TBC1D24 emerges as an important contributor to progressive postlingual dominant hearing loss

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Several TBC1D24 variants are causally involved in the development of profound, prelingual hearing loss (HL) and different epilepsy syndromes inherited in an autosomal recessive manner. Only two TBC1D24 pathogenic variants have been linked with postlingual progressive autosomal dominant HL (ADHL). To determine the role of TBC1D24 in the development of ADHL and to characterize the TBC1D24-related ADHL, clinical exome sequencing or targeted multigene (n = 237) panel were performed for probands (n = 102) from multigenerational ADHL families. In four families, TBC1D24-related HL was found based on the identification of three novel, likely pathogenic (c.553G>A, p.Asp185Asn; c.1460A>T, p.His487Leu or c.1461C>G, p.His487Gln) and one known (c.533C>T, p.Ser178Leu) TBC1D24 variant. Functional consequences of these variants were characterized by analyzing the proposed homology models of the human TBC1D24 protein. Variants not only in the TBC (p.Ser178Leu, p.Asp185Asn) but also in the TLDC domain (p.His487Gln, p.His487Leu) are involved in ADHL development, the latter two mutations probably affecting interactions between the domains. Clinically, progressive HL involving mainly mid and high frequencies was observed in the patients (n = 29). The progression of HL was calculated by constructing age-related typical audiograms. TBC1D24-related ADHL originates from the cochlear component of the auditory system, becomes apparent usually in the second decade of life and accounts for approximately 4% of ADHL cases. Given the high genetic heterogeneity of ADHL, TBC1D24 emerges as an important contributor to this type of HL.

Abbreviations
ABRs  Auditory brainstem responses
ARTA  Age-related typical audiogram
ATD  Annual threshold deterioration
ADHL  Autosomal dominant hearing loss
OAE  Otoacoustic emissions
nHL  Normal hearing level
cVEMP  Cervical vestibular evoked myogenic potentials
oVEMP  Ocular vestibular evoked myogenic potentials

Increasing use of high throughput DNA sequencing methods has significantly improved the detection rate of genetic alterations causative of hearing loss (HL). It has resulted in discovering novel HL variants and genes and assigning new inheritance patterns to known HL genes1–3. Several HL genes are causally involved in the development of both autosomal recessive and dominant forms of hereditary HL. Another level of complexity is

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provided by the diversity of clinical presentation. Some HL genes may lead to isolated HL but also syndromes that do not necessarily include HL as one of their phenotypic features. This phenomenon is well-exemplified by the TBC1D24 gene.

Recessive variants detected in TBC1D24 may cause a spectrum of phenotypes, beginning with a mild form of familial infantile myoclonic epilepsy (FIME; OMIM #605021) and encompassing early-infantile epileptic encephalopathy 16 (EIEE16; OMIM #615338) and progressive myoclonic epilepsy (PME) that represent a combination of epilepsy with other variable features and ending with DOORS syndrome (deafness, onychodystrophy, osteodystrophy, mental retardation and seizures; OMIM #220500), a syndromic form of HL. Alterations in TBC1D24 were also found in patients with isolated HL inherited in autosomal recessive (DFNB86; OMIM #614617) or autosomal dominant manner (DFNA65; OMIM #616044). While the involvement of TBC1D24 in the development of a recessive form of HL is documented, with ten pathogenic variants identified so far, only two TBC1D24 pathogenic variants have been reported in the context of autosomal dominant HL (ADHL).

