

The *TECTB*-C225Y Variant Causing Autosomal Dominant Deafness in a Nicaraguan Family Enhances Sensitivity to Noise-Induced Hearing Loss in Mice

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Abstract

Identifying new genes responsible for non-syndromic hearing loss remains a critical goal, as many individuals with hereditary deafness still lack a molecular diagnosis despite comprehensive genetic testing. The tectorial membrane (TM) is a specialized, collagen-rich, acellular matrix of the inner ear, essential for stimulating mechanosensitive hair cell bundles during sound transduction, and its structural integrity is critical for frequency tuning and auditory sensitivity. Although mutations in genes encoding a number of non-collagenous proteins found in the TM (*TECTA*, *CEACAM16*, *OTOG*, *OTOGL*) have been identified as deafness genes, definitive evidence implicating β -tectorin (*TECTB*) in human hearing loss has been lacking.

Here, we present multiple lines of genetic and experimental evidence linking a missense variant in *TECTB* (c.674G>A, p.Cys225Tyr) to autosomal dominant, non-syndromic hearing loss in a multigenerational family. The variant alters one of eight highly conserved cysteines present within the zona pellucida (ZP) domain of *TECTB* and is predicted to disrupt protein folding and matrix assembly. Using a *Tectb*-C225Y knock-in mouse model, we show that homozygous animals exhibit severe hearing loss and profound disruption of TM morphology, while heterozygote animals display decreased matrix content within the TM and increased susceptibility to noise-induced hearing loss—despite normal auditory thresholds.

These findings identify *TECTB* as a novel human deafness gene, further elucidate its structural role in maintaining TM integrity, and highlight its contribution to resilience against environmental and age-related auditory decline.

Key words: Hearing loss, deafness, tectorial membrane, beta-tectorin (*TECTB*), striated sheet matrix.

Introduction

Hearing loss is the most prevalent human sensory deficit (Armstrong et al., 2022) and can result from disruptions at any point along the auditory pathway. Amplification and transduction of sound stimuli are mediated by hair cells, the mechanosensory cells of the inner ear (Robles 2001, Corey 1979). The hair cells are arrayed along the tonotopic axis of the cochlea and specific regions are tuned to respond best to different sound frequencies.

Housed between the basilar and tectorial membranes and located within the organ of Corti, the hair cells respond locally to frequency-specific mechanical stimuli. The outer hair cells facilitate amplification of sound-induced basilar membrane motion through electromotility – rapid cycles of contraction and elongation – while inner hair cells convert mechanical stimuli into neural signals that are transmitted via the auditory nerve to the brain (Dallos, 2008). The defining feature of hair cells is a bundle of stereocilia, a set of actin-rich hair-like projections on the apical surface of each cell. Bundle deflection generates tension in links interconnecting the stereocilia, thereby opening mechanosensitive ion channels at the tips of all but the tallest stereocilia (Corey & Hudspeth, 1979; Robles & Ruggero, 2001). The tectorial membrane (TM) shears relative to the apical surface of the organ of Corti in response to sound waves, applying force to the hair bundles and facilitating mechanoelectrical transduction (Hakizimana & Fridberger, 2021; Verpy et al., 2011). The structural integrity of the TM is therefore critical for cochlear sensitivity, frequency discrimination – the sharpness of tuning along the tonotopic axis – and gain (Legan et al., 2000).

The TM is a specialized structure essential for auditory transduction that is composed, in mammals, of collagen fibers that are organized by and imbedded in a matrix formed by a number of non-collagenous glycoproteins including CEACAM16, α -tectorin (TECTA) and β -tectorin (TECTB) (Goodyear & Richardson, 2018). These proteins interact to form the striated sheet matrix (SSM), an acellular structure critical for effectively coupling mechanical sound stimulation to hair cell stereocilia deflection (Goodyear & Richardson, 2002). Among them, TECTA is the largest and most structurally complex, with a predicted molecular mass of ~239 kDa and 33 potential sites for N-glycosylation, while TECTB is smaller (~36 kDa) with just 4 potential N-glycosylation sites. Despite these differences, TECTA and TECTB contain a zona pellucida (ZP) domain (Jovine et al., 2005), a domain primarily defined by 8 highly conserved cysteine residues that form intrachain disulphide bonds and is responsible for polymerization in many proteins (Bokhove & Jovine, 2018).

The structural integrity of the TM must be preserved throughout life to ensure proper auditory function (Richardson et al., 2008). Key components of the TM such as TECTA and TECTB are predominantly expressed during early development (Rau et al., 1999), and the TM itself undergoes progressive deterioration with age, eventually becoming less dense (Bullen et al., 2020). This age-related degradation is exacerbated in mice lacking CEACAM16—a TM protein first expressed just before the onset of hearing in mice at ~postnatal day 12—highlighting the importance of ongoing molecular maintenance by specific structural components (Goodyear et al., 2019). In addition to aging, noise exposure has been shown to disrupt TM structure across multiple species, with significant morphological alterations and associated hearing loss (Adler et al., 1995; Canlon, 1987; Froymovich et al., 1995). Damage to the TM has also been documented in human temporal bone samples from individuals with histories of high noise exposure (Ishai, 2019). Although birds appear to exhibit limited TM regeneration following noise (Adler et al., 1995)—most notably within its basal lattice-like structures—similar regenerative capacity has not been observed in mammals.

Mutations in *TECTA* are a known cause of autosomal dominant and recessive non-syndromic hearing loss, recognizing it as a deafness gene (Verhoeven et al., 1998). Specifically, a dominant variant in *TECTA*, reported in a Spanish family and affecting the ZP domain caused mid-frequency hearing loss (Moreno-Pelayo et al., 2001), while several other variants within the same domain of TECTA are reported to cause different phenotypes, including prelingual non-progressive pan-frequency hearing loss and postlingual progressive high-frequency hearing loss (Moreno-Pelayo et al., 2001; Verhoeven et al., 1998). In studies using mouse models carrying *Tecta* variants identified in patients,

mutations within the ZP domain were reported to cause hearing loss and morphological abnormalities within the TM, including disruption of highly specific structural components (Legan et al., 2014; Legan et al., 2005).

Due to reported sensitivity to sound-induced seizures in several existing *Tecta* mutant mouse models, the effects of noise trauma could not be evaluated. Specifically, three knock-in lines carrying human *TECTA* variants linked to autosomal dominant hearing loss—*Tecta-C1619S/+*, *Tecta-C1837G*, and *Tecta-L1820F,G1824D/+*—all exhibited audiogenic seizures when exposed to octave-band white noise, even at low sound pressure levels (Legan et al., 2014), whereas wild-type littermates remained unaffected. This seizure sensitivity is likely attributable to the *S129SvEv* genetic background used in these models, as mice on this background are known to be more susceptible to audiogenic seizures than the commonly used, seizure-resistant *C57BL/6* mice. Another model, *Tecta-Y1870C* (Legan et al., 2005), was not explicitly tested for seizure sensitivity but displayed similar TM histological abnormalities, mild changes in basilar membrane tuning and distortion product otoacoustic emission (DPOAE) responses, alongside disproportionately large deficits in neural sensitivity and neural tuning. Thus, even subtle structural defects in the TM can profoundly alter cochlear function.

Although we previously reported that *Tectb* knockout mice (*Tectb*^{-/-}) display sharpened tuning, altered SSM organization and low-frequency hearing loss (Ghaffari et al., 2010; Russell et al., 2007), pathogenic variants in *TECTB* have not been previously linked to human hearing loss. In this study, we report a missense variant in *TECTB* NM_058222.3:c.674G>A, p.Cys225Tyr, hereafter abbreviated as *TECTB*-C225Y, in a multigenerational Nicaraguan family that segregates autosomal dominant, mild-to-severe hearing loss. The *TECTB*-C225Y substitution affects a conserved cysteine within the ZP domain—consistent with previously reported dominant missense *TECTA* variants that disrupt ZP-domain-mediated SSM assembly and cause autosomal dominant hearing loss in patients (Verhoeven et al., 1998). To study this variant, we developed a knock-in *Tectb*-C225Y mouse model and characterized auditory function and TM histology across the lifespan, and in response to noise trauma, assessing the extent and nature of the resulting deficit. Our findings establish *TECTB* as a novel human deafness gene and provide insight into the structural role of β -tectorin in TM integrity and cochlear function.

Results

The male proband (II:4) was born to healthy, non-consanguineous parents (**Fig. 1A**) and was in his late 60s at the time of his initial evaluation for this study. Three of the proband's five children have hearing loss, as well as his sibling and other distant family members. Newborn hearing screening was not performed. Hearing loss was reported to start between 6 and 15 years of age in all affected individuals. The proband and two of the affected family members underwent a follow-up evaluation more than a decade after enrolment in the study, providing an opportunity to assess hearing loss progression.

