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Article

Keywords: DFNA8/12, DFNB21, Hearing, hearing loss, splicing, TECTA

Posted Date: October 27th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11189368/v1>

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Additional Declarations: No competing interests reported.

Novel *TECTA* Variants Reveal Distinct Molecular Mechanisms in Dominant and Recessive Hearing Loss

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Abstract

Variants in *TECTA*, encoding the glycoprotein α -tectorin, a critical component of the cochlear tectorial membrane, have been linked to both autosomal dominant (DFNA8/12) and recessive (DFNB21) non-syndromic hearing loss (HL), depending on the affected protein domain. In this study, we identified and characterized five novel *TECTA* variants in seven families (five Iranian, two German), using whole exome sequencing, segregation analysis, and functional assays. We identified three autosomal recessive variants: two variants in the +5 position and one missense variant. Aberrant splicing was confirmed for variants at the +5 position: c.5272+5G>C (resulting in r.3544_4105del, p.(Val1182Leufs*14)) and c.4105+5G>C (leading to r.4977_5272del, p.(Arg1659Serfs5)), both associated with autosomal recessive inheritance. We also identified the autosomal recessive

c.5942A>G p.(Tyr1981Cys) variant, located in the vital zona pellucida domain. In the German families, we identified two autosomal dominant missense variants: c.2858T>A p.(Leu953His), and c.4437C>G p.(Cys1479Trp), both located in the zonadhesin-like domain. Established genotype-phenotype correlations are consistent with our identified variants. Our findings expand the mutational spectrum of *TECTA* and clarify the molecular mechanisms underlying HL. Our results support domain-specific mechanisms, splicing defects in recessive cases and predicted structural destabilization in dominant cases. These insights advance our understanding of *TECTA*-related HL, enabling precision diagnostics and potential therapeutic strategies for genetic HL.

Keywords: DFNA8/12, DFNB21, Hearing, hearing loss, splicing, *TECTA*

1 Introduction

Protein integrity is essential for the complex molecular interactions that enable auditory function. Deleterious variants in genes associated with hearing loss (HL) impact function or stability, resulting in HL, one of the most prevalent sensory disorders in humans (Morton and Nance 2006). HL associated proteins exhibit a broad spectrum of distinct domains, reflecting their diverse functional roles (Azaiez et al. 2018). Autosomal recessive and autosomal dominant inheritance account for roughly 80% and 20% of all genetic cases, respectively (Petersen and Willems 2006).

Some HL genes demonstrate both dominant and recessive forms of HL. A paradigmatic example is *TECTA*, where the mode of inheritance, dominant (DFNA8/12) or recessive (DFNB21), is determined by the variant's genomic location and functional consequences. Notably, the resulting HL phenotype correlates directly with the specific protein domain affected by the variant (Plantinga et al. 2006; Mustapha et al. 1999; Verhoeven et al. 1998; Naz et al. 2003).

TECTA, located on chromosome 11q23.3, spans 24 exons in a 7,353 base pairs (bp) transcript that translates into a 2,155 amino acid protein. This gene encodes α -tectorin, a glycoprotein and a key structural component of the non-collagenous matrix of the tectorial membrane (TM) within the cochlea (Verhoeven et al. 1998; Mustapha et al. 1999; Plantinga et al. 2006). The TM rests atop and contacts the outer hair cells, mechanically amplifying sound and activating their stereocilia. This deflection generates membrane potentials that convert sound into electrical signals (Legan et al. 2000).

Structurally, α -tectorin comprises three primary domains: an N-terminal nidogen-like domain (NIDO), previously called entactin domain (ENT), followed by a zonadhesin-like domain (ZA) domain containing one von Willebrand factor (vWF) type C domain, four type D domains, and three trypsin inhibitor-like domains, and a C-terminal zona pellucida (ZP) domain (Legan et al. 1997). Functionally, autosomal dominant variants within the ZA domain are predominantly associated with high-frequency HL, while those in the ZP domain typically manifest as mid-frequency HL

139 (Yamamoto et al. 2017). The structural complexity of α -tectorin highlights the poten-
140 tial for diverse variants to impact its function. Indeed, various *TECTA* variants have
141 been identified, each with distinct implications for hearing ability (Yang et al. 2025;
142 Yasukawa et al. 2019).

143 This study examines five newly identified *TECTA* variants in seven families,
144 elucidating their functional impact on protein structure and their role in hearing
145 impairment. Two of these variants are novel splice-altering variants, while the other
146 three are previously unreported missense variants. By characterizing these variants, we
147 aim to deepen the understanding of molecular mechanisms underlying HL and allow
148 precision medicine strategies for affected individuals. Such insights not only clarify
149 the genetic foundations of HL but also lay the groundwork for targeted therapeutic
150 interventions (Qi et al. 2025).

151

152 2 Results

153

154 2.1 Clinical and pedigree analyses

155

156 We describe a cohort comprising seven families (Fig. 1 A). None of the index patients
157 reported vestibular dysfunction, developmental delays or other syndromic features,
158 consistent with non-syndromic hearing loss (NSHL).

159 Families 1 to 5 were of Iranian descent, and diagnosed with autosomal recessive HL
160 (DFNB21). Families 6 and 7 were from Germany and exhibited autosomal dominant
161 inheritance (DFNA8/12). Audiograms were available from the German patients.

162 Family 1 comprised six available members, three of whom were affected by bilateral
163 NSHL: the index patient (II-2), his brother (II-3), and his nephew (III-1). Family 2
164 consisted of three available members with two individuals, namely, the index patient
165 (II-3) and her sister (II-2). The index patient showed congenital HL. Audiological
166 examination revealed bilateral, profound (>95 dB) sensorineural HL.

167 The index patients of families 3, 4, and 5 had a family history of parental consan-
168 guinity. In family 3 the history of NSHL included the index patient (V-1), his uncle
169 (IV-3) and the deceased grandfather (III-3). Similarly, the index patient of family 4
170 (V-1) presented with a family history of bilateral NSHL that includes his grandfather
171 (III-1).

172 Family 5 comprised five available members without a prior family history of NSHL.
173 Two affected individuals were siblings. The index patient (IV-1), aged 35, and his 43-
174 year-old brother (IV-2), both presented with severe, non-progressive congenital HL.
175 However, the brother's HL was described as milder compared to that of the index
176 patient.

177 The pedigree of family 6 showed hearing loss across four generations. NSHL was
178 present in the index patient's father (III-2), uncle (III-3), cousin (IV-2), grandfather
179 (II-3), and great-grandfather (I-1). The index patient (IV-1) exhibited bilateral mild
180 (PTA_{0.5-4kHz}: 35 dB) post-lingual sensorineural NSHL, which manifested at the age
181 of 3 (Fig. 1 B). Asymmetry in the high frequencies was observed, with the right ear
182 showing a shallow U-shaped audiogram and the left ear showing a gently sloping
183 high-frequency audiogram.

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Family 7 included 34 members, eight of whom were reported to have HL. The 17-year-old index patient (IV-1) exhibited mild bilateral mid-frequency U-shaped NSHL (PTA_{0.5-4kHz}: 25 dB) (Fig. 1 B). Her 14-year-old brother (IV-2) displayed mild high frequency HL (PTA_{0.5-4kHz}: 20 dB). The index patient's 39-year-old mother (III-5) had HL since the age of 10, which has been reported as progressive over time (PTA_{0.5-4kHz}: 45 dB). The index patient's aunt (III-7) and one of her uncles (III-9) were also affected. The aunt (III-7) and one of her unaffected sons (IV-3) also had diabetes. The audiogram of the 44-year-old uncle (III-9) revealed bilateral sloping mid-to high-frequency moderate HL (PTA_{0.5-4kHz}: 46 dB) with preservation of the low frequencies, whereas his six-year-old daughter had normal hearing (data not shown). The index patient's grandmother (II-6) has used hearing aids since the age of 40. Her audiometric measurements indicated a moderate level of HL (PTA_{0.5-4kHz}: 67 dB), with her frequency thresholds gently sloping from the lower (PTA_{0.5-1kHz}: 57.5 dB) to the higher frequencies (PTA_{4-8kHz}: 75 dB). The grandmother's now-deceased brother (II-14) had also used hearing aids from a young age. On the grandfather's side of the family (II-5), one nephew (III-1) was also affected by HL.

