouse and human genes and lies at position 36 and within exon 2 (**Figure 2**). The mouse sequence incorporates an unusual homorepeat of glutamines and, notably, does not have a bona fide pleckstrin homology (PH) domain seen in the human form.

Spnb5 mRNA tissue expression.

Spnb5 spectrin is expressed in most mouse tissues but at low levels. In ENCODE, the level of Spnb5 transcripts is an order of magnitude less abundant than the ubiquitous form Spnb2 (**Figure 1B**) (Yue et al., 2014). The peak abundance of Spnb2 approaches 50 RPKM while Spnb5 is 0.5 RPKM in testis (**Figure 1B, C**). Interestingly, the GTEX dataset shows high expression of Spnb5 in the peripheral (tibial) nerve (**Figure 1D**). To validate that mRNA is detected in mice, we tested RNA from the testis with primer sets that bridged exons. By standard rt-PCR, Spnb5 mRNA fragments encoding both the N-terminus and C-terminus were

amplified from testis and brain RNA, and its identity was confirmed by Sanger sequencing. In contrast to brain and testicular tissue, we could not detect Spnb5 mRNA in isolated OHCs and whole organ of Corti by both conventional and nested PCR (Table 1 details the gene-specific primer sets). Using rt-PCR from ~3000 OHCs and organ of Corti, we detect both Spnb1 and Spnb2. Using nested PCR, amplicons for these spectrins are robust, while we do not visualize any product for Spnb5 (**Figure 1E**). These data are in accord with data derived from the public data sets of transcriptome profiling for OHCs in gEAR (<https://umgear.org/>) (**See supplementary Figure1**).

Figure 1. (A) Spnb5 amino acid sequence differs from humans. Shown is the comparison of the deduced amino acid sequence of mouse and human Spnb5. Conserved amino acids are marked in red. There is divergence at the N and C termini. In addition, a region in the middle of the protein from position 817-922 shows significant divergence. The mouse version of this gene shares only 65.6 percent identity with the human form. **(B, C) Spnb5 is expressed in brain and testis.** The relative abundance of Spnb2 transcripts as compared to Spnb5 in mouse tissues. Shown are the expression of Spnb2 and 5 in RNA Seq. NCBI reference datasets (ENCODE; Bioproject PRJNA66167_(Yue et al., 2014). On average, the abundance of Spnb5 mRNA is an order of magnitude less abundant than Spnb2. Of the tissues examined, adult testis has the most mRNA for Spnb5. **(D) In human, Spnb5 is most expressed in peripheral nerve.** The relative abundance of Spnb5 in different tissues in GTEX database is presented (version 6) (Consortium, 2013). Of all the tissues examined Spnb5 is expressed in highest amounts in the peripheral nerve (tibial nerve). **(E) Spnb5 mRNA is not detectable in the organ of Corti or OHCs.** Shown are PCR amplifications from mouse brain (lanes 2-4), organ of Corti (lanes 6-8) and OHCs (lanes 10-12) of Spnb1 (lanes 2, 6 and 10), Spnb2 (lanes 3, 7 and 11) and Spnb5 (lanes 4, 8 and 12). The upper panel shows amplicons for these three mRNA transcripts using outer primers while the lower panel shows amplicons of primers using a nested set of primers. All three spectrins are detectable in brain. Spnb1 and 2 are detectable in both the organ of Corti and OHCs (lanes 6-7 and 10-11). Spnb5 however, is not detectable using either primer set (lanes 8 and 12).

Figure 1

Supplementary Figure 1.

Expression levels for each of the beta spectrins in OHCs.

Mining data from the gene Expression Analysis Resource (gEAR) we graphed the abundance of each of the five beta spectrin genes. This data set was derived from transcriptome analysis using sc-RNAseq of cell populations isolated from mouse cochleae (Li et al., 2018).

Targeted knockout of Spnb5 in mice using homologous recombination.

In order to properly target the β V spectrin gene in mice, we needed to know its precise gene structure. While the human gene has been well characterized and indexed since its discovery in 2000 (Stabach & Morrow, 2000), there has been ambiguity in the intron/exon usage in the mouse gene found on chromosome 2. The GENBANK entry for Spnb5 has been modified many times in the last several years, with the last significant annotation in 2007 (Benson et al., 2013). This annotation was derived by automated computational analysis using gene prediction algorithms. The exon prediction is made difficult because 1) the gene is very large, so PCR amplifying a full-length cDNA is not trivial, and 2) the conservation between human and mouse intron/exon boundaries is weak at the N-

and C-termini. The current and accepted annotated version in both the UCSC genome browser and in Ensembl shows Spnb5 spectrin mRNA in the mouse to be 14.4 kb with a 10,872 bp ORF that has 66 exons and codes for 3623 amino acids: NM_001370938: [NP_001357867.1](#). This version has been validated in the ENCODE project based on the robustness of next-generation sequencing efforts over the last several years (Yue et al., 2014).

Shown in the schematic in **Figure 2A** is the strategy for targeted deletion of exon 2 of Spnb5. The knockout strategy was based on the transcript ID: ENSMUST00000156159.3, and a targeting construct was generated in which three Lox P sites were inserted to flank the neomycin selection marker and exon 2 containing the start codon, resulting in disruption of the open reading frame upon Cre-mediated excision. Exon 2 contains the ATG start codon (with a consensus Kozak sequence) and an additional 37 amino acids at its N-terminus. Notably, the codon for the methionine residue corresponding to the human start codon is also contained within the second exon. A genomic bacterial artificial chromosome containing the Spnb5 locus was obtained, and genomic DNA fragments containing the first exons of Spnb5 were subcloned in pEasy (a gift from Sankar Ghosh). The targeting vector comprised a 4.3 kb long arm, a 4.1 kb short arm, and a middle selection cassette which contains exon two flanked by a PGK–neomycin resistance (Neo), two FRT sites, and the herpes simplex thymidine kinase gene (**Figure 2B and Primer Sets in Table 2**). The targeting plasmid DNA was linearized with Cla1 and electroporated into mouse embryonic stem cells (ESCs)