More than 40 different TBC proteins (TBC domain-containing proteins) are present in humans. TBC1D24 (and its homolog from D. melanogaster—Skywalker) contains a unique combination of TBC and TLDc domains. The TBC domain is considered to serve as GTPase-activating protein (GAP) that promotes GTP conversion to GDP in Rab proteins, which inactivates them. The Rab proteins are a crucial component of vesicular trafficking and are involved in vesicle formation, cargo transport along the cytoskeleton, and membrane fusion. In 2006, a dual-finger mechanism of the TBC-Rab activity was proposed by Pan et al. This mechanism, both the TBC domain and Rab supply residues crucial for GTP hydrolysis. Interestingly, a different mechanism was postulated for the TBC domain of Skywalker, as the region that supposedly interacts with Rab, is substantially different from that observed for other TBC proteins. This alternative hypothesis explains the TBC1D24 impact on vesicular traffic by the ability of its TBC domain to interact directly with the membrane. This hypothesis originates from the observation of the positively charged region unique for the TBC1D24 orthologs, constituting residues that are conserved among this family and are not observed in other TBC proteins. The binding is presumed to occur through direct interactions with phosphoinositides in the lipid bilayer as the TBC domain from Skywalker does not bind to membranes devoid of these lipids. The estimated $K_d$ (dissociation constant) for IP$_3$ binding is around 0.019 mM, similar to other phosphoinositide-binding proteins. Moreover, changes of the positively charged interface (e.g., Arg79Cys, Arg281Cys) significantly weaken the interactions with IP$_3$ ($K_i$ rises to 0.32 and 0.18 mM, respectively) and produce several phenotypes that can be linked to impaired vesicle transport, such as seizures. The role of TLDc, the second TBC1D24 domain is more obscure. However, oxidative stress sensing or resistance has been demonstrated in cell cultures.

For this study we carefully selected 102 HL families with pedigrees showing an autosomal dominant mode of inheritance. We found that in four of them, HL developed due to a TBC1D24 pathogenic variant. For three of the families, the identified variants were novel, not previously associated with hearing impairment. Stimulated by the relatively high frequency of TBC1D24 variants in the ADHL cohort, we focused on molecular aspects of detected TBC1D24 variants and clinical features of the TBC1D24-related HL. In this paper, we provide extensive characteristics of this type of HL.

Materials and methods

Ethics approval. All tested subjects gave informed consent for participation in the study following the tenets of the Declaration of Helsinki. The study was approved by the ethics committee at the Institute of Physiology and Pathology of Hearing (KB.IFPS.25/2017).

Study subjects. A total of 102 multigenerational families with HL occurring in at least three generations were recruited for the study. In all recruited families, HL was transmitted from at least one male family member to offspring, which strongly indicated an autosomal dominant mode of HL inheritance. From this group, four families with TBC1D24 pathogenic variants ($n = 29$ HL patients and $n = 22$ unaffected individuals) were selected for further evaluation (Fig. 1A–D).

Audiometry and hearing threshold data analysis. Pure-tone audiometry (PTA) was performed in individuals from the tested families. Hearing thresholds were measured with the AC40 clinical audiometer (Interacoustics, Middelfart, Denmark) for frequencies 125–8000 Hz with 10/5 dB descending-ascending threshold estimation procedure. The degree of HL was described as mild (21–40 dB), moderate (41–70 dB), severe (71–90 dB) or profound (> 90 dB). All available previous PTA results were also analyzed. Symmetry of hearing thresholds was validated, and mean binaural air conduction thresholds (dB hearing level, dBHL) were calculated. Patient III.10 from Family 2 has been excluded from further comparisons because his audiograms revealed steeply-sloping high-frequency HL different from all his family members. His HL most likely developed due to the acoustic trauma experienced in adolescence; none of his children had HL (Supplementary Fig. S1). Binaural hearing threshold data from families with TBC1D24-related HL were also collected.

Based on the obtained PTA data ($n = 81$ binaural hearing thresholds) from individuals with different TBC1D24 pathogenic variants, the age-related typical audiograms (ARTA) were constructed as described previously. A cross-sectional linear regression analysis of threshold on age was performed for every hearing frequency, and then characteristic hearing thresholds were predicted for fixed ages (10–80 years). The HL progression was determined and expressed as the annual threshold deterioration (ATD; dB per year). The progression was considered significant if the regression coefficient (slope) was significantly different from 0 at $p < 0.05$.

The ARTA were also constructed separately for TBC1D24 p.Ser178Leu (Family 1 together with the previously published data for this variant), as well as for p.Asp185Asn (Family 2) and p.His487Leu (Family 4). There...
Figure 1. Four families with TBC1D24 pathogenic variants. Black symbols indicate individuals affected by HL; open symbols indicate unaffected individuals; diagonal line denotes the deceased individuals. YOB year of birth. In Family 1 (A) TBC1D24 c.533C>T (p.Ser178Leu), in Family 2 (B) TBC1D24 c.553G>A (p.Asp185Asn), in Family 3 (C) TBC1D24 c.1461C>G (p.His487Gln) and in Family 4 (D) TBC1D24 c.1460A>T (p.His487Leu) were identified as causative for ADHL.
were no longitudinal PTA data for separate ARTA calculations for the remaining TBC1D24 p.His487Gln variant (Family 3). To evaluate differences between characteristic HL patterns observed in patients with different TBC1D24 variants, threshold features arrays were calculated. The data were plotted and compared using the chi-square goodness of fit test with significance level p < 0.05.