2.2 Molecular Analysis

Next-generation sequencing (NGS) was performed for all index patients. In brief, exomes of families 1-4 and 6 were enriched using the Illumina Nextera Rapid Capture Enrichment protocol and sequenced on a NextSeq500, achieving an average coverage of 99% of the target regions (with at least 10 reads) and a mean sequencing depth of 50-fold. Exome sequencing of family 5 was performed using the Illumina NovaSeq 6000 platform, whereas family 7, including the index patient and both parents, was analyzed with the TruSight One panel on a MiSeq desktop sequencer. For variant confirmation and segregation analysis of parents and additional family members, Sanger sequencing was employed (Supplementary Fig. A1). All variants and their consequences at the RNA and protein level are shown in Figure 2 A-C.

2.2.1 Autosomal recessive variants

In family 1, exome analysis identified a homozygous c.4105+5G>C variant in the donor splice region of exon 12 of *TECTA* (NM_005422.4) in the index patient (II-2) (Fig. 2 A). Splice prediction programs suggested that this variant could cause aberrant splicing (SpliceAI scores: donor loss, 0.90; acceptor loss, 0.00), supporting its pathogenicity. Segregation analysis using Sanger sequencing (Supplementary Fig. A1 A) revealed that his affected brother (II-3) also had the homozygous variant, while the unaffected parents (I-1 & I-2) and brother (II-4) were heterozygous carriers. The affected nephew (III-1) was also found to be a heterozygous carrier (Fig. 1 A).

In family 2, the same c.4105+5G>C variant was identified in the index patient (II-3) in a homozygous state. Segregation analysis revealed that the affected sister (II-2) was also homozygous for the variant, while her unaffected daughter (III-1) was heterozygous (Fig. 1 A & Supplementary Fig. A1 B).

Exome sequencing of the index patient (V-1) from family 3 revealed a homozygous (c.5272+5G>C) variant in the donor splice region of exon 16. Splice prediction

software highlighted this variant as a likely cause of exon skipping (SpliceAI scores: acceptor loss, 0.27; donor loss, 0.78), prompting further investigation. Segregation analysis and variant confirmation were performed using Sanger sequencing (Supplementary Fig. A1 C). The c.5272+5G>C variant was confirmed to be homozygous in the index patient and heterozygous in her parents (IV-1 & IV-2) and sister (V-2). Given the consanguineous marriage of the parents and the presence of other affected family members (IV-3 & III-3), this finding strongly supports an autosomal recessive inheritance (Fig. 1 A).

During the analysis of family 4, the same c.5272+5G>C variant was identified. Similar to the family 3, family 4 displayed consanguinity and the index patient (V-1) carried the variant in a homozygous state (Fig. 1 A). Segregation analysis confirmed that the unaffected parents (IV-1 & IV-2) and sister (V-2) were heterozygous carriers of the variant (Supplementary Fig. A1 D).

In family 5, NGS revealed that the index patient (IV-1) was homozygous for the c.5942A>G variant (Fig. 1 A). This results in the substitution of a tyrosine at position 1981 by cysteine. Segregation analysis showed that his affected brother (IV-2) was also homozygous for this variant, while his other non-affected brother (IV-3) and parents (III-1 & III-2) carried the variant in a heterozygous state (Supplementary Fig. A1 E). Multiple *in silico* prediction tools, including AlphaMissense predicted this variant to be likely pathogenic (Supplementary Table A1).

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2.2.2 Autosomal dominant variants

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In family 6, whole exome sequencing (WES) of the index patient (IV-1) revealed a heterozygous c.2858T>A missense variant in exon 10, resulting in a p.(Leu953His) amino acid substitution. Multiple *in silico* prediction tools predicted this variant to be deleterious (Supplementary Table A1). Sanger sequencing confirmed the heterozygous variant in the patient (Supplementary Fig. A1 F).

Finally, in our largest family, family 7, we identified the c.4437C>G variant in exon 14 in the index (IV-1), resulting in a p.(Cys1479Trp) amino acid substitution. This variant was also predicted to be pathogenic by different prediction tools (Supplementary Table A1). Segregation analysis via Sanger sequencing further supported the variant's pathogenicity, as it was detected in all affected family members in a heterozygous state. This included the patient's sibling (IV-2), mother (III-5), grandmother (II-6), aunt (III-7), and uncle (III-9). In contrast, the patient's healthy father (III-4) and uncle (III-8) did not have this variant (Fig. 1 A & Supplementary Fig. A1 G).

All identified variants share a conserved sequence across multiple species, including mouse, chicken, and zebrafish (Supplementary Fig. A2).

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2.3 Functional analysis of the *TECTA* c.4105+5G>C and c.5272+5G>C splice site variants via minigene assay

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To study the effects of the *TECTA* c.4105+5G>C variant on splicing, an *in vitro* minigene assay was conducted (Fig. 3 A). Agarose gel electrophoresis of RT-PCR products revealed different band sizes for wild-type (800 bp) and variant (260 bp) constructs (Fig. 3 B); the products were subsequently Sanger sequenced and analyzed

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(Fig. 3 C). The splice product resulting from the variant showed skipping of exon 12. This would result in r.3544_4105del, p.(Val1182Leufs*14) and a frameshift affecting the von Willebrand factor type D domain 3 (vWFD3), trypsin inhibitor-like repeat 3, von Willebrand factor type D domain 4 (vWFD4) and ZP domain (Fig. 2 B & C).

Similarly, we used an *in vitro* minigene assay to study the effects of the c.5272+5G>C variant (Fig. 3 D). We identified a 300 bp difference between the wild-type and variant PCR-products on agarose gels, corresponding roughly to exon 16. (Fig. 3 E). Sanger sequencing confirmed the presence of exon 16 in the transcript of the wild-type construct and an aberrant splice product in the variant construct, characterized by exon 16 skipping (Fig. 3 F). This resulted in the variant r.4977_5272del, p.(Arg1659Serfs*5), indicating that the c.5272+5G>C variant disrupts splicing of *TECTA* exon 16, causing a frameshift that affects parts of the vWFD4 and ZP domains (Fig. 2 B & C).

2.4 Protein Structure Analysis

To better assess the impact of nucleotide substitutions in *TECTA*, we used AlphaFold2 to model the three-dimensional structures of the p.(Leu953His) (c.2858T>A, family 6), p.(Cys1479Trp) (c.4437C>G, family 7), and p.(Tyr1981Cys) (c.5942A>G, family 5) substitutions (Fig. 4). We further used DynaMut2 to calculate changes in protein stability ($\Delta\Delta G$).

In the α -tectorin model, Leu953 is situated within a hydrophobic interaction site formed by three α -helical structures in the ZA (Fig. 4 A & Fig. 2 C). Leu953 likely participates in a hydrophobic interaction network with Arg911, Leu943, Val957, and Phe979 (Fig. 4 B). The c.2858T>A variant found in family 6 substitutes Leu953 with histidine, a comparatively larger residue that clashes sterically with the surrounding amino acids, causing a reduction in stability by -0.72 kcal/mol (Fig. 4 C). Furthermore, histidine is a polar residue and a weak base under physiological conditions, and may therefore disrupt the hydrophobic core and subsequently destabilize the local hydrophobic interaction site between the α -helices.

At the Cys1479 position, located immediately before the vWFD4, the cysteine residue is predicted to form a disulfide bond with Cys1464, stabilizing a β -hairpin structure (Fig. 4 D & E). The c.4437C>G variant identified in family 7 results in substitution of Cys1479 with tryptophan, disrupting this disulfide bond, leaving Cys1464 without a bonding partner and thereby reducing structural stability by -0.92 kcal/mol (Fig. 4 F). The inserted amino acid forms aromatic interactions with the neighboring Tyr1441 while also displacing the Glu1468 (Fig. 4 F). Furthermore, in the oxidizing environment of the extracellular matrix, a free nucleophilic cysteine may form unintended crosslinks with other protein residues in the TM, reducing its integrity and proper formation.