(129SV/EV). ESCs were selected for neomycin/G418 resistance. Positive clones were identified by PCR and confirmed by Southern blot analysis using external and internal probes. A correctly targeted ESC clone was injected into C57BL/6J blastocysts to produce germline transmitting chimeric mice. These were then mated with actin-Cre C57BL/6J transgenic mice for generation of heterozygous Spnb5 transgenic mice with both the Neo cassette and exon two removed (Spnb5^(+/-)). The resulting Spnb5^(+/-) offspring were crossed to generate βV spectrin-deficient mice. The genotypes of these mice were confirmed by PCR amplification (**Figure 2C**). Chimeric mice were backcrossed onto the C57BL/6J strain. Litters from heterozygous breeders Spnb5^(+/-) / Spnb5^(+/-) were of normal number, and the mice of all genotypes were phenotypically indistinguishable with normal lifespans >2 yrs old. We confirmed decreased expression of Spnb5 in cDNA from testes of Spnb5^(-/-) mice, where we found equivalent expression of Spnb2 and Spna2 (**Figure 2D and Primer Sets in Table 3**).

Figure 2. Exon 2 of the *Sptnb5* gene was removed using homologous recombination. (A) Schematic of the strategy used to delete exon 2. The construct contained a short arm of exon 1 and a long arm of exons 3, 4, and 5 together with the intervening introns. Both the selection marker Neo and exon two are flanked by Lox P sites. Linearized plasmid was injected into ES cells and clones with homologous recombination and selected by neomycin (Neo)/ G418 resistance. Mice were then crossed with Actin-Cre mice to generate floxed mice lacking the Neo cassette and exon 2. **(B) Vector map of pEASY used to target *Sptnb5* deletion.** Exon 2 was subcloned between two Lox P sites adjacent to the Neo gene. These two genomic fragments were in turn placed between a 4.1kb short arm containing Exon 1 and a 4.3kb long arm containing exons 3, 4, and 5 and the intervening introns. The vector also contains a thymidine kinase and ampicillin gene allowing for selection. The plasmid was linearized with Cla1. **(C) PCR amplification of genomic DNA confirming deletion of exon two and flanking intronic sequence.** In genomic DNA from *Sptnb5*^(-/-) mice, primers that amplify a product no longer contain part of intron 1; exon 2; and part of intron 2/3. Lane 1 base pair standards, lane 2 *Sptnb5*^(+/-), lane 3 *Sptnb5*^(-/-), lane 4 *Sptnb5*^(+/+). The larger fragment of 665 bp consists of unexcised exon two while the smaller fragment consists of genomic DNA fragment from which exon two has been excised. **(D) Decreased expression of *Sptnb5* in *Sptnb5*^(-/-) testicular cDNA compared to *Sptnb2* and *Spna2*.** *Sptnb5* exons 58-60 in lanes 2-3, *Sptnb5* exon 53-55 in lanes 4-5; *Sptnb2* in lanes 7-8 (exons 28-29) and *Spna2* (exons 54-55) in lanes 10-11. WT in lanes 2, 4, 7 and 10. *Sptnb5*^(-/-) in lanes 3, 5, 8 and 11.

Figure 2

Auditory thresholds and OHC function are similar between *Spnb5*^(-/-) and control mice.

To investigate how the loss of *Spnb5* impacts auditory information processing, we compared the *in vivo* ABR of 2-month-old *Spnb5*^(+/+) and *Spnb5*^(-/-) mice. ABR thresholds were not altered in *Spnb5*^(-/-) mice at all sound frequencies tested from 2 to 32 kHz (**Figure 3A**). We also found that distortion product otoacoustic emissions (DPOAE) measurements, a measure specific to OHC function, are not affected by the loss of β V spectrin (**Figure 3B**). These findings with preserved DPOAE thresholds also extended to suprathreshold responses at sound intensities of 80-90 dB SPL (**Figure 3C**). Since spectrins have been implicated in membrane integrity and protein shuttling, we also assayed prestin expression in the lateral wall using its gating charge movement (NLC) at postnatal day 13 (when prestin expression increases in OHCs) and at two months (Abe et al., 2007). At both these time points, we found NLC to be unaffected in *Spnb5*^(-/-) mice (**Figure 4A**). Our measures of the different parameters of NLC, Q_{sp} , V_h , and z , were similar between WT and *Spnb5*^(-/-) mice (**Figure 4, A-D**).

Figure 3

213

Figure 3. ABR analysis suggests a neuronal and not OHC dysfunction in *Spnb5*^{-/-} mice. (A) thresholds at frequencies from 2-32 kHz reveal no change in *Spnb5*^{-/-} mice. ABR thresholds in *Spnb5*^{-/-} (open circle) and littermate WT (solid circle) mice at 2-month-old are plotted over a range of frequencies. There was not a significant difference between WT and *Spnb5*^{-/-} mice at all frequencies tested from 2 to 32 kHz. **(B) Similar DPOAE thresholds were observed in 2-month-old WT and *Spnb5*^{-/-} mice at all frequencies. (C) DPOAE input/output function are unaffected in *Spnb5*^{-/-} mice.** Input/output (I/O) function analysis of DPOAE responses in WT (solid circle) and *Spnb5*^{-/-} (open circle) mice as a function of sound intensity at 16 kHz at two months of age are graphed. DPOAE amplitudes in *Spnb5*^{-/-} mice showed no significant difference across all sound intensities compared to WT mice. **(D) Wave I amplitudes are significantly altered between *Spnb5*^{-/-} and WT at high stimulus intensities at frequencies 5.6-32 kHz, but not at frequencies 2-4 kHz.** Shown is the wave I amplitudes at 90 dB SPL. There is a consistent reduction in the amplitudes of wave I amplitudes at these suprathreshold intensities from 5.6-32 kHz. **(E) Wave I amplitudes were significantly reduced in *Spnb5*^{-/-} mice at higher sound intensities (75-90 dB SPL). (F) Wave I latencies at 16 kHz show significant delays at 90 dB SPL.** Latencies at all other frequencies are not significantly delayed (data not shown). Data are means $\pm$ SEM. The number (n) of animals per experiment is shown in each graph. Asterisks indicate $P < 0.05$.