Risk factor questionnaire

A detailed hearing loss risk factor questionnaire was completed by all available family members prior to genetic analysis based on affected status as determined by pure-tone audiometry (**Supplementary Table 1**). Although none of the affected individuals reported excessive noise exposure, accurate assessments of noise exposure are difficult and may be underestimated in rural agricultural settings. None of the individuals who completed the questionnaire reported a history of maternal infection, otorrhea, alcohol consumption during pregnancy or exposure to aminoglycoside antibiotics such as gentamicin. Half of the respondents reported exposure to pesticides, including four of six (66.7%) affected individuals (II:5, III:2, III:5, and III:6), although the timing and extent of exposure were not specified. None of the six affected individuals reported birth defects or prematurity. Four of six (66.7%) affected individuals (II:4, II:5, III:3, and III:6) reported balance difficulties, although these were not formally evaluated with videonystagmography or other clinical vestibular testing. One individual

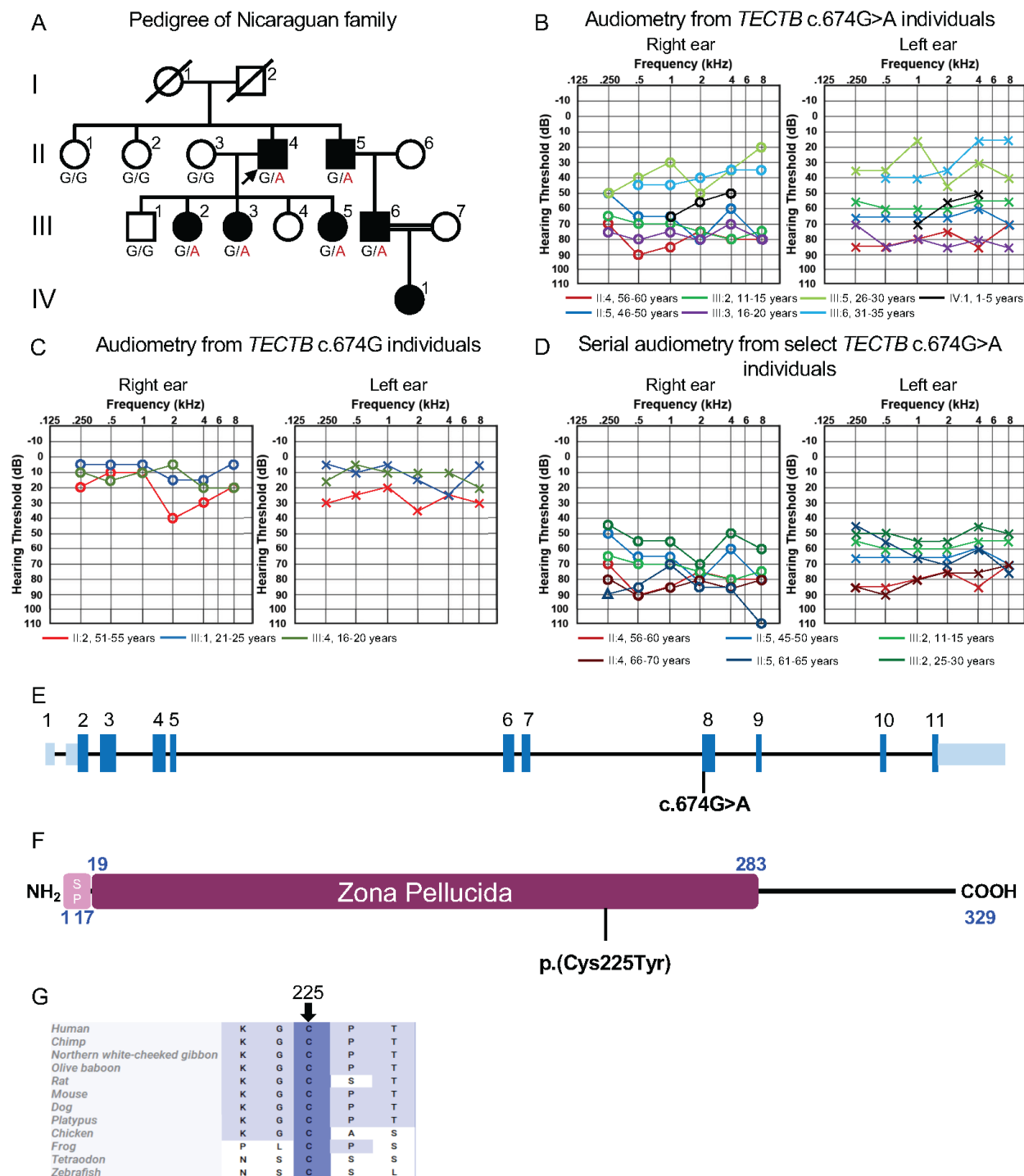


Figure 1. *TECTB*-C225Y Variant Segregates with Non-syndromic Hearing Loss in a Nicaraguan Family. (A) An abbreviated pedigree of the multigenerational family showing segregation of the heterozygous *TECTB* c.674G>A, p.Cys225Tyr variant. Filled symbols indicate affected individuals; open symbols indicate unaffected individuals. The proband II:4 is denoted with an arrow. Deceased individuals are indicated by a diagonal line. The variant nucleotide substitution “A” is shown in red. (B) Pure-tone audiograms of affected individuals II:4 (red), II:5 (dark blue), III:2 (dark green), III:3 (purple), III:5 (light green), III:6 (light blue), and IV:1 (black), with corresponding ages at the time of testing in the legend below. Air-conduction thresholds in dB hearing level are represented by circles (right ear) and crosses (left ear). (C) Pure-tone audiograms of unaffected individuals II:2 (red), III:1 (blue), and III:4 (green) carrying the reference allele, with ages at the time of evaluation presented in the legend below. Symbols as in panel B. (D) Follow-up pure-tone audiograms from II:4, II:5 and III:2 that span 12, 16, and 16 years, respectively. (E) Schematic representation of the *TECTB* (NM_058222.3), with coding exons shown in dark blue boxes and untranslated sequences in light blue. The identified variant maps to exon 8. (F) Domain structure of the *TECTB* protein, showing the signal peptide (SP, light maroon), and the conserved zona pellucida domain (ZP, maroon). The location of the C225Y substitution is indicated with a black bar. (G) Multiple sequence alignment of *TECTB* orthologues highlighting the conserved cysteine residue at position 225 (arrow), substituted in the affected family.

(II:5) reported a history of otitis media that resolved with treatment, and another (II:4) reported a temporary threshold shift following noise exposure (16.7% of affected individuals for each observation).

Pure-tone audiometry shows stable, mild to severe, sensorineural hearing loss

Pure-tone audiometry of participating individuals revealed symmetrical, bilateral sensorineural hearing loss (**Fig. 1B**). Follow up measurements are available from II:4, II:5 and III:2 that span 12, 16, and 16 years, respectively (**Fig. 1D**). At their initial assessment at age 56-60, the proband (II:4) showed severe sensorineural hearing loss (PTA_{0.5-4kHz} 81.25 dB HL), which had remained unchanged at the age of 66-70 (PTA_{0.5-4kHz} 80 dB HL). One of their children also demonstrated severe hearing loss (III:2, PTA_{0.5-4kHz} 73.75 dB HL) at age 11-15, which had improved to moderate hearing loss by age 26-30 (PTA_{0.5-4kHz} 51.25 dB HL). Another child (III:5, PTA_{0.5-4kHz} 31.25 dB HL) at the age of 21-25 years and a cousin (III:6, PTA_{0.5-4kHz} 32.50 dB HL) at the age of 26-30 presented with mild hearing loss. Moderate hearing loss was observed in another child (III:3, PTA_{0.5-4kHz} 58.75 dB HL) at age 16-20, a distant relative (IV:4, PTA_{1.0-4kHz} 58.33 dB HL) at age 1-5, and the proband's sibling (II:5, PTA_{0.5-4kHz} 63.75 dB HL) at age 46-50, which had remained unchanged at the age of 66-70 (PTA_{0.5-4kHz} 62.5 dB HL). Serial audiometry was not available for a subset of individuals but collectively, the available PTA_{0.5-4kHz} data reveal substantial intrafamilial variability, ranging from mild to severe hearing loss, without progression. Otoacoustic emissions were bilaterally absent in the most recent measurement from II:4, II:5 and III:2 at the age of 66-70, 61-65, and 26-30 years, respectively.

A *TECTB* variant segregates with autosomal dominant non-syndromic hearing loss

Gene panel sequencing and analysis of 74 hearing loss-associated genes was performed on genomic DNA from the proband but yielded no diagnostic findings, leaving the family genetically undiagnosed. To further investigate the possible genetic cause, two-point linkage analysis of the genome scan markers was conducted, revealing several loci with elevated logarithm of the odds (LOD) scores. A genome-wide multipoint LOD score representation of 22 autosomes identified five peaks above 2.0 on chromosomes 2, 9, 10, 14 and 15 (**Supplementary Figure 1A**), with a maximum multipoint LOD score of 2.2 at rs11195666 and rs12354968 on chromosome 10 (**Supplementary Figure 1B**) – a region that includes *TECTB* and four other genes (*GPAM*, *ACSL5*, *ZDHHC6*, and *VT11A*) (**Supplementary Figure 1C**).

Subsequent exome sequencing of the genomic DNA of the proband generated 195,746,072 total reads, of which 158,815,063 aligned with high quality, and 85.81% achieved a target coverage of 20x. Analysis of linkage intervals, as well as exome-wide analysis identified a heterozygous *TECTB* NM_058222.3:c.674G>A, p.Cys225Tyr missense variant in exon 8 (**Fig. 1E**, **Table 1**, **Supplementary Figure 2**). This variant results in an amino acid substitution of a small, polar cysteine to a larger aromatic tyrosine in the ZP domain (**Fig. 1F**), a region known to mediate extracellular matrix assembly. Multiple *in silico* prediction tools unanimously classified the variant as deleterious/damaging (**Table 1**), and phyloP analysis confirmed conservation of the affected nucleotide across multiple species (**Fig. 1G**). The *TECTB*-C225Y variant was absent from major population databases, including *gnomAD* v4.1.0 and the *All of Us* Data Browser.

Next, we performed a segregation analysis using Sanger sequencing (primers: *TECTB* Exon 8 F: 5'-CTCGGGAGCCTTGTGTGTAA-3' and *TECTB* Exon 8 R: 5'-CTCCTCCAATACTGGCACCC-3') revealing complete co-segregation of the *TECTB*-C225Y variant with the hearing loss phenotype across the family (**Fig. 1A**). Twelve additional rare variants identified in the exome sequencing data were excluded based on lack of segregation or predicted pathogenicity (**Supplementary Table 2**).

Among the three unaffected individuals carrying only the reference allele – II:2 (age 51-55), III:1 (age 21-25) and III:4 (age 16-20) – PTA_{0.5-4kHz} were within normal limits (**Fig. 1C**). Of note, individual II:2 showed elevated thresholds at 2 kHz (40 and 35 dB in right and left ears, respectively), though these findings fall within the expected range of age-related hearing decline and are not considered disease-associated.