Tyr1981 is located within the ZP, at the outer surface of an eight-stranded β -barrel like structure (Fig. 4 G). There, it interacts most notably with the residues Phe1995 and His2039 forming a Π - Π network that stabilizes the local secondary structure (Fig. 4 H). The Tyr1981Cys substitution caused by the c.5942A>G variant found in family 5 removes the Π -network as well as the loss of numerous hydrophobic interactions (Fig. 4 J). This results in a predicted stability change of -1.37 kcal/mol. The ZP is

indispensable for proper crosslinking of α -tectorin with other proteins. Consequently, the destabilization of this region may have severe effects on protein function.

2.5 Variant Interpretation

Family 1 and 2: The previously unreported c.4105+5G>C splice site variant was identified in two independent Iranian families. In both cases, affected siblings were homozygous for the variant, while an unaffected family member was heterozygous, consistent with autosomal recessive inheritance. This segregation pattern supports the application of PP1_Supporting, as recommended by [Oza et al. \(2018\)](#). In our minigene assay, the variant exhibited completely aberrant splicing and is predicted to undergo nonsense mediated decay (NMD). As the artificial splicing and cell system merely models the environment in which the variant is expected to exert its effect, we have classified it as PVS1_Strong (RNA) on the basis of the PVS1 decision framework described by [Walker et al. \(2023\)](#). Furthermore, the variant is absent from public databases such as gnomAD v4.1.0, for application of PM2_Supporting. Taken together, the c.4105+5G>C variant is classified as likely pathogenic according to the HL adapted American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines ([Oza et al. 2018](#)).

Family 3 and 4: The recessive inherited c.5272+5G>C variant has not been reported in the population database gnomAD v4.1.0, applying PM2_Supporting. Results from an *in vitro* minigene assay demonstrated a disruptive effect on pre-mRNA splicing, leading to a frameshift, premature stop codon, and presumably NMD, giving PVS1_Strong (RNA) ([Walker et al. 2023](#)). The c.5272+1G>A variant has previously been reported as pathogenic by [Behlouli et al. \(2016\)](#) and affects the same donor splice site region (exon 16) as the c.5272+5G>C variant. Both variants exhibit high SpliceAI scores for donor site disruption (0.99 for c.5272+1G>A and 0.78 for c.5272+5G>C) and identical acceptor loss scores (0.27), indicating a comparable impact on splicing. According to [Walker et al. \(2023\)](#), these similarities justify the application of PS1_Moderate. Although segregation analysis in two consanguineous pedigrees was conducted, it does not meet the requirements for PP1 ([Oza et al. 2018](#)). Based on this evidence, the c.5272+5G>C variant is classified as likely pathogenic according to the adapted ACMG/AMP guidelines ([Oza et al. 2018](#)).

Family 5: The novel c.5942A>G variant is absent from the population database gnomAD v4.1.0, for the application of PM2_Supporting. Notably, it has been previously reported in ClinVar (ID: 1315315) in 2021 and classified as a variant of uncertain significance (VUS). However, the pedigree allows the assignment of PP1_Supporting in line with [Oza et al. \(2018\)](#). Furthermore, the c.5941T>A variant causes the p.(Tyr1981Asn) amino acid exchange. An exchange of the same amino acid has been previously classified as pathogenic (ClinVar ID: 379299), providing PM5_Moderate evidence. AlphaMissense predicts this variant to be pathogenic with a score of 0.9467, allowing the application of PP3_Moderate (Supplementary Table A1) ([Bergquist et al. 2024](#); [Pejaver et al. 2022](#)). Given these findings, we therefore classify c.5942A>G as likely pathogenic according to the adapted ACMG/AMP guidelines ([Oza et al. 2018](#)).

Family 6: The identified missense variant c.2858T>A is absent from the population database gnomAD v4.1.0, fulfilling the PM2_Supporting criterion. Moreover,

AlphaMissense predicts the variant to be pathogenic with a score of 0.9384, meeting the criteria for PP3_Moderate according to Bergquist et al. (2024) and Pejaver et al. (2022) (Supplementary Table A1). Based on the available evidence, this variant is currently classified as a variant of uncertain significance (Oza et al. 2018).

Family 7: The c.4437C>G variant is supported by PP1_Strong evidence, derived from segregation analysis in an extended pedigree that includes six affected individuals (Oza et al. 2018). Its absence in gnomAD v4.1.0 supports the application of PM2_Supporting. In addition, AlphaMissense predicts this variant as pathogenic (0.9971), enabling the use of PP3_Strong (Supplementary Table A1) (Bergquist et al. 2024; Pejaver et al. 2022). Accordingly, the c.4437C>G variant is classified as pathogenic according to the adapted ACMG/AMP guidelines (Oza et al. 2018).

3 Discussion

In this study, we identified five novel variants in *TECTA* across seven families. Five families followed an autosomal recessive pattern of inheritance, while two showed an autosomal dominant pattern of inheritance. We utilized a combination of WES, *in vitro* splice assays, segregation analysis and molecular modeling to identify and classify these variants.

The c.4105+5G>C variant was identified in two independent Iranian families, following an autosomal recessive pattern. The HL in the index patient of family 1 was congenital, bilateral, and profound. An *in vitro* minigene assay was conducted and demonstrated skipping of exon 12, resulting in a frameshift and premature stop codon after fourteen amino acids. The stop codon is not located near the last exon or in a long exon (>400 nucleotides) (Lindeboom et al. 2016). Consequently, we expect the resulting mRNA to undergo NMD.

The biallelic c.5272+5G>C variant was identified in two independent Iranian families, families 3 and 4, both with consanguineous pedigrees. All affected members of these families have bilateral NSHL. An *in vitro* minigene assay demonstrated that the variant leads to skipping of exon 16, resulting in a frameshift and a premature stop codon after five amino acids. Given the location of the stop codon, this variant likely also triggers NMD.

Six variants in *TECTA* have so far been described as causing aberrant splicing (Hildebrand et al. 2011; Behlouli et al. 2016; Yang et al. 2024, 2025; Mustapha et al. 1999). Notably, Behlouli et al. have reported the c.5272+1G>A variant, which affects the same exon as the c.5272+5G>C variant reported here, in an Algerian family. In their study, the patients have exhibited prelingual, bilateral HL, with audiograms characterized as downsloping and hearing impairment severity ranging from moderate to severe. Autosomal recessive variants in *TECTA* have been reported to cause moderate to severe HL affecting mid-frequencies or all frequencies, with flat or U-shaped audiograms, irrespective of the variant's location (Mustapha et al. 1999; Meyer et al. 2007; Naz et al. 2003; Yamamoto et al. 2017). These findings underscore the variant heterogeneity of *TECTA* and its association with auditory pathologies. Understanding these variants is crucial for elucidating the molecular mechanisms underlying hearing impairment and for developing targeted therapeutic strategies.

415 The *TECTA* c.2858T>A variant was identified in a German family. Unfortunately,
 416 we were only able to investigate the heterozygous 4-year-old index patient; other family
 417 members were not available for genetic analysis. Missense variants in the ZA domain of
 418 *TECTA* are typically associated with autosomal-dominant high-frequency HL, which
 419 is consistent with the audiometric phenotype observed in one of the patient's ears (Bal-
 420 ciuniene et al. 1999; Plantinga et al. 2007, 2006; Yamamoto et al. 2017). The affected
 421 amino acid residue, Leu953, is conserved across multiple species, including human, rat,
 422 mouse, and zebrafish. The overall protein sequence identity of α -tectorin is approxi-
 423 mately 90% between humans and other mammalian species, and about 60% with the
 424 zebrafish. Molecular modeling suggests that the Leu953 substitution likely weakens
 425 hydrophobic interactions that are important for maintaining the local secondary struc-
 426 ture of α -tectorin. Although this specific mechanism has not yet been experimentally
 427 confirmed in *TECTA*, missense variants are known to result in structural defects of
 428 the TM in mutant mice (Legan et al. 2014).