Additional audiological and neurotological evaluation. For all probands (IV.1 Family 1; IV.12 Family 2; IV.7 Family 3 and IV.6 Family 4) additional audiological and neurotological examinations were performed. Assessment of auditory function comprised impedance audiometry, transient evoked otoacoustic emissions (TEOAE) and auditory brainstem responses (ABRs). Acoustic impedance measurements were performed with the Zodiac 901 instrument (Madsen Electronics, Copenhagen, Denmark). Stapedius reflex was analyzed for the frequencies 500, 1000, 2000 and 4000 Hz in the ipsilateral and contralateral modes. TEOAE were evoked by standard nonlinear click stimulus with an intensity of 80 ± 5 dB peSPL and recorded using the ILO-292 system (Otodynamics Ltd, Hatfield, United Kingdom).

ABRs were recorded using the Integrity V500 system (Vivosonic Inc., Toronto, Canada). The stimulus was 0.1 ms click with alternating polarity presented with the 90 dB normal hearing level (nHL) intensity at repetition rates of 11/s and 37/s. The amplifier bandwidth was 30–1500 Hz and analysis time 11 ms. The number of sweeps required for an averaged response was 1024.

The objective vestibular function was assessed based on the cervical and ocular evoked myogenic potentials (cVEMP, oVEMP) recorded following stimulation with a 500 Hz tone burst (2 ms rise/fall time, 2 ms plateau) presented through 3 M E-A-R TONE insert earphones at an intensity of 97 dB nHL and a presentation rate of 5.1/s. VEMP measurements were performed using the Interacoustics Eclipse module system (EclipseVemp, Interacoustics, Assens, Denmark). oVEMP responses were considered present if they were stronger than noise, and the N1 and P1 latencies replicated exactly across multiple collections. Responses were required to be larger than 1.5 mV to be considered present. oVEMP interaural amplitude was considered significantly symmetric and abnormal if there was over 33% side-to-side difference. cVEMP interaural amplitude was considered significantly asymmetric and abnormal, if there were over 36% side-to-side difference. To evaluate inner ear morphology and vestibulocochlear nerves anatomy, the temporal bone computed tomography was performed on a 64-slice CT scanner (Siemens CT Definition AS, Germany).

Multigene panels and Sanger sequencing. Genomic DNA was isolated from whole blood samples and buccal swabs of available family members. In probands from Families 1 (IV.1), 3 (IV.7) and 4 (IV.6) a custom multigene panel containing 237 HL genes was performed (SeqCap EZ Choice, Roche, Basel, Switzerland) and the DNA libraries were run on MiSeq using 2 × 75 bp paired-end reads. In the index patient from Family 2 (IV.12) clinical exome sequencing (TruSightOne, Illumina, Cambridge, UK) was performed according to the manufacturer’s protocol. The sample was run on MiSeq using 2 × 150 bp paired-end reads. Bioinformatics analysis was performed as described previously. Selected reads and candidate variants were verified with the Integrative Genomics Viewer (IGV).

The analysis pipeline included variant population frequencies from different population databases, i.e., the UK10K project (https://www.uk10k.org/), the NHLBI GO Exome Sequencing Project (ESP) (https://esp.gs.washington.edu/drupal/) and the Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org) (all accessed in 03/2021). Pathogenicity predictions for non-synonymous variants were performed using REVEL, CADD, LRT, PolyPhen-2, SIFT and MutationTaster computational algorithms. The potential effect of detected variants on TBC1D24 RNA splicing was assessed using SpliceSite Finder, MaxEntScan, NNSPLICE, GeneSplicer algorithms integrated with Alamut Visual Software v2.15 (Interactive Biosoftware, Rouen, Paris). The pathogenic potential of identified variants was evaluated according to standards and guidelines for interpreting sequence variants.