Table 1. Summary of *TECTB* variant and analysis results

Self-reported ancestry	Nicaraguan
Molecular genetics summary	
Chr10 g. position (GRCh37)	g.114057829G>A
Chr10 g. position (GRCh38)	g.112298071G>A
<i>TECTB</i> c. position (NM_058222.3)	c.674G>A
<i>TECTB</i> p. position (NP_478129.1)	p.(Cys225Tyr)
Zygosity	Heterozygous
<i>In silico</i> predictions	
phyloP [-19.0;11.0]	7.93
SIFT	Deleterious
PolyPhen-2	Probably Damaging
FATHMM	Damaging
MutationTaster	Deleterious
REVEL	Damaging
CADD	28.9
ClinPred	Damaging
Splicing Predictions	NR
gnomAD v4.1.0 MAF	0
All of Us Data Browser	0

Abbreviations: MAF, minor allele frequency; NR, Not remarkable

***Tectb*^{C225Y/C225Y} Mice Show a Severe Hearing Deficit**

The *Tectb*-C225Y mouse was created using the CRISPR-Cas9-mediated genome editing approach, introducing a c.674G>A (TGC to TAC) substitution in exon 8 of the *Tectb* gene to mimic the human C225Y variant (**Supplementary Figure 3**). The initial editing was performed in *C57Bl6N* background, which carries the *Cdh23*^{Ahl} allele known to cause early-onset age-related hearing loss. Following validation, the mutation was backcrossed onto a *C57Bl6-Cdh23*^{Ahl/+} mouse strain (JAX#002756) with the corrected *Cdh23* allele to preserve normal hearing. The resulting *TECTB*-C225Y mice appeared healthy, exhibited normal fertility, and produced offspring consistent with Mendelian ratios.

To evaluate the functional impact of the *TECTB*-C225Y substitution on cochlear function, *Tectb*^{C225Y/C225Y}, *Tectb*^{C225Y/+}, and wild-type mice were assessed using auditory brainstem response (ABR, **Fig. 2A**) and distortion product otoacoustic emission (DPOAE, **Fig. 2B**) testing at 5 weeks and at 3, 6, 9, 12, and 18 months of age. Across the assessed timepoints, *Tectb*^{C225Y/C225Y} mice exhibited severe, progressive sensorineural hearing loss, with average ABR threshold of approximately 60 dB SPL at 11.2 kHz (best frequency) at 5 weeks, and profound deafness in most animals by 12 months. In contrast, *Tectb*^{C225Y/+} mice retained normal ABR thresholds across all timepoints and frequencies, revealing levels of auditory performance similar to wild-type and consistent with normal age-related decline. We next assessed the peak 1 latency and amplitude of the ABR waves in response to 11.2 kHz pure tone stimulation in *Tectb*^{C225Y/+} and wild-type mice, revealing no statistically significant differences (**Supplementary Figure 4**).

Surprisingly, DPOAE thresholds revealed a milder phenotype compared to ABRs. Although *Tectb*^{C225Y/C225Y} mice displayed progressive elevation of DPOAE thresholds with age, especially at higher frequencies, this progression was slower and less severe than in ABR measurements. Notably, at younger ages and up to 12 months for some frequencies, DPOAE thresholds in *Tectb*^{C225Y/C225Y} mice remained partially preserved, suggesting some retained OHC function despite early ABR deficits. In *Tectb*^{C225Y/+} mice, DPOAE thresholds were nearly indistinguishable from wild-type controls at all ages, further underscoring the subtlety of the baseline phenotype in heterozygotes.

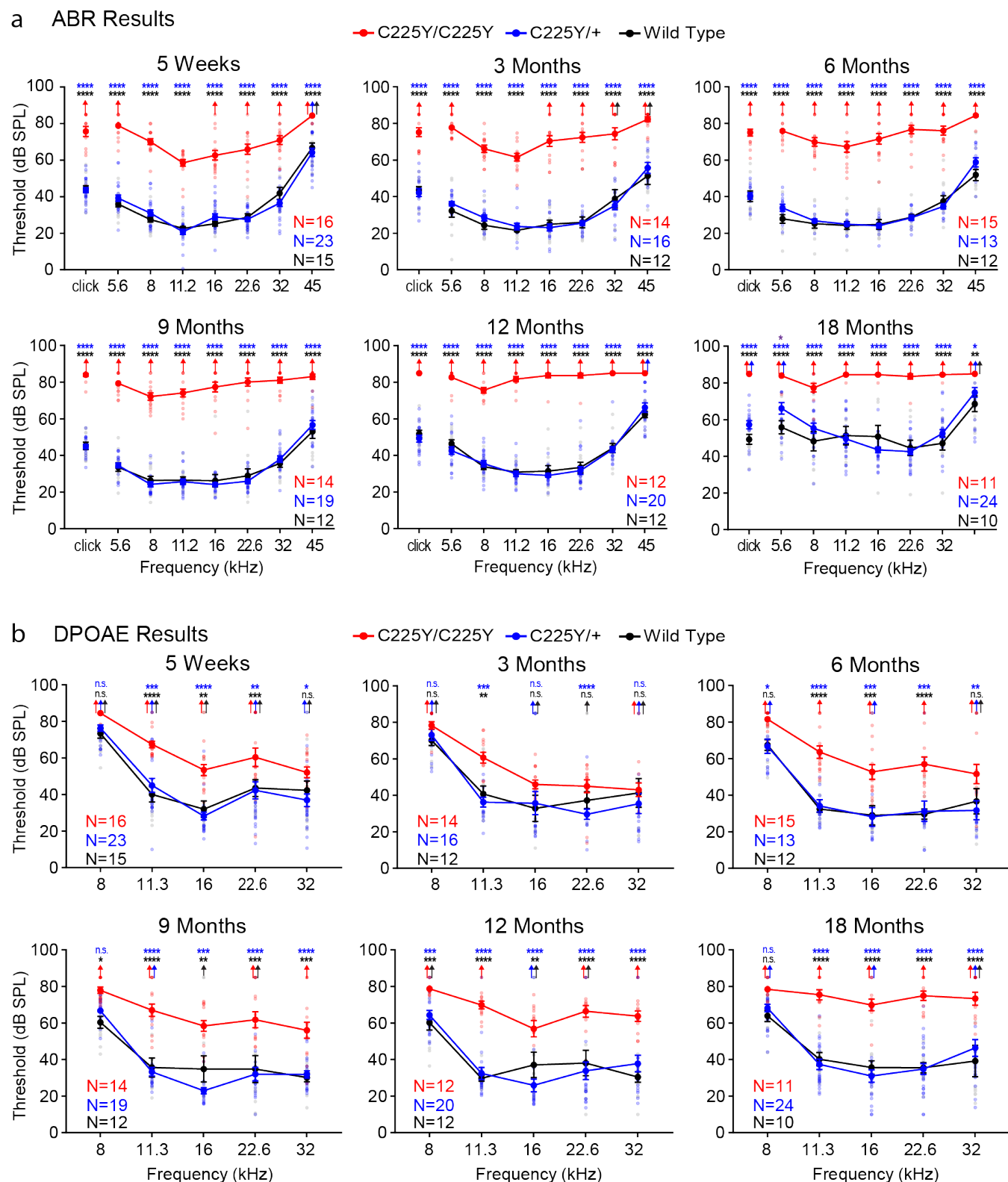


Figure 2. *Tectb*^{C225Y/C225Y} Mice Exhibit a Severe Hearing Deficit. (A) Auditory brainstem response (ABR) and (B) distortion product otoacoustic emission (DPOAE) thresholds of *Tectb*-C225Y mice tested at 5 weeks, and 3, 6, 9, 12, and 18 months of age. Data are shown as individual values with an overlaid mean $\pm$ SEM. Upward-pointing arrows indicate that at the highest sound pressure level tested (80 dB SPL), at least one mouse in the group had no detectable response at that frequency; these values were entered as 85 dB for analysis. *Tectb*^{C225Y/C225Y} mice exhibited significantly higher ABR and DPOAE thresholds than both *Tectb*^{C225Y/+} (blue asterisks) and wild type (black asterisks) mice across all tested ages. No significant differences were observed between *Tectb*^{C225Y/+} and wild-type mice at all but one data point (purple asterisk). The number of animals per group is indicated within each plot. Statistical analysis: two-way ANOVA with a matched design between frequencies for each mouse. Šidák's multiple comparison test was to evaluate differences between genotypes at each frequency; ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Overall, these findings differ from the dominant inheritance observed in the family segregating the *TECTB*-C225Y allele, in which a single copy of the variant allele is sufficient to cause significant levels of hearing loss. To explore the underlying cause of this discrepancy and to evaluate the validity of the mouse model in recapitulating the human phenotype, we next performed histological analysis of the TM in mice.

TECTB-C225Y Increases Tectorial Membrane Volume and Decreases Matrix Content

Following ABR and DPOAE testing, cochleae were fixed, resin-embedded and sectioned to allow morphological analysis of the TM. A previously established sectioning approach (Cheatham et al., 2014; Goodyear et al., 2019) using 1 μ m sections stained with toluidine blue was used to visualize TM profiles corresponding to the 4, 8, 20 and 40 kHz frequency regions of the cochlea on a single mid-modiolar cross-section (**Fig. 3A**).

At two months of age, *Tectb*^{C225Y/C225Y} cochleae exhibited, as expected, a gradient in TM size and shape from apex to base; however, severe structural abnormalities were already evident. The cross-sectional area of the TM was moderately enlarged in the 20 kHz region and was considerably increased in the 8 kHz region across all time points. Despite this enlargement, toluidine blue staining was more diffusely distributed in *Tectb*^{C225Y/C225Y} samples, suggesting the change in size reflected an increase in areas devoid of staining rather than an increase in TM material (**Fig. 3A**).