429 Only a few autosomal recessive missense variants in *TECTA* have been reported.
 430 One of the earliest examples has been documented in mouse models by Moreno-
 431 Pelayo et al. (2008), where they have characterized the spontaneous c.1046C>A
 432 p.(Ala349Asp). In their study, they have found, that the *Tecta*^{A349D/A349D} mouse
 433 line has displayed a phenotype similar to the deletion of the NIDO domain in the
 434 *Tecta* ^{Δ ENT/ Δ ENT} mouse line. Both lines have exhibited a TM that has been detached
 435 from the organ of Corti and spiral limbus, has lacked a striated-sheet matrix, and
 436 has been without otogelin and β -tectorin (*Tectb*). However, in the *Tecta*^{A349D/A349D}
 437 mouse line accumulations of mutated α -tectorin-A349D protein has still been detected
 438 within the remaining structure. This has demonstrated that the α -tectorin-A349D
 439 protein is not capable of interacting with β -tectorin or otogelin. We, therefore, hypoth-
 440 esize that a comparable sequence of event may occur for the recessive c.5942A>G
 441 variant described here, with an underlying pathomechanism more consistent with a
 442 loss-of-function phenotype than with a dominant-negative effect.

443 The autosomal dominant c.4437C>G variant was also identified in a German fam-
 444 ily (family 7), located in the ZA immediately preceding the vWFD4 domain. The
 445 17-year-old index patient displayed bilateral mid to high frequency HL, with the mid-
 446 dle frequencies being more pronounced. In contrast, other family members displayed a
 447 more pronounced HL in the higher frequencies. This is in line with previous reported
 448 genotype-phenotype correlation, where variants in the ZA domain typically affect the
 449 higher frequencies (Balciuniene et al. 1999; Plantinga et al. 2007, 2006; Yamamoto
 450 et al. 2017). The c.4437C>G variant resulted in the exchange of Cys1479 to a tryp-
 451 tophan, causing in a broken disulfide bond. In previous studies, variants affecting
 452 cysteine residues have been reported to result in a progressive form of HL (Hildebrand
 453 et al. 2011; Balciuniene et al. 1999; Pfister et al. 2004; Moreno-Pelayo et al. 2001).
 454 The index patient's mother (III-5) further reported a hearing deterioration.

455 Several studies have proposed that the mechanism by which dominant *TECTA*
 456 variants cause autosomal dominant non-syndromic hearing loss (ADSNHL) is via
 457 dominant-negative interference (Yasukawa et al. 2019; Yang et al. 2025; Asgharzade
 458 et al. 2017; Verhoeven et al. 1998). In a study by Hildebrand et al. (2011), the first
 459 homozygous patient with a dominant variant has been described. As expected, this
 460

patient has exhibited a more severe phenotype compared to older heterozygous carriers. One hypothesis originates from a recently identified splicing variant that evades NMD. This variant is hypothesized to produce an aberrant α -tectorin protein in the inner ear, disrupting the assembly of the fibrillar network (Yang et al. 2025).

According to Legan et al. (2014), haploinsufficiency is unlikely to be the mechanism by which dominant *TECTA* variants lead to HL. In their study, three mouse models have been generated, each with a different *Tecta* missense variant. Their findings have shown that α -tectorin protein has still been present in the TM of both heterozygous and homozygous knock-in mice. Earlier work by the same team has further supported this conclusion. They have detected α -tectorin in heterozygous mice carrying either a *Tecta* ^{Δ ENT/ Δ ENT} allele or a full knockout of *Tecta*. Importantly, the structure of the TM in these mice has closely resembled that of wild-type mice, reinforcing that haploinsufficiency has not been the underlying mechanism for HL in these cases. (Legan et al. 2014, 2005). These findings suggest that alternative mechanisms may underlie the pathogenicity of dominant *TECTA* variants like the c.4437C>G.

α -tectorin is thought to undergo post-translational cleavage into three distinct fragments, which are linked by disulfide bridges (Killick and Richardson 1997; Legan et al. 1997; Verhoeven et al. 1998). Variants affecting cysteine residues may destabilize the TM through two non-mutually exclusive pathways. First, the loss of critical disulfide cross-links could weaken the structural integrity of the TM, leading to disorganization, a mechanism that may underlie the pathogenic effect of the here described the c.4437C>G, p.(Cys1479Trp) variant. This mechanism has previously been described in a study by Legan et al. (2014). Second, unbound cysteines might act as nucleophiles, forming aberrant covalent bonds with other proteins within the TM. Similarly, dominant-negative mechanisms resulting from aberrant disulfide bond formation have been proposed and demonstrated for other proteins such as NOTCH3, fibulin-3, and progranulin (Lee et al. 2023; Woodard et al. 2022; Wang et al. 2010a). Whether this occurs in *TECTA* remains to be clearly demonstrated.

In summary, our study identified and characterized four novel *TECTA* variants, highlighting their roles in both autosomal dominant and recessive HL. Using NGS, *in vitro* splice assays, and *in silico* modeling, we elucidated the molecular mechanisms of these variants, emphasizing their impact on aberrant splicing and protein structure. These findings underscore the genetic heterogeneity of *TECTA* and its critical role in auditory pathologies. Understanding these mechanisms is essential for developing targeted therapeutic strategies to improve the quality of life for those affected by genetic hearing impairments.

4 Material and Methods

4.1 Patient enrolment and ethical approval

This study was approved by the ethics committees at the Medical Faculty of the University of Würzburg (approval number 131/21 and 226/18). Informed consent from the parents or legal guardians of the participants was obtained for the publication of their data. Families were ascertained in Germany and Iran as part of a large ethnically diverse Iranian population rare disease study. From an initial pool of over 600

507 individuals with HL who were negative for *GJB2* and *STRC* variants/copy number
508 variations, we selected, post hoc to genetic testing, cases with a *TECTA* diagnosis.
509 Audiometry and medical information were collected from participating individuals.

510

511 4.2 Sample collection and clinical evaluation

512

513 Blood samples were collected from all participants after obtaining informed consent
514 from patients or their parents. Genomic DNA was extracted using standard protocols.
515 Severity of HL was determined using pure-tone audiometry that measured hearing
516 thresholds at frequencies of 0.25, 0.5, 1, 2, 4, and 8 kHz. Severity of HL was determined
517 by calculating pure tone averages of frequencies between 0.5 to 4 kHz ($PTA_{0.5-4kHz}$)
518 following established guidelines to determine the severity of HL as mild (21-40 dB),
519 moderate (41-70 dB), severe (71-95 dB), or profound (>95 dB) according to established
520 guidelines ([Mazzoli et al. 2003](#)).

521

522 4.3 Whole exome sequencing

523

524 Families 1-4 and 6: exome library preparation of the index patients was performed
525 according to the Illumina Nextera Rapid Capture Enrichment protocol (Illumina, San
526 Diego, California, USA) using 50 ng of genomic DNA. Prepared libraries were sub-
527 sequently subjected to 2x76 bp paired-end sequencing on an Illumina NextSeq500
528 platform with a v2 reagent kit. Sequence reads were aligned to the human refer-
529 ence genome (GRCh37/hg19) using the Burrows–Wheeler Aligner. Variant calling and
530 downstream analyses were carried out with GensearchNGS software (PhenoSystems
531 SA, Braine le Chateau, Belgium). Variants were filtered out if they showed coverage
532 below 10, a Phred-scaled quality score lower than 15, an allele frequency under 15, or
533 a minor allele frequency (MAF) greater than 2%.

534 For family 5, exome sequencing was performed on the DNA sample of the index
535 patient by Macrogen (Seoul, Republic of Korea). Briefly, the sample was subjected to
536 exome enrichment with the SureSelect Target Enrichment v6 kit (Agilent Technologies,
537 Santa Clara, CA, USA), followed by sequencing with Illumina NovaSeq 6000 (Illumina,
538 San Diego, CA, USA) using standard protocols. Then, short reads were aligned to the
539 GRCh38 using BWA ([Li and Durbin 2009](#)) and duplicate reads were marked using
540 Picard ([Pic 2019](#)). GATK ([van der Auwera 2020](#)) and ANNOVAR ([Wang et al. 2010b](#))
541 were used for variant detection and annotation, respectively.

542 For family 7 the index patient and his parents were sequenced using the TruSight
543 One panel, following the manufacturer's protocol (Illumina, San Diego, CA, USA).
544 Sequencing was performed using 2x150 bp paired-end reads on a MiSeq desktop
545 sequencer, with alignment to the human reference genome (GRCh37, hg19) (Illumina).
546 The resulting BAM files were analyzed using GensearchNGS software (PhenoSystems
547 SA, Wallonia, Belgium). Variants were filtered based on the following criteria: coverage
548 exceeding 20 reads, variant frequency above 20%, minor allele frequency below 0.01,
549 and variant types such as missense, nonsense, synonymous, and splice-site variants.
550 The remaining variants were further refined by comparing them to parental data.