235

Figure 4

236

237 **Figure 4. Spnb5^{-/-} mice have similar nonlinear capacitance characteristics as controls at**
 238 **two age groups.** NLC was measured at postnatal day 13, a midpoint of NLC development, and at
 239 two months when hearing is mature. **(A) There are no significant effects of OHC NLC in Spnb5^{-/-}**
 240 **mice.** Shown are representative traces of NLC measurement in Spnb5^{-/-} and WT mice. There
 241 are no differences in NLC between Spnb5^{-/-} and WT mice at these two time points. **B, C, and D.**
 242 **NLC parameters are unaffected in Spnb5^{-/-} mice.** Shown are means (+/- SEM) of NLC
 243 parameters in Spnb5^{+/+} and Spnb5^{-/-} mice at 13 days and two months, with no significant
 244 differences ($P > 0.05$) in all of these measures (Q_{sp} , V_h and z). **(B) Q_{sp} , a specific measure of net**
 245 **charge movement per unit of membrane corrected for linear capacitance. (C) V_h , a measure**
 246 **of the voltage dependence of charge movement. (D) z , a measure of the slope in NLC voltage**
 247 **dependence and indicative of the unitary charge movement.**

248

Loss of β V spectrin affects hearing sensitivity in a manner suggesting a neural processing disorder.

The ABR is characterized by a series of electrical waves representing the progressive transfer of the auditory signal from the periphery to the CNS (Song, McGee, & Walsh, 2006). Wave I represents the summated response from the spiral ganglion and auditory nerve (Melcher et al., 1996). While we see no differences in ABR thresholds, we note that Wave I amplitudes from 5.6 kHz and upwards are significantly altered between *Spnb5*^(-/-) and WT at high stimulus intensities (suprathreshold). We also analyzed the latency of wave I to investigate any potential changes in the rate of propagation of neural transmission (**Figure 5**). Wave I latency at 16 kHz was progressively delayed at high sound intensities reaching significance at the highest sound intensity (90 dB SPL). Latencies at other frequencies were not significantly different from WT.

***Spnb5* plays a critical role in sensorineural processing.**

What could be the cause of the differences in amplitudes of wave I between WT and *Spnb5*^(-/-) mice? We sought to exclude synaptopathy as a cause of the decrease in wave I amplitudes. For instance, spectrins have been shown to be important in targeting structural elements of the synaptic apparatus as well as maintaining the integrity and compartmentalization of axons and dendrites ((Hammarlund, Jorgensen, & Bastiani, 2007), (Stankewich et al., 2010), (Efimova et al., 2017; Stankewich et al., 2011), (Lorenzo et al., 2019). We compared the numbers of ribbon synapses at the base, middle, and apex of the organ of Corti

between *Spnb5*^{-/-} and WT mice. We used antibody labeling of the marker protein CtBP-2 (Bramhall, McMillan, Kujawa, & Konrad-Martin, 2018), a major component of the ribbon synapses, and a well-defined marker of synaptopathy (Sergeyenko, Lall, Liberman, & Kujawa, 2013). We found no differences in CtBP-2 labeled synaptic boutons of IHC in the three cochlea turns between WT and *Spnb5*^{-/-} mice (**Figure 5I**). We then sought to identify morphological differences in afferent nerve numbers as a possible cause of the reduced wave I amplitudes. We found significant reductions in neurofilament labeled, nonmyelinated, afferent fibers in the habenular in all three turns as well as efferent fibers in the basal and middle turns of the cochlea (**Figure 5G, H**).

Figure 5

Figure 5. *Spnb5*^{-/-} mice show evidence of neuropathy and no evidence of a synaptopathy. Shown are representative z axis projections of confocal micrographs of WT and β V spectrin KO mice in which synaptic boutons are labeled with antibodies to CtBP-2 (**B-G**). Afferent (**I-N**) and efferent (**P-U**) nerve fibers are labeled with antibodies to neurofilament. The scale bar

equates to 20 microns. There is a significant reduction in the density of afferent fibers at the habenular in all three turns and efferent fibers in the cochlear canal of Spnb5^(-/-) mice. In contrast, there were no differences in CtBP-2 positive bouton counts in all three turns of the cochlea. **(A, H, O) Shown are the mean (+/- SEM) synaptic (A) counts, afferent (H) and efferent (O) neuronal and in all three turns of the cochlea.** The differences in afferent and efferent counts were significant (* P<0.05, n = 4 cochlea) between WT and Spnb5^(-/-) mice in all three turns of the cochlea (afferent), and in the middle and basal turns of the cochlea (efferent). CtBP-2 counts between WT and Spnb5^(-/-) mice were not significantly different (P>0.05).

DISCUSSION

In this paper, we elucidate the function of β V spectrin in hearing using a mouse gene deletion model. Removal of exon 2 containing the initiator methionine and an N-terminal 37 amino acids, which, in part, compose its actin-binding domain, results in a mouse with normal ABR thresholds, and normal measures of OHC function including DPOAE and NLC. Typical DPOAEs are a strong indication of normal OHC electromotility (Shera & Guinan, 1999). The presence of normal NLC measures further reiterates this interpretation. Moreover, we were unable to detect Spnb5 mRNA in the organ of Corti or OHCs.

In contrast, we find a reduction in wave I amplitudes that are concordant with our finding of reduced numbers of nerve fibers. These data point to β V spectrin as critical for the maintenance and function of afferent and efferent nerve fibers. The normal ABR thresholds in the presence of reduced wave I amplitudes we observe in mice have been seen previously in mutations of other proteins and, in particular, in the recovery phase of noise-induced hearing loss (Maison, Pyott, Meredith, & Liberman, 2013) (Kujawa & Liberman, 2009).