By contrast, the TM morphology in *Tectb*^{C225Y/+} cochleae was grossly similar to that of wild-type controls. However, an increase in regions devoid of toluidine blue staining was consistently observed in the central body of the TM, particularly in the apical, low-frequency region of the cochlea. The lower matrix density observed in regions of the TM in young *Tectb*^{C225Y/+} mice resembles that seen to a greater extent in older mice by 14 months of age. Whether this is a consequence of early-onset degradation or failure in matrix formation/deposition remains to be resolved (**Fig. 3A**).

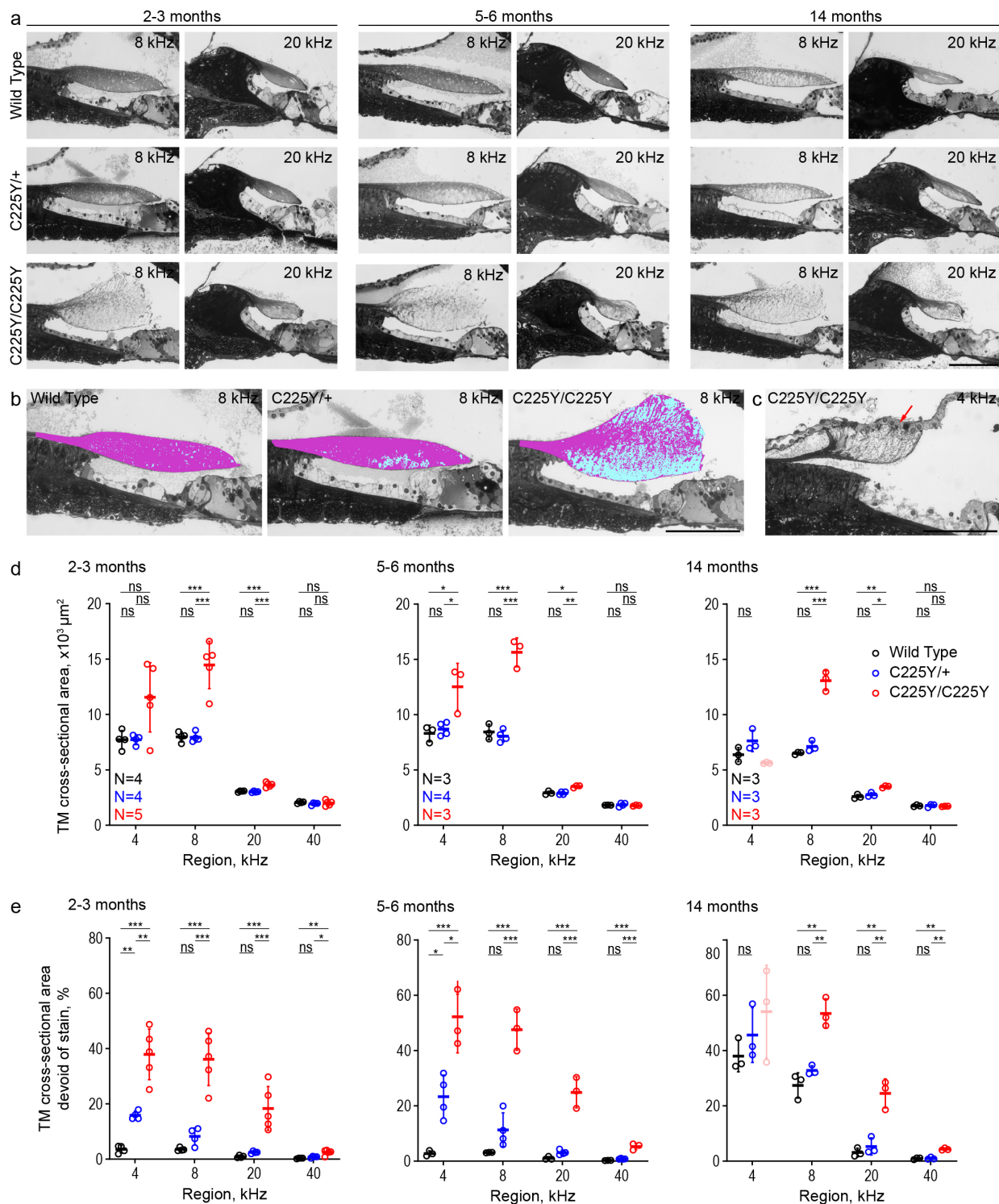
To quantitatively assess TM morphology, we performed blinded morphometric analysis using adaptive thresholding, measuring cross-sectional area and staining intensity, with the latter used as a measure of matrix content (**Fig. 3B**). For each image, the outer border of each TM was manually traced to calculate the area (**Fig. 3D**). A threshold was then applied to mask the stained regions of the TM, allowing quantification of unstained area (see **Fig. 3B** compared to **Fig. 3A** images of 2-3-month-old TMs at 8 kHz), expressed as a percentage of the total TM cross-sectional area (**Fig. 3E**). This metric served as a proxy measure of matrix content within the TM.

At the apical end of *Tectb*^{C225Y/C225Y} cochleae, the TM was frequently found to be completely disintegrated or displaced and bound to Reissner's membrane (**Fig. 3C red arrow**), precluding reliable quantification in this region. Therefore, measurements from this location of *Tectb*^{C225Y/C225Y} cochleae were excluded from further analysis. Quantification of all other images revealed a significant increase in TM cross-sectional area in *Tectb*^{C225Y/C225Y} mice relative to wild type mice across all cochlear locations except the extreme basal, 40 kHz region (**Fig. 3D**). *Tectb*^{C225Y/+} mice, by contrast, showed no significant difference in TM area compared to wild-type mice.

In contrast to area measurements, the proportion of the TM cross-section lacking stain—an indirect measure of TM matrix content—was elevated in *Tectb*^{C225Y/C225Y} and to a lesser extent in *Tectb*^{C225Y/+} mice as compared to wild-type controls (**Fig. 3E**). Notably, this increase was especially prominent in the apical, low-frequency regions of the cochlea, even in *Tectb*^{C225Y/+} mice that otherwise displayed normal TM area values. An age-associated increase in cross-sectional area devoid of stain was also observed in wild-type mice at later time points, suggesting that the *TECTB*-C225Y variant might accelerate structural degradation of the TM (**Fig. 3E**).

TECTB-C225Y disrupts Hensen's Stripe, the Marginal Band, and the Striated Sheet Matrix

In addition to the observed changes in TM thickness and matrix content, the *TECTB*-C225Y variant also caused subtle but consistent disruptions in structural and ultrastructural features of the TM (**Fig. 4**). Specifically, abnormalities were noted in the morphology of the marginal band and Hensen's stripe



(Fig. 4A, arrows and arrowheads, respectively)—two key features of the TM—in both *Tectb*^{C225Y/+} and *Tectb*^{C225Y/C225Y} mice. These differences were most pronounced in the basal, high-frequency region of the TM, where such structural features are most readily identified in cross-sections.

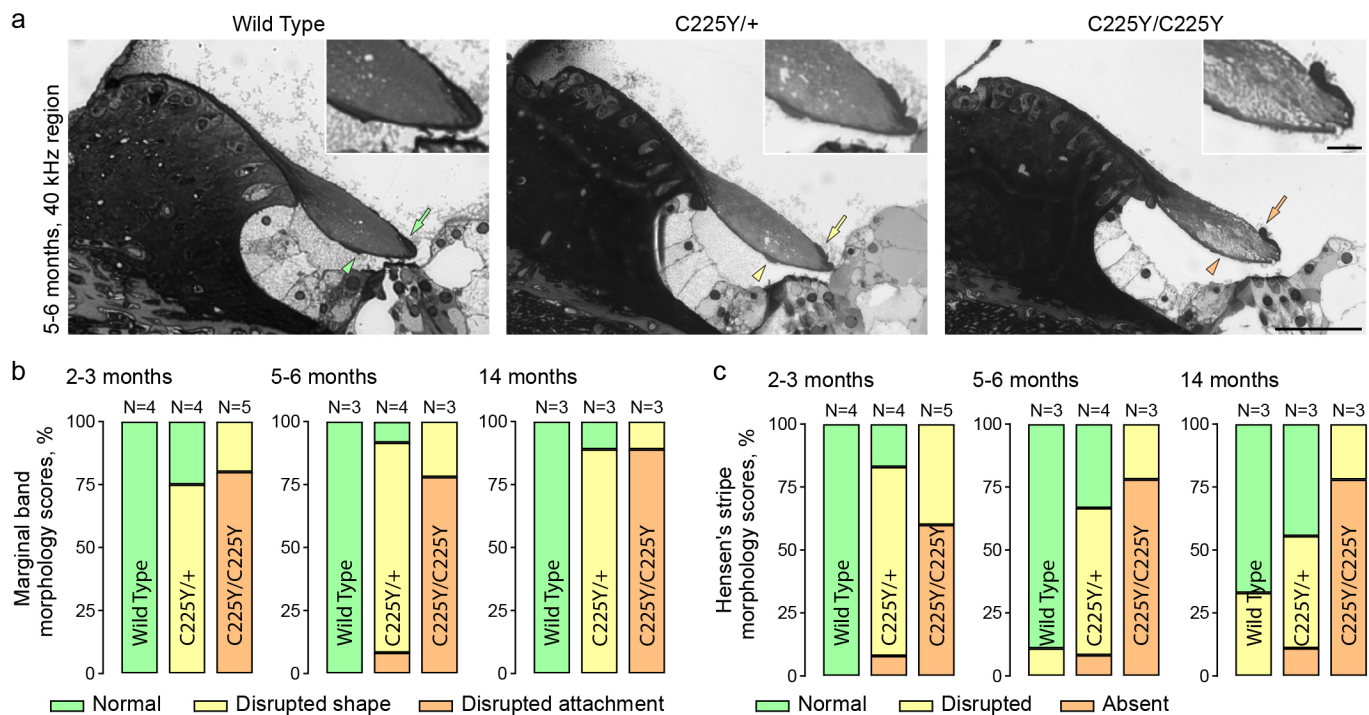


Figure 4. TECTB-C225Y disrupts TM ultrastructure. (A) Representative micrographs of toluidine blue-stained 1 μ m resin sections from the 40 kHz region of wild-type, *Tectb*^{C225Y/+} and *Tectb*^{C225Y/C225Y} cochleae. Arrowheads indicate Hensen's stripe, arrows indicate the marginal band. (B) and (C), pooled morphology scores from blinded reviewers assessing the integrity of the marginal band (B) and Hensen's stripe (C) in TM cross-sections from the 40 kHz region. Scoring was based on structural continuity and deviation from typical shape. The number of animals per group is indicated within each plot.