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4.4 Variant classification

Initial variant prioritization was conducted using Alamut Visual software (Interactive Biosoftware, Rouen, France), integrating in silico prediction tools including SIFT (Pertea et al. 2001), MutationTaster (Schwarz et al. 2014), and PolyPhen-2 (Adzhubei et al. 2010). The possible effects of variants on pre-mRNA splicing were evaluated with SpliceSiteFinder-like, MaxEntScan (Yeo and Burge 2004), NNSPLICE (Reese et al. 1997), GeneSplicer (Pertea et al. 2001), and SpliceAI (Jaganathan et al. 2019). Databases like OMIM (Onl 29.08.26), HGMD (Stenson et al. 2014), Deafness Variation Database (DVD) (Azaiez et al. 2018) and ClinVar (Landrum et al. 2018) were used for acquiring gene and variant background information. Population frequencies were retrieved from gnomAD (v4.1.0, 807,162 individuals, released on 04/19/2024) (Chen et al. 2024). Variant classification followed the HL adapted ACMG/AMP criteria (Oza et al. 2018). Splice interpretation applied the ACMG/AMP criteria from the ClinGen SVI Splicing Subgroup (Walker et al. 2023). Variant nomenclature was verified using Mutalyzer (Lefter et al. 2021).

4.5 Sanger sequencing

To validate variants identified by NGS and perform segregation analysis, in available family members, Sanger sequencing was employed. PCR amplification was carried out using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA) under standard cycling conditions. Primers, detailed in Supplementary Table A2, were designed using Primer3. Sequencing was performed on an ABI 3130xl 16-capillary sequencer (Life Technologies, Carlsbad, CA, USA), and data were analyzed using Gensearch 4.3 software (PhenoSystems SA, Wallonia, Belgium).

4.6 Minigene assay

For variants predicted by SpliceAI to impact splicing (splicing scores >0.50), specifically the *TECTA* variants c.5272+5G>C and c.4105+5G>C, *in vitro* minigene assays were performed, as described previously (Doll et al. 2020). Briefly, wild-type and variant versions of *TECTA* exon 16 or exon 12, along with their surrounding regions, were PCR-amplified from both the index patient's and a control individual's genomic DNA using primers with XhoI and NotI restriction sites (Supplementary Table A2). These PCR products encompassed the exon and approximately 400 bp of flanking sequences both upstream and downstream. Post-amplification, the products were purified, subjected to restriction enzyme digestion, and subsequently cloned into the linearized pSPL3b-cam exon-trapping vector (Burn et al. 1995). The resulting constructs were then transformed into DH5 α competent cells, purified through mini-prep according to manufacturers protocol, and the sequences of both wild-type and variant vectors were verified by Sanger sequencing.

HEK 293T cells were transfected with 2 μ g of the respective pSPL3b-cam vector, either wild-type or variant, at a density of 2.25×10^5 cells/mL, using 6 μ L of FuGENE®HD Transfection Reagent (Promega). Controls included the empty pSPL3b-cam vector and negative transfection reactions. After 24 hours, the transfected cells were harvested for RNA using the QIAzol Lysis Reagent (Quiagen) according

599 to manufacturers protocol. Approximately 1 μ g of RNA was reverse transcribed into
600 cDNA using the FIREScript®RT cDNA synthesis kit (Solis BioDyne), following the
601 manufacturer’s instructions. The resulting cDNA was PCR-amplified using vector-
602 specific primers SD6 and SA2 (Supplementary Table A2). The PCR products were
603 visualized on a 1% agarose gel and confirmed by Sanger sequencing.

604

605 4.7 Structure prediction and molecular modeling

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607 To evaluate the potential structural and functional impacts of the amino acid substitu-
608 tions c.2858T>A, c.4437C>G and c.5942A>G, AlphaFold2 was employed to generate
609 3D structure models, as no experimentally determined crystal structure is currently
610 available for α -tectorin (Jumper et al. 2021). Structure visualization and analysis
611 were performed using PyMOL (Schrödinger 2015). To model changes in amino acid
612 interactions and to calculate $\Delta\Delta G$ values DynaMut2 was used (Rodrigues et al. 2021).

613

614 5 Declarations

615

616 5.1 Ethics approval and consent to participate

617 Informed consent to participate was obtained from all the participants and/or their
618 legal guardians. This study was approved by the ethics committees at the Medical
619 Faculty of Würzburg University, Germany (approval number 131/21 and 226/18) and
620 complied with the Declaration of Helsinki.

621

622 5.2 Consent for publication

623

624 Written informed consent for publication of their clinical details and/or clinical images
625 was obtained from the patients and/or their legal guardians.

626

627 5.3 Availability of data and material

628 All variants have been submitted to ClinVar and are publicly available. The ClinVar
629 submission number is SUB16447368, and the accession numbers are SCV007670376 -
630 SCV007670380.

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632 5.4 Competing interests

633

634 The authors declare no competing interests.

635

636 5.5 Funding

637 B.V. is supported by the German Research Foundation (DFG) Heisenberg program VO
638 2138/8-1 grant 543719215 and the DFG Collaborative Research Center 1690 (Project
639 A03).

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641 5.6 Authors’ contributions

642
643 D.B., T.H. and M.A.H.H. designed and organized the study. M.A.H.H, D.L. and T.H.
644 supervised this study. D.B. and W.E.P. designed, visualized, and performed functional

studies. A.R., B.O., E.K., N.C., M.Z., H.G. contributed to the patient recruitment 645
and clinical data. M.D. performed the bioinformatic WES data analysis. C.G. and 646
D.B. performed the molecular modeling. M.A.H.H., J.D., L.S., S.K. and B.V. analyzed 647
the WES data. A.B. provided the resources to conduct this study. D.B. wrote the 648
manuscript, and all the coauthors reviewed the manuscript. 649

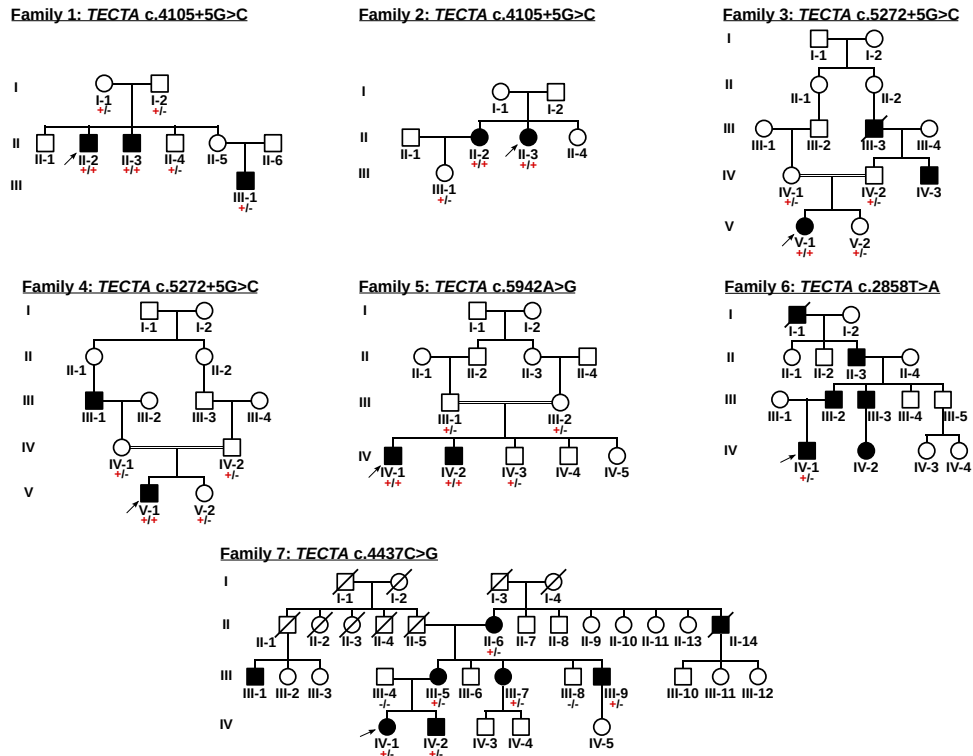
5.7 Acknowledgements 650
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We thank the families for their participation and for sharing relevant information. 652
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5.8 Authors' information 654
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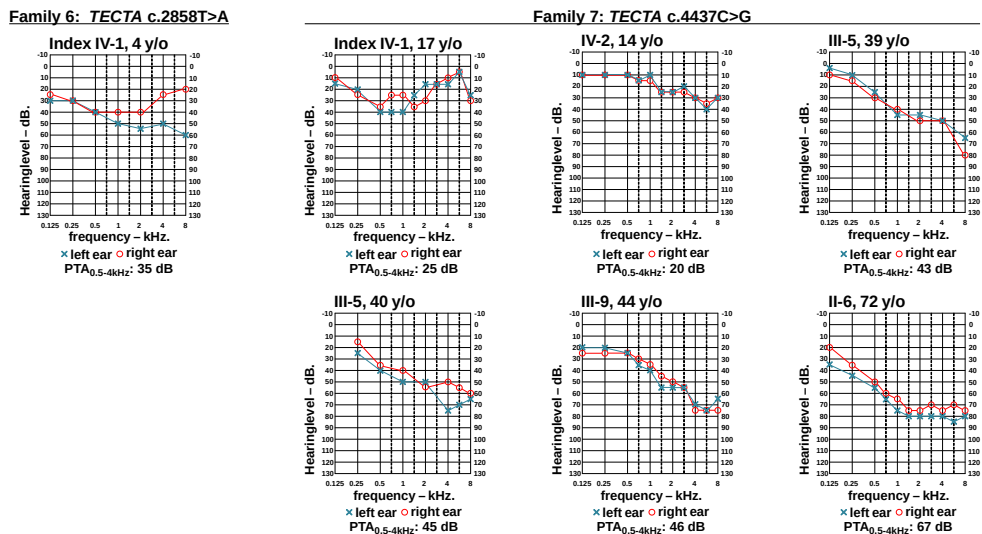