How do we explain the preserved ABR thresholds in the presence of reduced wave I amplitudes? Loss of neurons of up to 80% has been shown to be associated with normal thresholds as long as hair cells were functional (Schuknecht & Woellner, 1953). Although speculative, other authors have reasoned that a small increase in sound pressure of 5 dB results in wider recruitment of neurons adjacent to the index sound frequency that could compensate for a 50% loss in afferent neurons using insensitive measures such as compound action potentials and ABRs (Taberner and Liberman 2005), (Lin, Furman, Kujawa, & Liberman, 2011). An important distinction in our data is that, unlike in noise-induced hearing loss models, we see no differences in CtBP-2 counts (Liberman & Kujawa, 2017). Our CtBP-2 counts and preliminary analysis using nerve counts suggest that the functional effects in these mice lie in the nerve. Ongoing work in our lab includes detailed analysis of high and low threshold fiber counts in these animals, together with studies that will better determine the exact molecular mechanisms which underlie the reduction in dendritic and axonal numbers.

We find absent effects on OHC function in *Spnb5*^(-/-) mice that is consistent with the undetectable expression of these transcripts in the cochlea and OHC in particular. We note similar low/absent *Spnb5* RNA expression upon interrogation of both OHC single-cell RNA and OHC RNA expression datasets of adult mice that have been published (Li et al., 2018) and gEAR portal (Shen, Scheffer, Kwan, & Corey, 2015), (Liu, Chen et al. 2018), (Hoa et al., 2020). Together, these data question the validity of β V spectrin antibody staining of OHCs, the principal reason

for ascribing its presence in OHCs (Legendre et al., 2008), (Cortese et al., 2017). These papers led to the belief that β V spectrin had an integral role in the cytoskeleton of hair cells. Our assertion that β V spectrin is not important for OHC function is based, in part, on the absent phenotype in the knockout mice. It is reasonable to ask if the knockout mouse resulted in an actual loss of function in the gene. We feel confident that the deletion of the second exon in *Spnb5*^(-/-) resulted in a silencing of the gene. In addition to the neuronal phenotype we describe herein with the *Spnb5*^(-/-) mice, we were also unable to detect *Spnb5* transcript from the testis of knockout mice that may relate to degradation of nonsense transcripts and a phenomenon observed in deletion of other genes (Baker & Parker, 2004). While the phenotype of mice is consistent with the absent expression of *Spnb5* transcript in OHCs, we have to be mindful that normal OHC function in these mice could be due to other beta spectrins substituting for *Spnb5* spectrin. In this context, the presence of a clear auditory phenotype in the *Spnb2* spectrin knockout mice would suggest that such an interchange and substitution by other spectrins is, however, not likely. Moreover, *Spnb4*^(qv/qv) spectrin has a neuronal phenotype, similar to *Spnb5*^(-/-).

In conclusion, we present data in this study that β V spectrin plays an important role in auditory function. Our data confirm that β V spectrin is necessary to maintain the integrity or function of afferent and efferent nerves of the peripheral auditory system. Importantly, our data show no evidence that β V spectrin has a function in OHCs, as has been previously suggested.

MATERIALS AND METHODS

Mice.

All procedures conducted with mice were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine. C57BL/6J mice were housed under pathogen-free conditions and maintained as a breeding colony. In our studies, C57BL/6J mice were engineered to harbor a gene knockout of Spnb5 (β V spectrin). The floxed exon 2 of Spnb5 was excised with Cre-recombinase from embryonic stem (ES) cells in culture before injecting them into mouse blastocysts under the guidance of the Yale Gene Targeting Center. This construct was designed to create a constitutive loss of β V spectrin. For genotyping animals, tail snips were collected from mice at their weaning age. Genomic DNA was prepared by an overnight 56°C incubation in tail lysis buffer 50mM Tris pH 8.0, 50mM KCL, 2.5mM EDTA, 0.4% Igepal, 0.4% Tween20 supplemented with Proteinase K (400 μ g/ml). gDNA was assayed by PCR using the following primer set: Forward 5'-CTGTCCGCCAAAGAGCAGGGGCCATG-3' Reverse 5'-GATGTGGGGTCAGCCCAGCATCTCC-3' and PCR amplicons were visualized on a 2% agarose gel.

Organ of Corti and OHC isolation.

Prestin-YFP mice that were 2-3 months old were euthanized and the cochleae extracted. The organ of Corti was dissected and treated with pre-warmed 0.05% trypsin for 8 minutes at 37°C. The organ of Corti was dissociated by triturating the tissue 20-30 times in a 1 ml pipet tip. The cell suspension was spun at 1500 g for

10 minutes. The cell pellet was resuspended in DMEM supplemented with 10% FBS and then filtered through a 40-micron cell strainer. The cells were FACS sorted, and YFP positive OHCs were collected.

mRNA detection.

Total RNA from mouse testis and the whole brain was either obtained from Takara or prepared from 100 mg of testis tissue from 3 month WT and β V KO C57BL/6J animals with Trizol reagent (Chomczynski & Mackey, 1995). cDNA was prepared with Superscript IV reverse transcriptase (ThermoFisher Scientific, Waltham, MA,) and quantified with a Nanodrop spectrophotometer. Standard procedures followed reagent manufactures directions for first-strand synthesis. The organ of Corti and OHCs (prepared as described above) were isolated and proteolyzed with 1 unit of proteinase K. After inactivating at 95°C for 5 minutes, non-purified RNA was treated with Superscript IV to generate cDNA. Gene-specific PCR primers bridging consecutive exons were designed to ensure the exclusion of genomic DNA from amplification in **Table 1**.

Auditory brainstem responses (ABR).

ABR experiments were conducted in a manner similar to that described by Song *et. al.* (El-Hassar et al., 2019). Briefly, animals were anesthetized with 480 mg/kg, i.p., chloral hydrate, and all recordings were conducted in a sound-attenuating chamber (Industrial Acoustics Corp., Bronx, NY). A customized TDT3 system (Tucker-Davis Technologies Inc., Alachua, FL) was used for ABR recordings.

Subdermal needle electrodes (Rochester Electro-Medical, Lutz, FL) were positioned at the vertex (active, noninverting), the infra-auricular mastoid region (reference, inverting), and the neck region (ground). Differentially recorded scalp potentials were bandpass filtered between 0.05 and 3 kHz over a 15 ms epoch. A total of 400 trials was averaged for each waveform for each stimulus condition.