To quantify these observations, three investigators independently evaluated marginal band and Hensen's stripe morphology in cross-sections at the 40 kHz region while blinded to genotype. The pooled scores are presented in Fig. 4B-C. While individual cross-sections showed some variability, morphology scores consistently segregated by genotype, suggesting a genotype-dependent structural deficit.

In *Tectb*^{C225Y/+} mice, the TM marginal band frequently appeared less smooth and less rounded compared to the uniform, curved morphology observed in wild-type mice (Fig. 4A, arrows). These deficits were further exaggerated in *Tectb*^{C225Y/C225Y} mice, where the marginal band was often raised or deformed at its upper edge. Graded disruption was also evident in Hensen's stripe, ranging from altered morphology in *Tectb*^{C225Y/+} mice to complete absence in some *Tectb*^{C225Y/C225Y} sections, and disruption to stereotyped morphology in other *Tectb*^{C225Y/C225Y} TMs (Fig. 4A, arrowheads). Notably, occasional disruption of Hensen's stripe morphology was also observed in older wild-type mice (Fig. 4C), further suggesting a potential age-related degradation component, or that these phenotypes could be indicative of accelerated aging of the TM in *Tectb*^{C225Y/+} mice.

Finally, the TM cross-sections were evaluated using transmission electron microscopy (TEM). Ultrastructural analysis revealed that *Tectb*^{C225Y/C225Y} mice lacked the stereotypical SSM architecture characteristic of wild-type and *Tectb*^{C225Y/+} tectorial membranes, indicating a profound disruption of matrix organization (Fig. 5). Interestingly, anti-TECTB immunolabeling in cochlear cryosections demonstrated that TECTB-C225Y protein is still present in the TM of *Tectb*^{C225Y/C225Y} mice (Supplementary Fig. 5), despite the complete absence of the SSM, suggesting that the C225Y substitution does not prevent protein expression or localization to the TM, but likely disrupts its ability to assemble into the striated-sheet matrix. This severe ultrastructural phenotype in *Tectb*^{C225Y/C225Y} mice

is consistent with their profound ABR and DPOAE deficit. In contrast, *Tectb*^{C225Y/+} mice showed only mild morphological disruptions in the TM, while reporting no apparent ABR and DPOAE deficit. The mild structural phenotype accompanied by normal auditory thresholds in *Tectb*^{C225Y/+} mice prompted us to test whether environmental stress factors, such as noise exposure, might unmask latent auditory vulnerability.

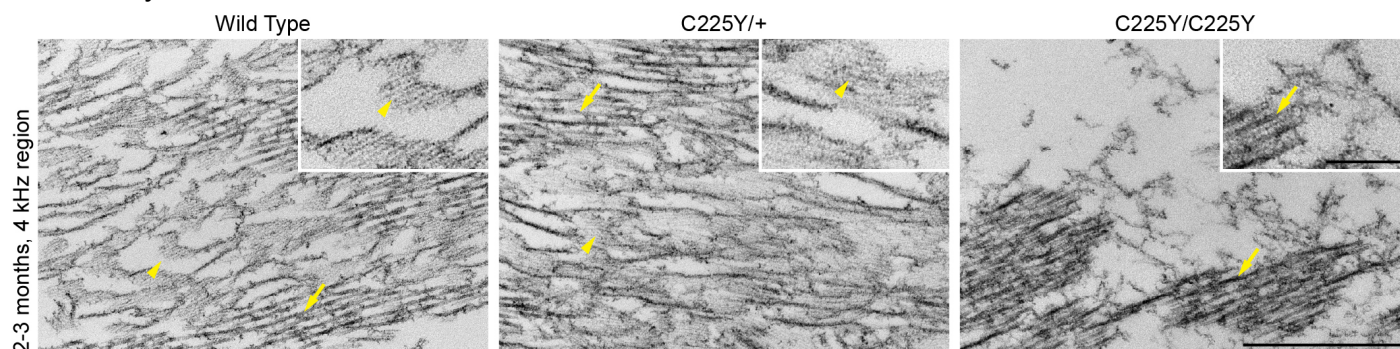


Figure 5. *Tectb*^{C225Y/C225Y} tectorial membranes lack striated sheet matrix. Transmission electron micrographs from the central body of tectorial membranes in the 4 kHz region of 2–3-month-old mice. Arrows point to collagen fibers; arrowheads point to striated sheet matrix. Scale bars, 400 nm (main panels) and 100 nm (insets).

***Tectb*^{C225Y/+} Mice Exhibit Increased Susceptibility to Noise Insult**

The Nicaraguan family exhibited a clear autosomal-dominant phenotype. In contrast, *Tectb*^{C225Y/+} mice raised in a relatively quiet, controlled animal facility environment displayed normal ABR and DPOAE thresholds despite mild morphological defects in the TM. We reasoned that, unlike laboratory mice, humans are routinely exposed to environmental noise, which might unmask a latent vulnerability conferred by the *TECTB*-C225Y variant, potentially explaining the apparent species discrepancy.

To test this hypothesis, we exposed cohorts of 16-week-old and 18-month-old mice to 8-16 kHz octave-band noise at 100 dB SPL for 2 hours, reported as the highest non-neuropathic noise level known to induce temporary threshold shifts (TTS) in mice of this age (Jansen et al., 2015). ABRs and DPOAEs were measured before exposure and again at 2 days and 2 weeks post-exposure (**Fig. 6**). At both ages, and at both post-exposure time points, *Tectb*^{C225Y/+} mice exhibited significantly greater ABR threshold shifts than wild-type littermates. While 16-week-old wild-type mice exhibited near-complete recovery by two weeks—a pattern consistent with TTS—*Tectb*^{C225Y/+} mice failed to recover, instead developing a permanent threshold shift (PTS) of up to 28.46 ± 5.13 dB SPL. In the 18-month-old cohort, both wild-type and *Tectb*^{C225Y/+} mice exhibited a PTS, though the threshold shifts were significantly larger in *Tectb*^{C225Y/+} group.

Importantly, DPOAEs were comparable between genotypes at all time points, suggesting that the enhanced vulnerability is not driven by OHC dysfunction but rather by compromised TM integrity. Given the high variability in DPOAE threshold shift measurements—particularly at low and high frequencies, which we attribute to equipment limitations at these edge frequencies—we next analyzed DPOAE peak amplitude growth as a function of sound intensity for the 22.4 kHz stimulus (**Supplementary Figures 6-7**). This analysis revealed slight, if any, differences between genotypes that did not reach statistical significance. These findings reconcile the human–mouse phenotype discrepancy and demonstrate that the *TECTB*-C225Y variant renders the cochlea more susceptible to environmental noise.

To investigate whether this increased susceptibility is accompanied by structural changes in the TM, we analyzed inner ears collected from 14-month-old *Tectb*^{C225Y/+} and wild-type mice used in the noise exposure experiments described above. Using the same light microscopy and quantification pipeline as in Figure 3, we found no significant differences in cross-sectional area or toluidine blue staining density between the two genotypes (data not shown). These results suggest that the functional vulnerability is not accompanied by further detectable structural disorganization detectable at the light microscopy level.