Fig. 1 Pedigrees of families 1 to 7 and available audiograms. (A) Pedigrees of families 1 to 7 and segregation results. Index patients are indicated by arrows. Genotypes are denoted as: +/+ (homozygous), +/- (heterozygous), and -/- (wild-type). Variant alleles are highlighted in red. Squares, males; circles, females; black symbols, affected individuals; white symbols, unaffected individuals. (B) Audiograms for members of family 6 and 7. Left ear marked by blue crosses, right ear by red rings

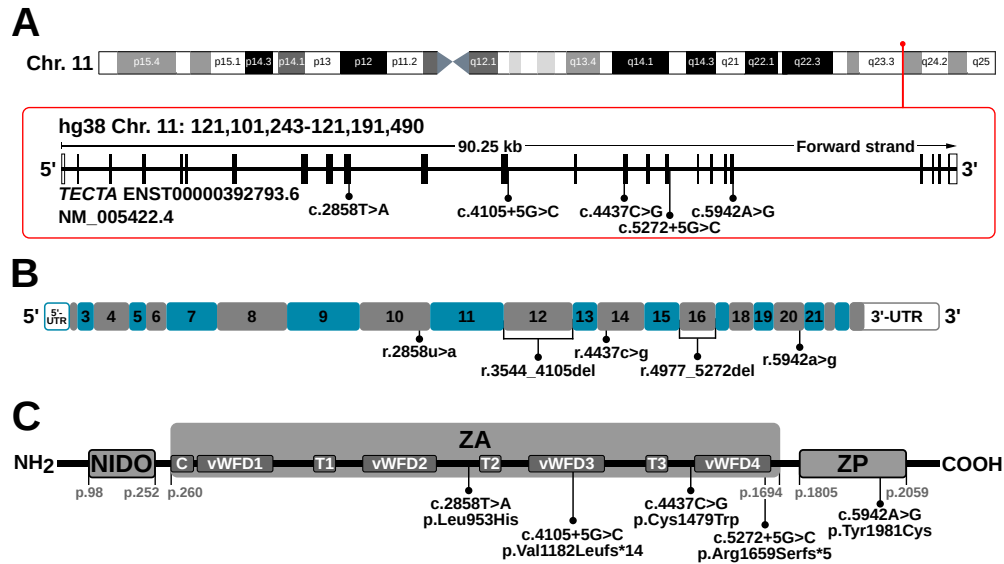


Fig. 2 Genomic structure of the human *TECTA* gene, including exons, encoded protein, and identified variants. (A) Genomic organization and chromosomal location of *TECTA*, adapted from [Cunningham et al. \(2022\)](#). Genetic variants are mapped to the NM_005422.4 transcript of *TECTA*. (B) Exon composition of *TECTA*. (C) Structural domains of α -tectorin, based on UniProt entry O75443 ([The UniProt Consortium 2025](#)). Abbreviations: NIDO (Nidogen-like domain), ZA (zonadhesin-like domain), C (von Willebrand factor type C domain), vWFD1-4 (von Willebrand factor type D domain 1-4), T1-3 (trypsin inhibitor-like repeats 1-3), ZP (zona pellucida domain)

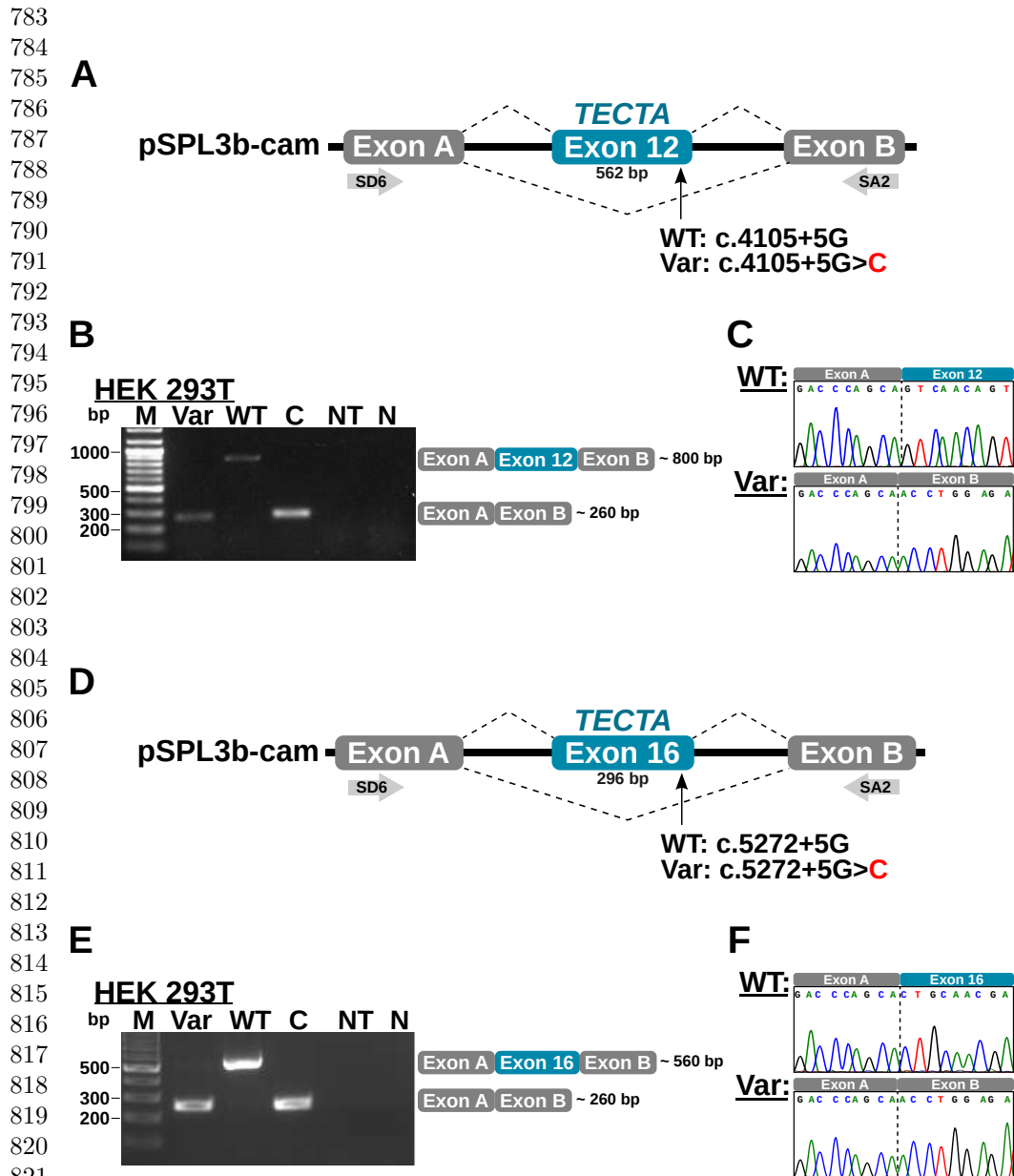


Fig. 3 Splicing analysis of the *TECTA* variants c.4105+5G>C and c.5272+5G>C. (A, D) Schematic representation of the splicing behavior in wild-type and variant constructs. (B, E) Agarose gel electrophoresis of RT-PCR products comparing wild-type and variant constructs. (C, F) Sanger sequencing results of the RT-PCR products shown in panels B and E. Abbreviations: WT (wild-type), Var (variant), C (control), NT (not transfected), N (negative control), bp (base pairs).