Symmetrically shaped tone bursts were 3 ms long (1 ms raised cosine on/off ramps and 1 ms plateau). All acoustic stimuli were delivered free field via a speaker (Part FF1 2021, Tucker-Davis Technologies) positioned 10 cm from the vertex. Stimulus levels were calibrated using a 0.5-inch condenser microphone (model 4016, ACO Pacific, Belmont, CA) positioned at the approximate location of the animal's head during recording sessions and are reported in decibels of sound pressure level (SPL; referenced to 20 μ Pa). Stimuli of alternating polarity were delivered at a rate of $\sim$ 20/s. Tone burst responses were collected in half-octave steps ranging from 32 to 2.0 kHz. The effects of the level were determined by decreasing stimulus intensity in 5 dB steps up to a maximum stimulus level of 90 dB SPL. The ABR threshold was defined as the lowest intensity of sound level capable of evoking a reproducible, visually detectable response. Latencies and amplitudes of the initial ABR peaks were measured from sound stimuli delivered at a rate of 20/s and at a frequency of 16 kHz, one of the most sensitive frequency ranges of hearing in mice. Latencies and amplitudes of wave I at 90 dB SPL were also measured at all the other frequencies. The analysis was performed in BioSig software (BioSig Technologies, Westport, CT) on traces with discernible peaks by setting cursors

at the maxima and minima of the peaks. Latency was defined as the time from the onset of the stimulus to the peak, while amplitude was measured by taking the mean of the ΔV of the positive and negative deflections of the ABR wave.

Distortion product otoacoustic emissions (DPOAE).

The acoustic stimuli were produced, and responses were recorded using a customized TDT3 system (Tucker-Davis Technologies Inc., Alachua, FL) controlled by BioSig, digital signal processing software. DPOAE was measured by inserting a probe into anesthetized mouse external ear canal to deliver sound stimuli, and emissions of sound were collected by a microphone and recorded for subsequent analysis. To deliver acoustic stimuli and record the responses, two speakers (f_1 and f_2) (MF1 Multi-Field Magnetic Speakers, TDT) and a microphone (ER-10B, Etymotic Research, Elk Grove Village, IL) were connected to a short PVC tube, and tapered plastic probe that was inserted into the external auditory meatus of the mice. Two simultaneous continuous pure tones (f_1 and f_2) that have a frequency ratio of 1.2 (f_2/f_1) and equal sound level ($L_1 = L_2$) were delivered at center frequencies of 8, 12, 16, 24 and 32 kHz with sound levels from 80 to 20 dB SPL in 10 dB decrements. Stimuli duration was 83.88 ms at a repetition rate of 11.92 per second. The acquired DPOAE responses were averaged 100 times. The DPOAE of interest was at $2f_1-f_2$, the largest and most prominent DPOAE. The DPOAE threshold was defined as the lowest sound intensity capable of evoking a visually detectable $2f_1-f_2$ signal above the noise floor. DPOAE amplitudes were analyzed offline in BioSigRP by setting cursors at the peak of the $2f_1-f_2$ signals.

Nonlinear capacitance (NLC).

Nonlinear capacitance was conducted in a fashion much like that previously described in the Methods Mol. Biol. 2016 Song and Santos-Sacchi (Song & Santos-Sacchi, 2016). WT C57BL/6J and β V spectrin KO littermates at postnatal day 13 and at 2-month-old were compared. Apical turns of the organ of Corti were isolated from each and recorded with ionic current blocking solutions to minimize interference on measures of membrane capacitance. The extracellular solution contained (in mM): 100 NaCl, 20 tetraethylammonium (TEA)-Cl, 20 CsCl, 2 CoCl₂, 1 MgCl₂, 1 CaCl₂, 10 HEPES, pH 7.2. The intracellular solution contained (in mM): 140 CsCl, 2 MgCl₂, 10 HEPES, and 10 EGTA, pH 7.2. Pipettes had resistances of 3-5 M Ω . Gigohm seals were made, and stray capacitance was balanced out with amplifier circuitry prior to establishing whole-cell conditions. A Nikon Eclipse E600-FN microscope with 40 $\times$ water immersion objective lens was used to observe cells during voltage clamp. Whole-cell voltage-clamp recordings were performed from all three rows of OHCs on whole-mount organ of Corti. OHCs were recorded at room temperature using jClamp software and an Axopatch 200B amplifier. Data were low pass filtered at 10 kHz and digitized at 100 kHz with a Digidata 1320A. In order to extract Boltzmann parameters, capacitance-voltage data were fit to the first derivative of a two-state Boltzmann function.

$$C_m = NLC + C_{sa} + C_{lin} = Q_{\max} \frac{ze}{k_B T} \frac{b}{(1+b)^2} + C_{sa} + C_{lin} \quad (\text{eq.1})$$

where $b = \exp\left(-ze \frac{V_m - V_h}{k_B T}\right)$, $C_{sa} = \frac{\Delta C_{sa}}{(1+b^{-1})}$

$Q_{\max}$ is the maximum nonlinear charge moved, V_h is the voltage at peak capacitance or equivalently, at half-maximum charge transfer, V_m is Rs-corrected membrane potential, z is valence, C_{lin} is linear membrane capacitance, e is the electron charge, k_B is Boltzmann's constant, and T is the absolute temperature.

Immunocytochemistry (ICC).