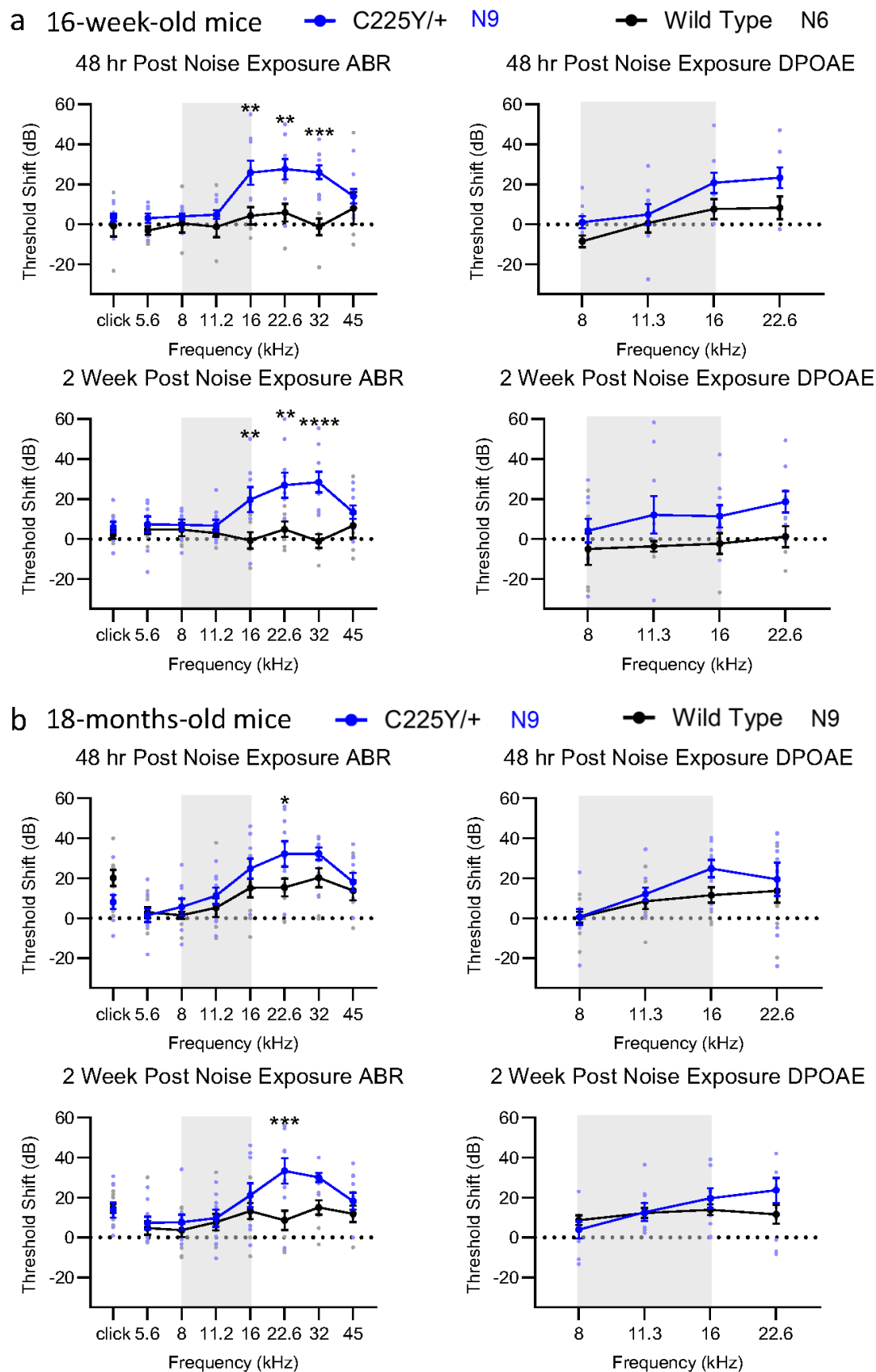


Figure 6: *Tectb*^{C225Y/+} Mice show increased vulnerability to noise damage. Threshold shifts in ABR and DPOAE measurements at 16 weeks (A) and 18 months (B) of age following noise exposure. Threshold shifts were calculated by subtracting pre-exposure values from those obtained 2 days and 14 days post-exposure. Wild-type and *Tectb*^{C225Y/+} mice were exposed for 2 hours to 8–16 kHz octave-band noise at 100 dB SPL, indicated by the shaded area on each graph. When no response was detected at 80 dB SPL, the threshold was entered as 85 dB, one interval above the system's test ceiling. Data are shown as mean $\pm$ SEM. *Statistical Analysis:* two-way ANOVA followed by Šidák's multiple comparisons test between genotypes at each frequency. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Discussion

This study identifies *TECTB* as a human deafness gene and reveals a previously unrecognized role for β -tectorin in providing the cochlea with resilience to age-related and noise-induced hearing loss in mice. A missense c.674G>A, p.Cys225Tyr *TECTB* variant, located in the conserved ZP domain, segregates with autosomal-dominant, non-progressive sensorineural hearing loss in a multigenerational Nicaraguan family. Evidence for deleterious variant impact was predicted with bioinformatic tools. Furthermore, the variant impacted a strongly conserved amino acid residue, and segregated in a large family. To validate its impact *in vivo*, we generated a knock-in mouse model expressing the orthologous mutation.

In mice, the *Tectb*-C225Y allele shows a gene-dosage effect. *Tectb*^{C225Y/C225Y} mice exhibited profound deafness with gross ultrastructural deficits of the TM, including complete loss of the SSM. These features resemble the phenotype of *Tectb* knockout mice (Russell et al., 2007), consistent with the conclusion that the TECTB-C225Y substitution severely impairs β -tectorin function. In contrast, *Tectb*^{C225Y/+} mice exhibit normal auditory thresholds under standard housing conditions despite a subtle reduction in TM matrix density and altered morphology of key structural features such as the marginal band and Hensen's stripe. These results suggest that under unstressed conditions, the *Tectb*-C225Y allele alone is insufficient to cause measurable auditory dysfunction in mice, a finding at odds with the human phenotype where hearing loss is evident in all heterozygous carriers. There are several examples of gene discovery studies describing human autosomal dominant non-syndromic hearing loss that identified a phenotype in mouse models with biallelic disruption (Azaiez et al., 2015; Yan et al., 2013; Zhang et al., 2020). We recognize that both inter-species differences and environmental factors may contribute to this discrepancy.

To reconcile it, we considered species-specific differences in environmental exposure. Humans are routinely exposed to a wide range of sound environments throughout life, while laboratory mice are typically housed in quiet, controlled conditions. We therefore hypothesized that noise exposure might unmask latent cochlear vulnerability in *Tectb*^{C225Y/+} mice. Indeed, after exposure to a noise trauma paradigm that caused TTS in wild-type mice, *Tectb*^{C225Y/+} mice showed significantly larger and permanent ABR threshold shifts, likely recapitulating aspects of the human phenotype. These findings reveal a gene-environment interaction in which the *Tectb*-C225Y variant sensitizes the cochlea to noise insult, leading to long-term functional consequences.

Importantly, DPOAEs were similar between wild-type and *Tectb*^{C225Y/+} mice as they aged, and largely similar after exposure, suggesting that OHC function was mostly preserved. The divergence between ABR and DPOAE results could indicate that the noise vulnerability phenotype is not driven by OHC dysfunction, perhaps arising from impaired force transmission through the TM to IHCs. This hypothesis is further supported by morphological evidence of structural disorganization in the TM and its key components, as well as reduced optical density (interpreted as matrix content) in stained TM sections. The TM's ability to efficiently couple OHC- and IHC-driven motion may be particularly sensitive to structural disruptions under conditions of stress and warrant more attention in future studies.

The disruption of the ZP domain in TECTA is characterized as mid- or pan-frequency hearing impairment in patients with DFNA8/12 (Verhoeven et al., 1998). These individuals present highly variable audiogram profiles including mild-to-severe and have an age of onset ranging from early childhood to adolescence (Chen et al., 2022; Yasukawa et al., 2019). Multiple studies have reported both, progression (Moteki et al., 2012) and non-progression (Iwasaki et al., 2002) of hearing loss for patients with variants disrupting the ZP domain. In those with progressive hearing loss, deterioration was not accelerated by particular TECTA variants but rather by age (Yasukawa et al., 2019).

The *TECTB*-C225Y substitution occurs at a highly conserved cysteine within the ZP domain, a motif critical for the polymerization and stabilization of many extracellular matrix proteins (Bokhove & Jovine, 2018). The absence of the SSM in *Tectb*^{C225Y/C225Y} mice, along with our indirect measures reporting reduced TM matrix density in both *Tectb*^{C225Y/+} and *Tectb*^{C225Y/C225Y} mice, suggests that the variant

interferes with the correct incorporation of β -tectorin into the TM matrix and/or its stability once incorporated, leading to functional deficit. These observations align with prior studies of TECTA mutations (Chen et al., 2022; Yasukawa et al., 2019), some of which are also predicted to affect ZP-domain interactions and lead to dominant or recessive hearing loss depending on the nature and position of the variant (Iwasaki et al., 2002; Moteki et al., 2012). Notably, pathogenic TECTA ZP-domain variants often produce mid-frequency or flat hearing loss with variable progression, similar to the audiometric phenotype observed in our patient cohort, and have also been associated with TM structural abnormalities resembling those we observed in *Tectb*^{C225Y/+} mice (Legan et al., 2014; Legan et al., 2005; Xia et al., 2010).

Audiometric data from the Nicaraguan family showed mostly stable hearing loss ranging from mild to severe, even among siblings, and no evidence of progression over time, as confirmed by re-testing three family members over a decade after the initial hearing test was administered, adding more certainty to our report. This variability in phenotype expression despite a shared genotype supports a model in which environmental modifiers, particularly noise exposure, are likely to contribute to disease manifestation. Our findings in *Tectb*^{C225Y/+} mice provide direct experimental support for this hypothesis and suggest that the TECTB-C225Y variant creates a sensitized cochlear environment—likely to be functionally silent under ideal conditions but prone to early decompensation under stress.

In addition to the direct reporting on the TECTB-C225Y variant, our results highlight the importance of mouse strain genetic background in auditory phenotyping. Earlier studies of TM protein mutants, including *Tecta* knock-ins, were constrained by seizure susceptibility in certain mouse strains, which precluded noise exposure experiments (Legan et al., 2014). By generating the *Tectb*-C225Y mouse model on a C57BL/6J background with a corrected *Cdh23*^{Ahl/+} allele, we avoided these limitations and enabled the interrogation of both baseline and environmentally induced phenotypes following noise insult. The ability to model gene-environment interactions in this setting was critical for uncovering the additional effects conferred by the TECTB-C225Y variant and may be relevant to other deafness genes whose phenotypic impact might also be conditional on environmental stressors that remain untested.

The distinction between ABR and DPOAE responses is an unexpected finding that may provide mechanistic insight. While DPOAEs reflect the electromotility of OHCs, ABRs integrate responses across the full auditory pathway, from cochlear mechanics to neural signaling. Thus, largely preserved DPOAEs alongside more degraded ABRs suggest that the TM may still support OHC function but fails to efficiently transmit motion to IHC stereocilia. This divergence between ABR and DPOAE measurements could suggest that the functional impairment in *Tectb*^{C225Y/C225Y} mice may be more pronounced in IHC stimulation or, perhaps, cochlear mechanics, rather than OHC mechanotransduction per se. This is consistent with previous observations in *Tecta*-Y1870C and *Otoa*-EGFP knock-in mice, which demonstrated a critical role for the TM in driving IHC stimulation, with isolated TM defects leaving OHC function largely unaffected (Legan et al., 2005; Lukashkin et al., 2012). This differential vulnerability could underlie the ABR/DPOAE dissociation observed in both aging and noise-exposed *Tectb*^{C225Y/+} mice. While TECTB immunolabeling in the cochlea has only been observed in the TM (Russell et al., 2007), suggesting that its role in hearing is primarily localized to this structure, we cannot entirely exclude the possibility of additional or indirect functions elsewhere in the cochlea. Furthermore, the greater ABR threshold shifts observed in noise-exposed *Tectb*^{C225Y/+} mice point to a failure in cochlear resilience—a likely result of compromised or weakened TM architecture, rendering it more susceptible to noise insult.