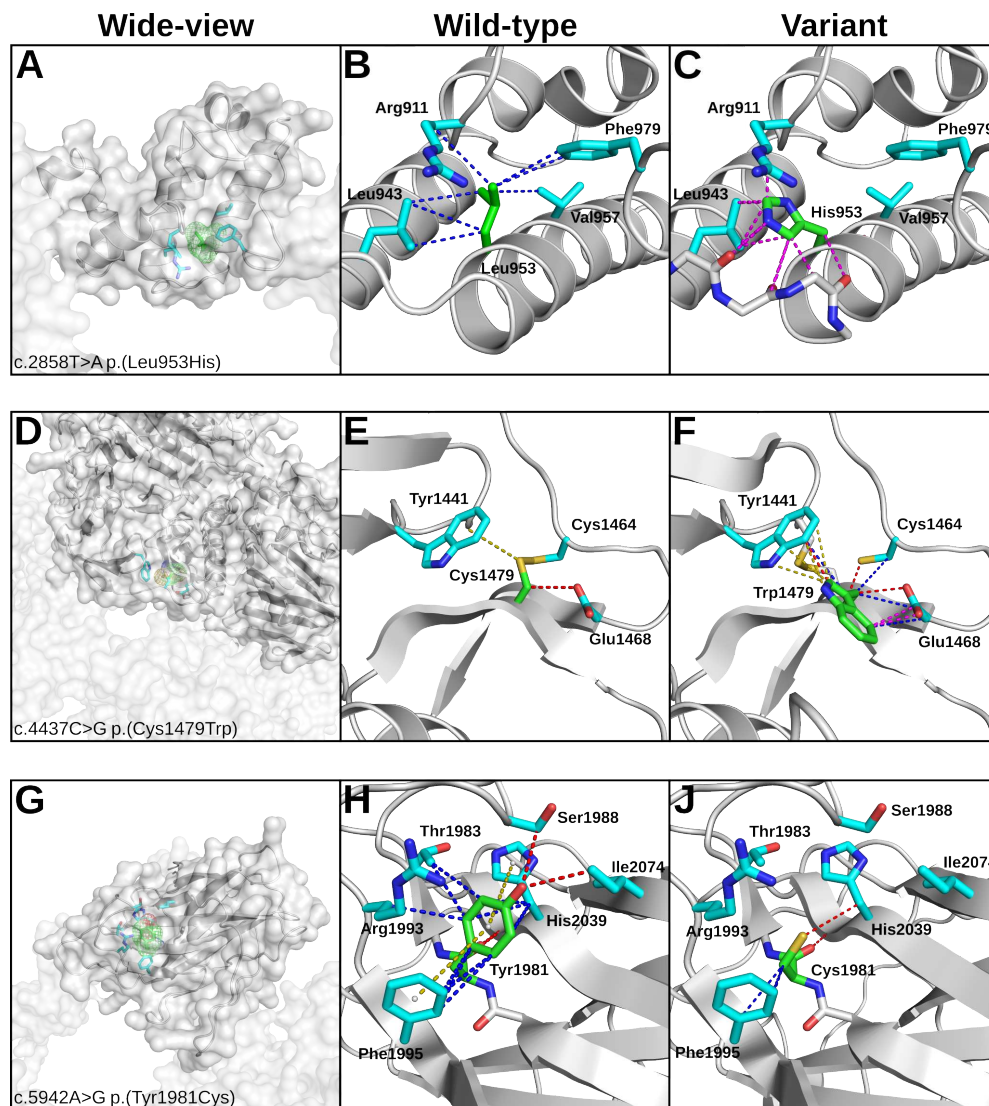


Fig. 4 Ribbon representations of α -tectorin structure predictions using AlphaFold2 and DynaMut2 for the c.2858T>A p.(Leu953His), c.4437C>G p.(Cys1479Trp) and c.5942A>G p.(Tyr1981Cys) variants. Affected residues are highlighted in green, while interacting residues are shown in light blue. Interactions are color-coded as follows: red (hydrogen bonds), blue (hydrophobic), yellow (aromatic), and pink (clashes). (A, D, G) Wide-view visualization of residues Leu953, Cys1479, and Tyr1982 within the broader protein structure. (B, E, H) Close-up of the wild-type structure, illustrating predicted residue interactions. (C, F, J) Variant models depicting the p.(Leu953His) ($\Delta\Delta G$ stability: -0.72 kcal/mol), p.(Cys1479Trp) ($\Delta\Delta G$ stability: -0.92 kcal/mol), and p.(Tyr1981Cys) ($\Delta\Delta G$ stability: -1.37 kcal/mol) substitutions, along with changes in amino acid interactions.