For ICC, staining was performed on fixed and decalcified 3 turns of the organ of Corti to compare the number of neuronal fibers and synapses between all 3 turns organ of Corti in KO and WT mice. Temporal bones were fixed overnight in 4°C and then decalcified in 120 mM ethylenediaminetetraacetic acid (EDTA) for 2 days. Whole-mount of the organ of Corti was dissected, washed in PBS 0.05% Tween 20, 0.05% Triton-X 100 (incubation buffer) three times for 3 minutes, and incubated with primary antibody in incubation buffer overnight at 4°C with mouse anti-CtBP-2 antibody (1:50, BD Transduction, San Jose, CA, cat# 612044), chicken anti-neurofilament (NF) antibody (1:500, MilliporeSigma, Billerica, MA, cat# AB5539). After three washes in incubation buffer, the tissue was incubated with secondary antibody in incubation buffer for 1 hour at room temperature. The secondary antibodies used included donkey anti-rabbit Alexa Fluor 568/647 (1:200; Thermo Fisher Scientific, cat # A-10042/A-31573), goat anti-mouse Alexa Fluor 568/647 (1:200; Thermo Fisher Scientific, cat # A-21124/A-21235) and goat anti-chicken Alexa Fluor 488/594 (1:200; Thermo Fisher Scientific, cat # A-11039/A-11042). After antibody treatment, cells were washed in incubation buffer and

mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA), and viewed using a Zeiss confocal laser-scanning microscope (Oberkochen, Germany).

Nerve (NF positive) and synaptic vesicle (CtBP-2 positive) counts were performed posthoc in Fiji (ImageJ). Briefly, z stacks of images were separated by probes, background-subtracted (using a 50-pixel rolling ball radius), and z sections containing the images of interest identified for further analysis (slice keeper). CtBP-2 labeled IHCs and NF labeled afferent fibers surrounding IHCs in proximity to the habenula and efferent fibers in the cochlear canal were counted. Z projections (sum) of the respective sections were obtained and automated thresholds determined (max entropy). Counts of particles (synaptic vesicles) immunopositive for CtBP-2 larger than 0.1 microns were scored. Neuronal numbers were established by counting the peaks in a profile plotted perpendicular to neural fibers in the cochlea canal. For afferent nerve counts the profile was plotted at the midpoint of nerve densities.

Statistical Analysis.

Data were analyzed using FIJI, Microsoft Excel, SigmaPlot and GraphPad Prism. The measures of variability are depicted as error bars +/- the SEM and stated numerically in the figure legends. Unpaired, one or two-tailed Student's t-test were performed using both Excel and GraphPad Prism to make comparisons between WT and Spnb5^(-/-) data points. P-values are indicated in the figure legends to two significant figures. The sample size (n) is stated in the figures.

524

525 **COMPETING INTERESTS**

526 The authors declare no competing interests.

527

528 **AUTHOR CONTRIBUTIONS**

529 MCS, JPB, JSM, DN and JSS conceptualized the project. PRS and JSM
530 generated the Spnb5 deleted mice. MCS and JPB conducted animal breeding and
531 genotyping. MCS, JPB, SK, and DN performed gene expression and
532 immunohistochemistry analysis. JPB and LS performed assays to assess hearing
533 function. MCS and DN wrote the manuscript, and all authors contributed to its
534 editing and refinement.

535

536 **ACKNOWLEDGEMENTS**

537 These studies were supported by grants R01 DC 008130 and R01 DC 008130
538 from the National Institute of Health to JSS and DN and grants P01 NS35476
539 (project 4) and R01 HL28560 to JSM. Other support includes the Raymond Yesner
540 Endowment to Yale University (JSM). We also would like to thank Winston Tan,
541 Alexei Surguchev and Lan Ji for their technical assistance.

542

REFERENCES

- Abe, T., Kakehata, S., Kitani, R., Maruya, S., Navaratnam, D., Santos-Sacchi, J., & Shinkawa, H. (2007). Developmental expression of the outer hair cell motor prestin in the mouse. *J Membr Biol*, 215(1), 49-56. doi:10.1007/s00232-007-9004-5
- Bai, J. P., Surguchev, A., Ogando, Y., Song, L., Bian, S., Santos-Sacchi, J., & Navaratnam, D. (2010). Prestin surface expression and activity are augmented by interaction with MAP1S, a microtubule-associated protein. *J Biol Chem*, 285(27), 20834-20843. doi:10.1074/jbc.M110.117853
- Baker, K. E., & Parker, R. (2004). Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Curr Opin Cell Biol*, 16(3), 293-299. doi:10.1016/j.ceb.2004.03.003
- Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2013). GenBank. *Nucleic Acids Res*, 41(Database issue), D36-42. doi:10.1093/nar/gks1195
- Bramhall, N. F., McMillan, G. P., Kujawa, S. G., & Konrad-Martin, D. (2018). Use of non-invasive measures to predict cochlear synapse counts. *Hear Res*, 370, 113-119. doi:10.1016/j.heares.2018.10.006
- Chomczynski, P., & Mackey, K. (1995). Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. *Biotechniques*, 19(6), 942-945. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/8747660>
- Consortium, G. T. (2013). The Genotype-Tissue Expression (GTEx) project. *Nat Genet*, 45(6), 580-585. doi:10.1038/ng.2653
- Cortese, M., Papal, S., Pisciotano, F., Elgoyhen, A. B., Hardelin, J. P., Petit, C., . . . El-Amraoui, A. (2017). Spectrin betaV adaptive mutations and changes in subcellular location correlate with emergence of hair cell electromotility in mammals. *Proc Natl Acad Sci U S A*, 114(8), 2054-2059. doi:10.1073/pnas.1618778114
- Efimova, N., Korobova, F., Stankewich, M. C., Moberly, A. H., Stolz, D. B., Wang, J., . . . Svitkina, T. (2017). betaIII Spectrin Is Necessary for Formation of the Constricted Neck of Dendritic Spines and Regulation of Synaptic