Our data support a semidominant model for the mouse *Tectb*-C225Y allele. *Tectb*^{C225Y/C225Y} mice display a severe, congenital phenotype with gross TM deficits and early hearing loss. *Tectb*^{C225Y/+} mice exhibit structural TM abnormalities and a functionally silent phenotype under baseline conditions but display an increased sensitivity to noise insult. This model echoes prior findings from *Tecta* ZP-domain mutants, where dominant phenotypes often arise from structural disorganization rather than protein loss *per se*. Furthermore, it supports the notion that subtle TM disruptions in humans may contribute to

complex hearing phenotypes that are currently underdiagnosed, particularly those with unexplained increased noise sensitivity or age-related declines.

Although we did not directly assess the TM's biophysical properties, our electrophysiological and morphological findings are consistent with altered extracellular matrix architecture and impaired function. The specific interactions of β -tectorin with other TM proteins remain to be defined, but future studies leveraging structural biology and protein chemistry may elucidate the relevant binding interfaces and clarify how disease-associated variants disrupt matrix assembly.

Taken together, this study provides evidence that *TECTB* is a *bona fide* human deafness gene and demonstrates that a single missense mutation within its ZP domain compromises TM integrity and cochlear resilience to noise insult. The findings also illustrate the value of cross-species modeling and the importance of considering environmental context in interpreting genotype–phenotype relationships. Finally, these results argue for the inclusion of *TECTB* in diagnostic panels for hereditary hearing loss.

Materials and Methods

Family recruitment and clinical assessment

This study focusing on the genetic etiology of hereditary hearing loss in rural Nicaraguan families was approved by the institutional review board of Dartmouth-Hitchcock Medical Center (No. 22289) and the Nicaraguan Ministry of Health. Written informed consent was obtained from participating members (II:1, II:2, II:3, II:4, II:5, III:1, III:2, III:3, III:4, III:5, III:6 and IV:1) of a Nicaraguan family. Genomic DNA of participating family members was extracted from peripheral blood leukocytes.

Participating individuals completed a risk factor questionnaire that reviewed perinatal and birth history, neonatal or perinatal infections, maternal exposure to rubella, exposure to ototoxic drugs or pesticides, other congenital or significant medical problems, ear infection history, and noise exposure. Detailed physical examination included an otoscopic and craniofacial examination and assessment of dysmorphic features including the craniofacial development and examination of the pinna, otoscopic examination of the ear, and examination of other physical features known to be associated with syndromic hearing loss (ocular exam, skin pigmentation abnormalities, limb abnormalities). Audiometric data included pure-tone thresholds for 0.5, 1, 2, 4, and 8 kHz, which were collected as previously reported (Saunders et al., 2007). Both air and bone thresholds were obtained. Severity of hearing loss was determined by averaging pure-tone thresholds over 0.5, 1, 2, and 4 kHz ($PTA_{0.5-4\text{kHz}}$) for the better ear. The severity of hearing loss in the better ear was defined as mild for thresholds averaging 20-40 dB hearing level (HL), moderate for 41-70 dB HL, severe for 71-95 dB HL, and profound in excess of 95 dB HL. Progressive hearing loss was defined as a deterioration of >15 dB HL in the average over the frequencies of 0.5, 1, and 2 kHz within a 10-year period. Otoacoustic emissions were assessed in individuals II:4, II:5, and III:2.

Genetic testing

High-throughput sequencing using the OtoGenome® 74-gene panel (**Supplementary Table 3**) was performed on the proband's DNA sample using oligonucleotide-based target capture (Agilent SureSelect) followed by Illumina HiSeq sequencing. Variant calls were generated using the Burrows-Wheeler Aligner followed by GATK analysis. Variants were mapped to human genome reference build hg19. This test detected >99% of substitution variants (95% CI = 98.5-100) and 95% of small insertions and deletions (95% CI = 83.1 - 98.6). Following an uninformative result, the proband's sample was subjected to 2 x 75 bp paired-end exome sequencing v3 (Broad Institute, Cambridge, MA, USA). Variant calls were annotated by Annovar (Wang et al., 2010).

Variant assessment and validation

Exome data were filtered to remove variants with an allele frequency of 0.01 or more in the population frequency databases gnomAD v4.1.0 and All of Us Data Browser (All of Us Research

Program Genomics, 2024; Chen et al., 2024). Coding and splice site variants were retained for analysis. Deleteriousness of missense variants was assigned according to the prediction from multiple prediction tools (SIFT, PolyPhen-2, FATHMM, MutationTaster, REVEL, CADD, and ClinPred) and supported by evolutionary conservation of the affected amino acid as assessed by phyloP. Splicing predictions utilized the splice tools embedded in Alamut Visual Plus as well as Splice AI Visual (de Sainte Agathe et al., 2023). Both heterozygous and homozygous variants were analyzed. Variants suspected of having potential clinical significance related to the probands' hearing loss were confirmed by Sanger sequencing in family members to assess segregation with the disorder.

Genotyping, copy number variation calling and linkage analysis

Whole genome genotyping using the genomic DNA samples was performed using the HumanOmni2.5-8v1 SNP array (Illumina, San Diego, CA, USA) at Partners Research Core. Copy number variation (CNV) calls were visualized using GenomeStudio following calling with pennCNV and CNVPartition (Illumina). Mendelian errors due to genotype errors were checked with Pedcheck and erroneous genotypes were removed (O'Connell & Weeks, 1998). A parametric model was used, as the pedigrees showed autosomal dominant inheritance. Multipoint linkage analysis was performed using Allegro version 2.0 (Gudbjartsson et al., 2005).

Generation of the *Tectb*-C225Y knock-in mouse line

All procedures and protocols were approved by the Institutional Animal Care and Use Committees of Mass Eye and Ear and of the University of Nebraska Medical Center. For work conducted at the University of Sussex, animals were bred and maintained in accordance with UK Home Office guidelines, and the experiments were performed in accordance with the Home Office Animals (Scientific Procedures) Act 1986, following approval by the Animal Welfare Ethical Review Board at the University of Sussex. All aspects of animal care, use, and experimental procedures were conducted in compliance with relevant institutional and national ethical regulations governing animal research.

CRISPR strategy. The *Tectb*-C225Y mouse line was created using the CRISPR-Cas9 system. The guide RNA sequence used for changing the codon TGC to TAC was TGTCTCATCGGTGGG **GC**/AGCTGG. The guide is in antisense orientation. Codon 225 (TGC) is bold-faced, and the PAM sequence is shown in italics. The guide cut site is shown with the symbol "/".

CRISPR reagents. The guide RNA was synthesized by annealing two ultramer oligonucleotides (atatcggatcccTAATACGACTCACTATAGGTGTCTCATCGGTGGGGCAGcGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT T and AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATT TTAAGTTGCTATTTCTAGCTCTAAAACgCTGCCCCACCGATGAGACACCTATAGTGAGTCGTATTA gggatccgatat) followed by in vitro transcription using MEGAshortscript T7 kit (Ambion: AM 1354). The *Tectb* repair DNA (ultramer) sequence was GAGCCTTTTTGGAGCTCTCTGGTGTGATGTTT CTTCTCCCTTCCCTCTGCATCCCAGCT**AC**CCCCACCGATGAGACAGTCCTCGTGCATGAGAACG GCAAAGACCACAGGGCCACTTTCCA. The ultramers were purchased from IDT (Coralville, Iowa, USA). Codon 225 Y (TAC) is underlined and the nucleotide incorporated is shown in bold-faced letter. The Cas9 mRNA was synthesized using the pBGK plasmid (Harms et al., 2014; Miura et al., 2018) as a template for invitro transcription using mMESSAGE mMACHINE T7 ULTRA kit (Ambion: AM 1345). Cas9 mRNA.

Pronuclear injection. C57BL/6 mice were used as embryo donors. Detailed descriptions of CRISPR/Cas9-mediated mouse genome editing are described in (Quadros et al., 2015). Briefly, the injection mix contained 10 ng/μL each of sgRNA, repair-DNA ultramer (ssDNA) and Cas9 mRNA.

Genotyping of offspring and nucleotide sequencing. Genomic DNAs were extracted from the offspring using Qiagen Gentra Puregene Tissue Kit and the target region was PCR-amplified using primers F1 (CATCCATTCCCGTGGCTCTG) and R1 (GACTGGAGGAGCCGGATTTG). The wild-type sequence contains a *PvuII* Restriction Endonuclease site and successful homologous recombination

results in the loss of a *PvuII* site. The PCR products were genotyped by Restriction Fragment Length Polymorphism (RFLP, see **Supplementary Figure 3A**). Primers F2 (GCTCCTCACAAGCCTAGCAG) and R2 (GCGACAGTGAGAAGCTCTCC) were then used to amplify products that were Sanger sequenced with the R2 primer to confirm presence of the mutant allele (**Supplementary Figure 3B**).

The *Tectb*-C225Y allele was then transferred to a C57BL/6J-*Ch23^{Ahl+}* strain (Jax cat# 002756) which retains the line's background while correcting the *Cdh23^{Ahl}* mutation known to cause age-related hearing loss in the C57BL/6J line. Test groups consisted of mixed male and female mice from shared large litters, which were aged for testing and tissue collection at pre-determined time points on site. This approach ensures standardized experimental conditions and evaluates both genders to control for any potential sex-specific effects. Animals were randomly allocated to experimental groups.

Auditory testing

ABR and DPOAE measurements were performed in a sound-attenuating and electrically shielded chamber using the *EPL Cochlear Function Test Suite* (EPL Acoustic System, Eaton-Peabody Laboratories) at Mass Eye and Ear. Recordings were collected at six timepoints: 5 weeks, and 3, 6, 9, 12, and 18 months of age. Each mouse underwent both ABR and DPOAE testing in a single session per timepoint.

Mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (10 mg/kg). Artificial tears were applied to prevent corneal drying. Body temperature was maintained at 37 °C throughout the procedure using a thermostatically controlled heating pad. Tympanic membranes were visually inspected for obstructions or anomalies prior to testing. Subdermal needle electrodes were inserted at the vertex (non-inverting), post-auricularly behind the stimulated ear (inverting), and near the base of the tail (ground).