Appendix A Supplementary information

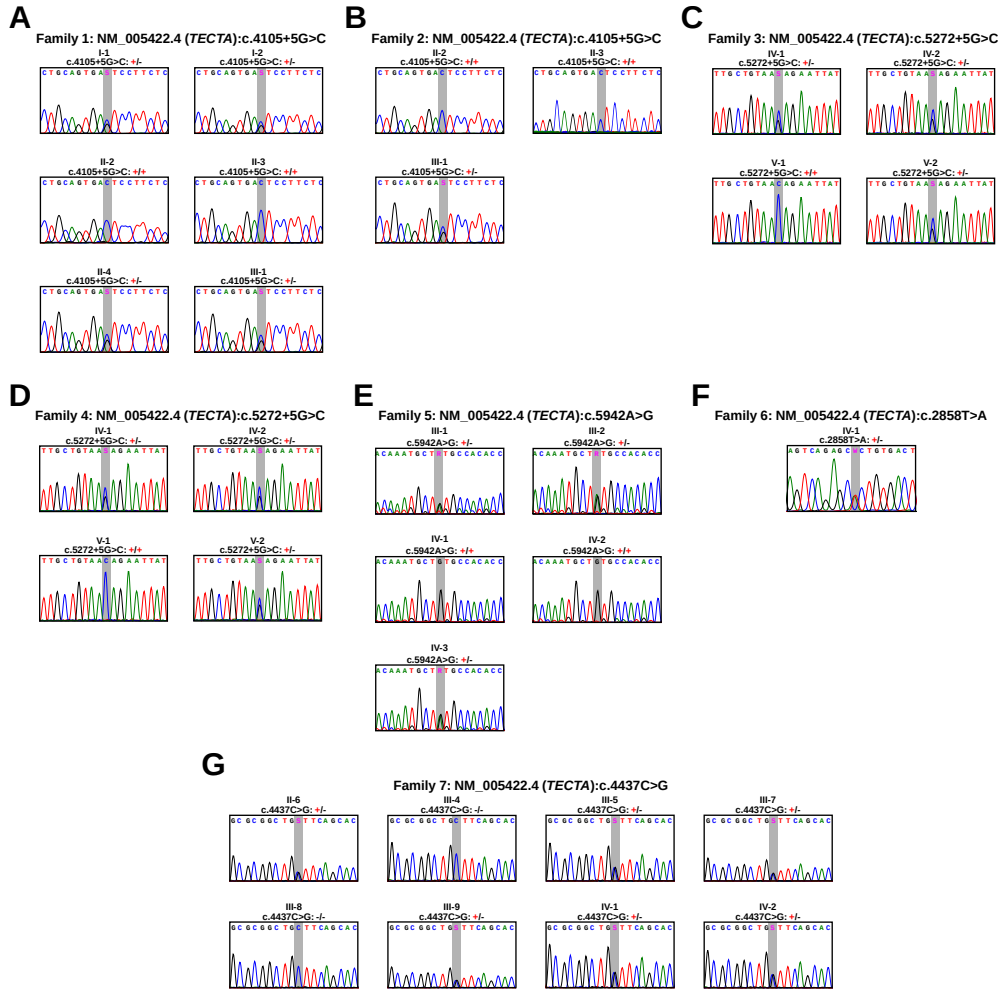


Fig. A1 Segregation analysis using Sanger sequencing for all available family members. (A, B) Sanger sequencing chromatograms for the c.4105+5G>C variant in families 1 and 2; (C, D) Sanger sequencing chromatograms for the c.5272+5G>C variant in families 3 and 4; (E) Sanger sequencing chromatograms for the c.5942A>G variant in family 5; (F) Sanger sequencing chromatograms for the c.2858T>A variant in family 6; (G) Sanger sequencing chromatograms for the c.4437C>G variant in family 7. Genotypes are denoted as: +/- (heterozygous), and -/- (wild-type). Variant alleles are highlighted in red.

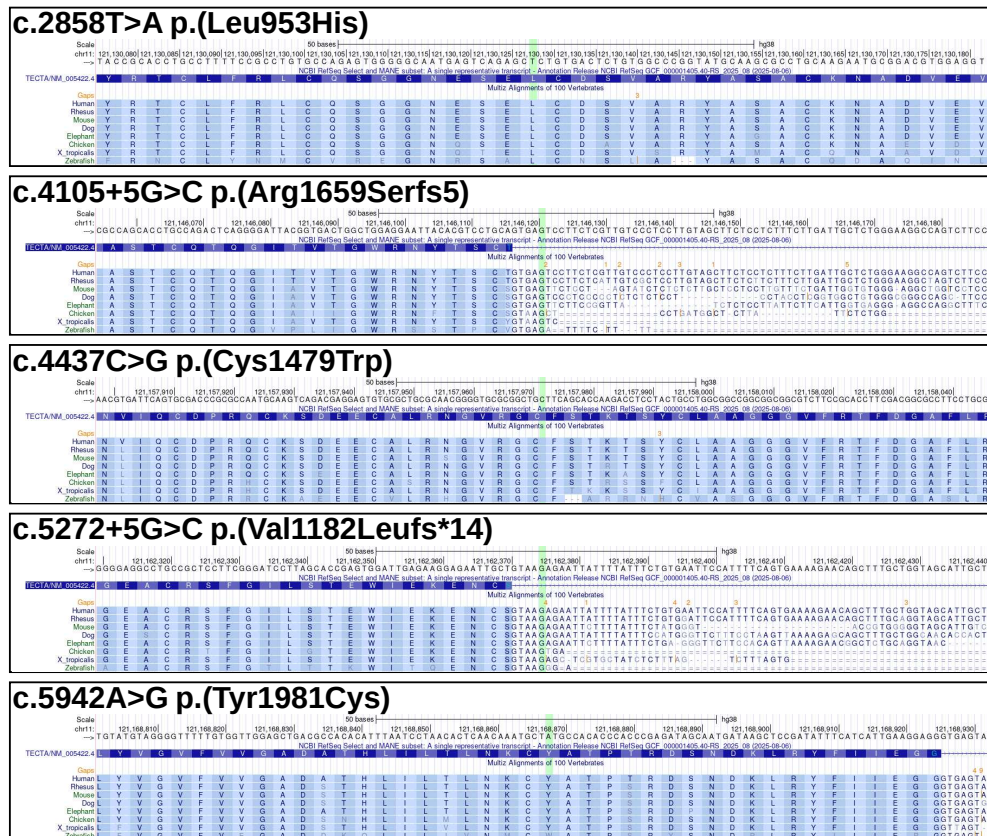


Fig. A2 Conservation analysis of the identified *TECTA* variants. Variants are highlighted in green. Based on data from the UCSC genome browser (<http://genome.ucsc.edu/>)

Table A1 Results of *in silico* prediction tools for all missense variants discussed. The gene specific threshold for PrimateAI-3D: 0.77.

Variant	Method	Score
NM_005422.4(<i>TECTA</i>):c.2858 T>A	AlphaMissense	0.9384
	CADD	27.6
	PrimateAI-3D	0.87
	REVEL	0.936
	SIFT (max)	0
NM_005422.4(<i>TECTA</i>):c.4437C>G	AlphaMissense	0.9971
	CADD	28.4
	PrimateAI-3D	0.97
	REVEL	not available
	SIFT (max)	0
NM_005422.4(<i>TECTA</i>):c.5942A>G	AlphaMissense	0.9467
	CADD	29.3
	PrimateAI-3D	0.85
	REVEL	0.675
	SIFT (max)	0.003

Table A2 Primer used in this study. Overhangs with respective restriction sites are highlighted by the use of (*).

Primer	Sequence (5'->3')
TECTA_c.5942A_fw	AATGGTAGGTAATTGAATAGG
TECTA_c.5942A_rev	GAATGTTGTCTATAAAGAGGC
SD6-Primer	ATTTAGGTGACACTATAG
SA2-Primer	ATCTCAGTGGTATTTGTGAGC
TECTA_5272+5_seq_rev_minigen	CACAGCTCTCTACTATCCAATCC
TECTA_5272+5_XhoI_fw	*TCATACTCGAG*GTGGAAGTGGCTTAGAAACC
TECTA_5272+5_NotI_rev	*TTCATTGCGGCCGC*TCGCTTTCTGATAGAGGACGG
TECTA_4105+5_seq_rev_minigen	ACTCAGTTCCAAAGTGCATATCC
TECTA_4105+5_XhoI_fw	*TCATACTCGAG*ACCAAGGATAGTCATTTGCGTC
TECTA_4105+5_NotI_rev	*ATCATTGCGGCCGC*CCAAGAGGTTACATGTCATGC
TECTA_c.4437_fw	GCCAAGCCTGATAAAAGACG
TECTA_c.4437_rev	TAAGCTGGAAGGAGATGTCG
TECTA_c.2858_fw	GGGCAGACCGTGTCTTTATC
TECTA_c.2858_rev	TTGAGCTCAGTCCCACCTG

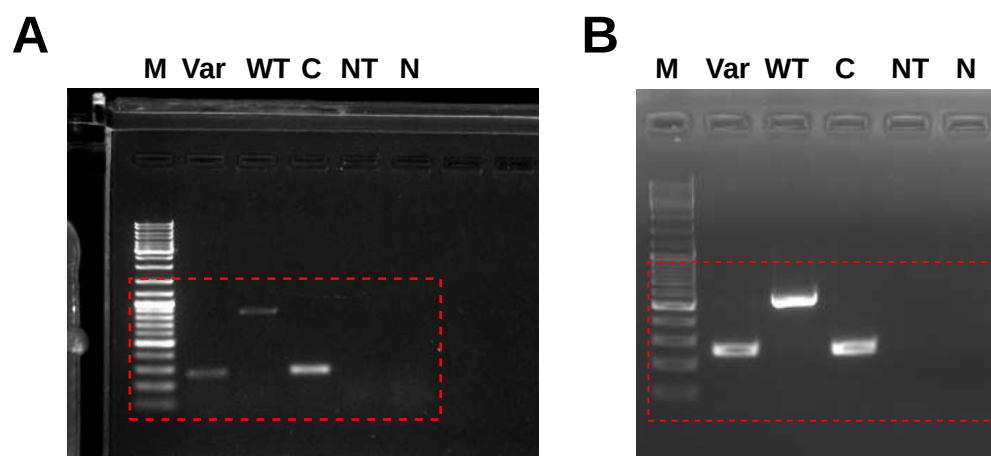


Fig. A3 Full images of agarose gel electrophoresis displaying RT-PCR products for wild-type and variant samples, with the cropped regions marked in red and presented in Figure 3. (A) Uncropped image of Figure 3 B. (B) uncropped image of Figure 3 E. Abbreviations: WT (wild-type), Var (variant), C (control), NT (not transfected), N (negative control), bp (base pairs).

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