- 576 Activity in Neurons. *J Neurosci*, 37(27), 6442-6459.
577 doi:10.1523/JNEUROSCI.3520-16.2017
- 578 El-Hassar, L., Song, L., Tan, W. J. T., Large, C. H., Alvaro, G., Santos-Sacchi, J.,
579 & Kaczmarek, L. K. (2019). Modulators of Kv3 Potassium Channels
580 Rescue the Auditory Function of Fragile X Mice. *J Neurosci*, 39(24), 4797-
581 4813. doi:10.1523/JNEUROSCI.0839-18.2019
- 582 Hafidi, A., Despres, G., & Romand, R. (1990). Cochlear innervation in the
583 developing rat: an immunocytochemical study of neurofilament and
584 spectrin proteins. *J Comp Neurol*, 300(2), 153-161.
585 doi:10.1002/cne.903000202
- 586 Hammarlund, M., Jorgensen, E. M., & Bastiani, M. J. (2007). Axons break in
587 animals lacking beta-spectrin. *J Cell Biol*, 176(3), 269-275.
588 doi:10.1083/jcb.200611117
- 589 Hoa, M., Olszewski, R., Li, X., Taukulis, I., Gu, S., DeTorres, A., . . . Kelley, M.
590 W. (2020). Characterizing Adult Cochlear Supporting Cell Transcriptional
591 Diversity Using Single-Cell RNA-Seq: Validation in the Adult Mouse and
592 Translational Implications for the Adult Human Cochlea. *Front Mol*
593 *Neurosci*, 13, 13. doi:10.3389/fnmol.2020.00013
- 594 Holley, M. C., & Ashmore, J. F. (1990). Spectrin, actin and the structure of the
595 cortical lattice in mammalian cochlear outer hair cells. *J Cell Sci*, 96 (Pt
596 2), 283-291. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/2211870>
- 597 Holley, M. C., Kalinec, F., & Kachar, B. (1992). Structure of the cortical
598 cytoskeleton in mammalian outer hair cells. *J Cell Sci*, 102 (Pt 3), 569-
599 580. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/1506434>
- 600 Kujawa, S. G., & Liberman, M. C. (2009). Adding insult to injury: cochlear nerve
601 degeneration after "temporary" noise-induced hearing loss. *J Neurosci*,
602 29(45), 14077-14085. doi:10.1523/JNEUROSCI.2845-09.2009
- 603 Legendre, K., Safieddine, S., Kussel-Andermann, P., Petit, C., & El-Amraoui, A.
604 (2008). α III-betaV spectrin bridges the plasma membrane and cortical
605 lattice in the lateral wall of the auditory outer hair cells. *J Cell Sci*, 121(Pt
606 20), 3347-3356. doi:10.1242/jcs.028134

- 607 Lewis, M. A., Nolan, L. S., Cadge, B. A., Matthews, L. J., Schulte, B. A., Dubno,
608 J. R., . . . Dawson, S. J. (2018). Whole exome sequencing in adult-onset
609 hearing loss reveals a high load of predicted pathogenic variants in known
610 deafness-associated genes and identifies new candidate genes. *BMC*
611 *Med Genomics*, 11(1), 77. doi:10.1186/s12920-018-0395-1
- 612 Li, Y., Liu, H., Giffen, K. P., Chen, L., Beisel, K. W., & He, D. Z. Z. (2018).
613 Transcriptomes of cochlear inner and outer hair cells from adult mice. *Sci*
614 *Data*, 5, 180199. doi:10.1038/sdata.2018.199
- 615 Liberman, M. C., & Kujawa, S. G. (2017). Cochlear synaptopathy in acquired
616 sensorineural hearing loss: Manifestations and mechanisms. *Hear Res*,
617 349, 138-147. doi:10.1016/j.heares.2017.01.003
- 618 Lin, H. W., Furman, A. C., Kujawa, S. G., & Liberman, M. C. (2011). Primary
619 neural degeneration in the Guinea pig cochlea after reversible noise-
620 induced threshold shift. *J Assoc Res Otolaryngol*, 12(5), 605-616.
621 doi:10.1007/s10162-011-0277-0
- 622 Liu, Y., Qi, J., Chen, X., Tang, M., Chu, C., Zhu, W., . . . Zhong, G. (2019).
623 Critical role of spectrin in hearing development and deafness. *Sci Adv*,
624 5(4), eaav7803. doi:10.1126/sciadv.aav7803
- 625 Lorenzo, D. N., Badea, A., Zhou, R., Mohler, P. J., Zhuang, X., & Bennett, V.
626 (2019). betaII-spectrin promotes mouse brain connectivity through
627 stabilizing axonal plasma membranes and enabling axonal organelle
628 transport. *Proc Natl Acad Sci U S A*, 116(31), 15686-15695.
629 doi:10.1073/pnas.1820649116
- 630 Maison, S. F., Pyott, S. J., Meredith, A. L., & Liberman, M. C. (2013).
631 Olivocochlear suppression of outer hair cells in vivo: evidence for
632 combined action of BK and SK2 channels throughout the cochlea. *J*
633 *Neurophysiol*, 109(6), 1525-1534. doi:10.1152/jn.00924.2012
- 634 Melcher, J. R., Knudson, I. M., Fullerton, B. C., Guinan, J. J., Jr., Norris, B. E., &
635 Kiang, N. Y. (1996). Generators of the brainstem auditory evoked potential
636 in cat. I. An experimental approach to their identification. *Hear Res*, 93(1-
637 2), 1-27. doi:10.1016/0378-5955(95)00178-6
- 638 Papal, S., Cortese, M., Legendre, K., Sorousch, N., Dragavon, J., Sahly, I., . . . El-
639 Amraoui, A. (2013). The giant spectrin betaV couples the molecular