ABRs were recorded in response to both broadband clicks and pure tone stimuli at 5.6, 8, 11.2, 16, 22.6, 32, and 45 kHz. Each tone pip was 5 ms in duration, with a 0.5 ms rise-fall time, delivered in alternating polarity at a rate of 30 stimuli per second. Stimulus intensity ranged from 20 to 80 dB SPL in 5 dB steps. Responses were amplified 10,000×, filtered between 0.3 and 3 kHz, and averaged across 512 repetitions, as previously reported (Strelkova et al., 2024). ABR thresholds were determined using the *ABR Peak Analysis* tool (Suthakar & Liberman, 2019), then visually inspected and corrected as needed. ABR thresholds were defined as the lowest stimulus intensity that evoked repeatable and identifiable waveform peaks.

DPOAEs were recorded in response to two simultaneous pure-tone stimuli, f1 and f2 ($f2/f1 = 1.2$), where f2 frequencies matched those used in ABR testing (5.6, 8, 11.2, 16, 22.6, 32, and 45 kHz). Primary tone levels were swept from 10 to 80 dB SPL for f2 in 5 dB increments, with L1 set to L2 + 10 dB. DPOAEs were recorded at the 2f1–f2 frequency using a low-noise microphone mounted within the APL acoustic system assembly. Iso-response contours were interpolated from plots of 2f1–f2 amplitude versus f1 level. The DPOAE threshold was defined as the f1 level required to elicit a 2f1–f2 distortion product of 0 dB SPL. DPOAE thresholds were reported only for 8, 11.2, 16, 22.6, and 32 kHz, as measurements at 4, 5.6, and 45 kHz were highly variable across groups, likely due to equipment limitations and calibration inconsistencies at these edge frequencies.

In cases when the strongest sound stimulus (80 dB SPL) resulted in no detectable response, the threshold was reported as 85 dB SPL and indicated with an upward-pointing arrow on the graphs. All responses were then analyzed and plotted in GraphPad Prism. In addition to thresholds, ABR peak I amplitudes and latencies were analyzed for certain age groups and genotypes as reported in the manuscript.

Noise exposure

Test cohorts were established at two time points: a young age of 16 weeks and an advanced age of 18 months, utilizing mice that had recently undergone longitudinal auditory testing. Following baseline ABR and DPOAE assessments (as described above), awake, unrestrained mice were placed

individually in small cages and exposed to octave-band noise (8–16 kHz) for 2 hours at 100 dB SPL—identified as the highest non-neuropathic noise level known to induce TTS in this age group (Jensen et al., 2015). The noise was generated from a white-noise source, filtered, amplified, and delivered via a horn mounted atop a reverberant tabletop exposure booth. Exposure levels were confirmed in each cage using a 0.25-inch Brüel and Kjær condenser microphone.

ABRs and DPOAE thresholds were then reassessed 2 days and 2 weeks after exposure to evaluate auditory recovery. Threshold shifts from pre-exposure levels were calculated for each frequency and time point in each animal. Three animals were excluded from the analysis (one WT and one *Tectb*^{C225Y/+} mouse at 16 weeks, and one 18-month-old *Tectb*^{C225Y/+} mouse) due to undetectable pre-exposure ABR or DPOAE responses at one or more reported frequencies. DPOAE threshold shifts were reported only for 8, 11.2, 16, and 22.6 kHz, as measurements at 4, 5.6, 32, and 45 kHz were highly variable across groups, likely due to equipment limitations and calibration inconsistencies at these edge frequencies.

Tissue processing and imaging

Cochleae were removed and placed in petri dishes containing PBS. The oval and round windows were opened, and a small hole was made through the bone at the apical end of each cochlea. A small volume (~20 µL) of fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 (EMS 15960), containing 10 mg/mL tannic acid (Sigma 403040)) was slowly perfused through the oval window and a further 20 µL of fixative was delivered through the hole at the apical end. Further holes were subsequently made in the lateral walls of the middle and basal turns. Cochleae were then immersed for ~12 h in fixative at 4°C on a rocker. Following three washes in 0.1 M sodium cacodylate, cochleae were post-fixed in 1% osmium tetroxide (EMS 19150) in 0.1 M sodium cacodylate pH 7.4 for 4 h at room temperature. Cochleae were then washed a further three times in 0.1 M sodium cacodylate and decalcified in 0.5 M EDTA (Millipore 324504) containing 0.1% glutaraldehyde (EMS 16019) for 5 days at 4°C on a rocker. Following a wash in distilled water, cochleae were dehydrated through an ascending ethanol series, equilibrated in propylene oxide and infiltrated and embedded in Epon 812/Araldite 502 resin (EMS). After curing at 60°C for 48-72 h with the cochleae positioned so that the oval and round windows faced upwards, cochleae were mounted with the windows facing downwards and sectioned until profiles of the ~4, 8, 20, and 40 kHz regions could be obtained from a single section.

For light microscopy, 1 µm sections were stained with 1% (w/v) Toluidine blue containing 1% (w/v) borax, prior to widefield imaging. For TEM, ultrathin sections (~80 nm) were collected onto formvar/carbon-coated slot grids. Sections were stained on grids with 1% uranyl acetate and lead citrate, then imaged on a Hitachi TH7800 TEM operated at 100 kV at the Center for Nanoscale Systems, Harvard University. Images were processed in Adobe Creative Suite.

For immunostaining, the inner ears were immersion fixed after removal of the oval and round windows in 3.7% formaldehyde (10% formalin) in 0.1 M sodium phosphate buffer pH 7.4 for 2-3 hours at room temperature, washed 3 x in PBS and decalcified at 4°C in 0.5 M EDTA pH 8.0 for 3 days. After washing 3x with PBS to remove the EDTA, samples were equilibrated with 30% sucrose in PBS, imbedded in 1% low-gelling temperature agarose in PBS containing 18% sucrose, mounted onto cryotome chucks coated with a thin layer of OCT compound, fast frozen using Cryospray and cryosectioned at a temperature of ~-30°C and a thickness of 15 microns. Cryosections were mounted on gelatin-coated glass slides, dried overnight at 37°C, preblocked with 10% heat-inactivated horse serum in PBS (PBS/HS) for 1 hour, and stained overnight at room temperature with rabbit anti-chick TECTB (R7, (Knipper et al., 2001)) at a dilution of 1:1000. Sections were washed, labelled for 1 hour with Texas-Red conjugated phalloidin (Invitrogen) and Alexa-488 goat anti-rabbit Ig, both at a dilution of 1:500 in PBS/HS, washed in PBS, mounted in Vectashield and imaged using a Zeiss Axioplan2 equipped with a Spot RT camera and a 20× lens.

Staining and quantification of TM cross-section area

Adaptive thresholding was utilized in order to quantify the cross-sectional area of the TM and density of stained material as described previously (Cheatham et al., 2014). Briefly, while blinded to genotype,

the outer border of TMs were traced in Adobe Photoshop CC, and area calculated. A threshold level that excluded all but pixels devoid of stain was identified for each image utilizing the Image J adaptive threshold tool, and the area of these pixels was calculated as a percentage of TMs cross-sectional area.

Quantification of ultrastructure deficits

Marginal band and Hensen's stripe morphology in cross-sections of the 40 kHz region was scored in a random order, blind to genotype. Morphology scores were pooled from 3 independent investigators. Marginal bands were scored as 1-normal, 2-mishapen, 3-severely misshapen. Hensen's stripes were scored as 1-normal, 2-disrupted, 3-absent.

Statistical Analysis

TM cross-sectional area and TM unstained area. One-way ANOVAs were carried out at each frequency with Šidák's multi-comparison tests between each genotype, followed by Benjamin Hochberg correction for all comparisons within each age group. Proportions of TM sections devoid of stain (%) underwent arcsin square root transformation prior to statistical analysis in order to better satisfy the requirements of a One-way ANOVA. All statistical analyses were carried out in GraphPad Prism, with the exception of Benjamin Hochberg corrections which were calculated manually.

ABR and DPOAE Results. Two-way ANOVAs were carried out to compare each frequency between genotypes at each age point. Aging data comparing three genotypes were analyzed with Tukey's multiple comparisons test. Noise exposure data comparing two genotypes were analyzed with Šidák's multi-comparisons test. All statistical analyses were carried out in GraphPad Prism.

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Author contributions

E.B.H.: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing-review and editing, visualization, funding acquisition; **B.V.:** Validation, formal analysis, investigation, data curation, visualization, writing – original draft, project administration, funding acquisition; **R.J.G.:** Conceptualization, methodology, validation, formal analysis, investigation, data curation, visualization, writing – review & editing; **R.T.O.:** Investigation, methodology, validation, formal analysis, data curation, writing-original draft; **S.S.A.:** Resources; **K.M.:** Validation, investigation, data curation; **R.V.M.:** Investigation; **K.C.:** Investigation; **K.L.G.:** Investigation; **R.Q.:** Investigation,

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Data availability

The source data used to generate the graphs in this manuscript are available in the supplementary data file. Further imaging data that support the findings of this study are available from the corresponding authors upon request.

Competing interests

The authors declare no competing interests.

Ethics approval

Human studies were approved by the institutional review board of Dartmouth-Hitchcock Medical Center (No. 22289) and the Nicaraguan Ministry of Health.

All procedures and protocols were approved by the Institutional Animal Care and Use Committees of Mass Eye and Ear and of the University of Nebraska Medical Center. For work conducted at the University of Sussex, animals were bred and maintained in accordance with UK Home Office guidelines, and the experiments were performed in accordance with the Home Office Animals (Scientific Procedures) Act 1986, following approval by the Animal Welfare Ethical Review Board at the University of Sussex. All aspects of animal care, use, and experimental procedures were conducted in compliance with relevant institutional and national ethical regulations governing animal research.

Consent to participate and publish

Written informed consent was obtained from all participants including permission to publish.

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