- 640 motors to phototransduction and Usher syndrome type I proteins along
641 their trafficking route. *Hum Mol Genet*, 22(18), 3773-3788.
642 doi:10.1093/hmg/ddt228
- 643 Parkinson, N. J., Olsson, C. L., Hallows, J. L., McKee-Johnson, J., Keogh, B. P.,
644 Noben-Trauth, K., . . . Tempel, B. L. (2001). Mutant beta-spectrin 4 causes
645 auditory and motor neuropathies in quivering mice. *Nat Genet*, 29(1), 61-
646 65. doi:10.1038/ng710
- 647 Schuknecht, H. F., & Woellner, R. C. (1953). Hearing losses following partial
648 section of the cochlear nerve. *Trans Am Laryngol Rhinol Otol Soc*, 58(57th
649 Meeting), 369-393; discussion, 393-364. Retrieved from
650 <https://www.ncbi.nlm.nih.gov/pubmed/13146770>
- 651 Sergeyenko, Y., Lall, K., Liberman, M. C., & Kujawa, S. G. (2013). Age-related
652 cochlear synaptopathy: an early-onset contributor to auditory functional
653 decline. *J Neurosci*, 33(34), 13686-13694.
654 doi:10.1523/JNEUROSCI.1783-13.2013
- 655 Shen, J., Scheffer, D. I., Kwan, K. Y., & Corey, D. P. (2015). SHIELD: an
656 integrative gene expression database for inner ear research. *Database*
657 (Oxford), 2015, bav071. doi:10.1093/database/bav071
- 658 Shera, C. A., & Guinan, J. J., Jr. (1999). Evoked otoacoustic emissions arise by
659 two fundamentally different mechanisms: a taxonomy for mammalian
660 OAEs. *J Acoust Soc Am*, 105(2 Pt 1), 782-798. doi:10.1121/1.426948
- 661 Song, L., McGee, J., & Walsh, E. J. (2006). Frequency- and level-dependent
662 changes in auditory brainstem responses (ABRS) in developing mice. *J*
663 *Acoust Soc Am*, 119(4), 2242-2257. doi:10.1121/1.2180533
- 664 Song, L., & Santos-Sacchi, J. (2016). A Walkthrough of Nonlinear Capacitance
665 Measurement of Outer Hair Cells. *Methods Mol Biol*, 1427, 501-512.
666 doi:10.1007/978-1-4939-3615-1_28
- 667 Stabach, P. R., & Morrow, J. S. (2000). Identification and characterization of beta
668 V spectrin, a mammalian ortholog of Drosophila beta H spectrin. *J Biol*
669 *Chem*, 275(28), 21385-21395. doi:10.1074/jbc.C000159200

- 670 Stankewich, M. C., Cianci, C. D., Stabach, P. R., Ji, L., Nath, A., & Morrow, J. S.
671 (2011). Cell organization, growth, and neural and cardiac development
672 require alphaII-spectrin. *J Cell Sci*, 124(Pt 23), 3956-3966.
673 doi:10.1242/jcs.080374
- 674 Stankewich, M. C., Gwynn, B., Ardito, T., Ji, L., Kim, J., Robledo, R. F., . . .
675 Morrow, J. S. (2010). Targeted deletion of betaII spectrin impairs
676 synaptogenesis and generates ataxic and seizure phenotypes. *Proc Natl*
677 *Acad Sci U S A*, 107(13), 6022-6027. doi:10.1073/pnas.1001522107
- 678 Yue, F., Cheng, Y., Breschi, A., Vierstra, J., Wu, W., Ryba, T., . . . Mouse, E. C.
679 (2014). A comparative encyclopedia of DNA elements in the mouse
680 genome. *Nature*, 515(7527), 355-364. doi:10.1038/nature13992
681
682

Table1. Beta spectrin gene specific primers.

Primers				Amplimer (bps)	
Spnb1	forward	CAAAGAGCAGGAGGTGTCAGC	reverse	TCTAGGCAGGTGCTGAAGTTC	264
	nested forward	GATGCGTGTGCCGGCGCC	nested reverse	CTGGTATTGATCTCTGCCT	197
Spnb2	forward	CCGGTGGGAGTGGTTAAGAC	reverse	CATCCCAGGTGCTGCAGAC	188
	nested forward	GATTTGGAGGTCCATCAG	nested reverse	TCAAACGCCTCATGGCGCT	144
Spnb5	forward	CTCCGGAGCTTTGAGCAGGCTGC	reverse	CCTGAGCCCACGCTAGCAATTCC	439
	nested forward	GATACAGGAGAAGACCACC	nested reverse	TTCTGGACTTTGGCCTCC	319
Spnb5	forward	TGGCCTTCTCAGGGCCAAAGGT	reverse	GCACTCATCTGGATGCAGGGCTG	440
	nested forward	CTTGACAGGGAGGAGTTTGG	nested reverse	GCACTCATCTGGATGCAGGGCTG	313

Primer sets were used to detect for mRNA of Spnb1, Spnb2, and Spnb5 in the brain, organ of Corti, and isolated OHCs. The PCR amplimer sizes from mRNA are presented in the final column.

Table 2. Primers for the Spnb5 gene used to prepare the three DNA fragments that were subcloned into pEasy-Flox.

Fragment 1	forward	CAGCGGCCGACGGGGCTGCCTCCTACTGTACC	reverse	GAGCGGCCGCACTTGAATGACCCAGAGCCACCCCT
Fragment 2	forward	GGTCTAGACCTCTACTCGCTTAGGTTCCAATC	reverse	GCTCTAGATGACTCTACAGCAGACCCCAAGGTC
Fragment 3	forward	GACTCGAGCCTGTAGCTATTTCTGGCCTACCCCTCC	reverse	GACTCGAGGGAGGGAGGGTTACTGTAGTAGAAGGGC

The targeting vector for homologous recombination was constructed by cloning three PCR generated fragments into the pEASY-Flox vector. Fragment 1 is a 4115 bp product, fragment 2 is a 469 bp product that contains Spnb5 exon 2, and fragment 3 is 4371 bp of sequence that spans up to exon 5. The three fragments were sequence verified. The oligos contain common restriction sites at their 5' end for easy cloning into the targeting vector.

Table 3. Primers for rt-PCR in WT and Spnb5^{-/-} tissue.

Spnb5	exon 58/60	forward	CTCCGGAGCTTTGAGCAGGCTGC	reverse	CCTGAGCCCACGCTAGCAATTCC	439
Spnb5	exon 53/55	forward	AGGAGAAACACCAGAATTTGGAGAATG	reverse	TGTCCACTCTCCAGAAGGGCAACTG	412
Spnb2	exon 28/29	forward	GATACAGGAGAAGACCACC	reverse	TTCTGGACTTTGGCCTCC	144
Spna2	exon 54/55	forward	AACAGGAGTGACTGAGGAGG	reverse	CCTTCCTCAACCATTGGCAGA	144

These primer sets were tested on cDNA from testis isolated from WT and Spnb5^{-/-} 3-month-old mice. Because the C-terminus of these large genes tends to be more abundant than N-terminus exons, we designed primer sets that will amplify exons near the poly-A tail.

708
709
710
711
712
713
714
715
716
717
718