The presence of the candidate pathogenic variants was confirmed by Sanger sequencing and reported based on the TBC1D24 NM_001199107.1 and NP_001186036.1 reference sequences. The primer pairs 5'-GTCCTCGCGACATCTCCTCTCGTGCAGTGGGCTCTGTCATT as well as 5'-AGATGAAAAAGGTGTGTGGGCTCTCAG and 5'-CAGACCGTTGAACTCCTAGATAG were used for amplification of TBC1D24 exon 2 and 7, respectively. PCR products were labeled with BigDye Termination cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA) and sequenced with a 3500XL Genetic Analyzer (Applied Biosystems). Obtained results were analyzed using Variant Reporter Software v1.1 (Applied Biosystems).
family was also taken into consideration. The 3D model of the protein was built with MODELLER. A model quality assessment was carried out using the MolProbity webserver. Due to the presence of a nearly 40-residue long insertion in the human TLDc domain compared to the template (pdb|6r82), the model was proposed using the I-TASSER server rather than by MODELLER. To predict the putative conformation of TBC-TLDc complex in D. melanogaster, crystal structures of TBC and TLDc domains were used (pdb|5jhn and pdb|6r82, respectively). Protein–protein docking was carried out with the Hdock server using its default parameters. For the TLDc domain, amino acid conservation was calculated using the ConSurf Server and the manually curated MSA for this domain. Visualization was done with PyMol (www.pymol.org).

Table 1. Results of objective audiological, neurotological and imaging examinations of probands with TBC1D24-related ADHL. ABR auditory brainstem responses, CI cochlear implant, CT computed tomography, cVEMP cervical vestibular evoked myogenic potentials, F female, HAs hearing aids, M male, oVEMP ocular vestibular evoked myogenic potentials, PTA pure tone audiometry, TEOAE transient evoked otoacoustic emissions, y.o. years old.

| Patient | Family 1 | Family 2 | Family 3 | Family 4 |
|---------|----------|----------|----------|----------|
| IV.1    | Patient | (M, 29 y.o.) | Patient | (F, 39 y.o.) | Patient | (F, 24 y.o.) | Patient | (F, 39 y.o.) |
| Age at HL onset | 18 y.o. | 15 y.o. | 8 y.o. | 34 y.o. |
| PTA     | Bilateral, mild to moderate | Bilateral, moderate to severe | Bilateral, severe to profound | Bilateral, mild to moderate |
| TEOAE   | Absent | Absent | Absent | Absent |
| Stapedial muscle reflex | Increased | Increased | Absent | Increased |
| ABR     | Normal | Normal | Absent | Normal |
| cVEMP   | Normal | Normal | Normal | Normal |
| oVEMP   | Normal | Normal | Normal | Normal |
| Tinnitus/vertigo | Chronic tinnitus, sporadic vertigo | Chronic tinnitus, no vertigo | Sporadic tinnitus, no vertigo | Chronic tinnitus, no vertigo |
| HAs or CI usage | Binaural HAs | Binaural HAs | Unilateral CI | Binaural HAs |
| Temporal bone CT | Normal | Normal | Normal | Normal |

Informed consent. Written informed consent was obtained from all participants.

Consent for publication. Participants consented to publication of nonidentifiable data.

Results

Cochea involvement in patients with TBC1D24-related ADHL. TBC1D24 variants causative for HL were identified in four probands (4/102), which corresponds to an approx. 4%-prevalence of TBC1D24-related ADHL in our cohort. All affected individuals from the four TBC1D24 families were diagnosed with bilateral, postlingual, progressive sensorineural HL. Mean HL onset was at the earliest in the second decade of life, i.e., 19.5 y.o. in Family 1, 16.7 y.o. in Family 2, 16.3 y.o. in Family 3 and 35 y.o. in Family 4. In all families hearing thresholds had a similar down-sloping pattern and mainly mid and high frequencies were affected. Most HL patients routinely use HAs (Table 1).

As shown by average ARTA (Fig. 2A) TBC1D24-related HL starts as mild at low and mid frequencies and moderate at high frequencies. It progresses with age (up to 80 y.o.) and becomes moderate to severe at low and mid frequencies and profound at high frequencies. The ATD in patients with TBC1D24 pathogenic variants is statistically significant at all frequencies (p < 0.001) and ranges from 0.61 (8 kHz) to 0.75 (0.25 and 0.5 kHz) dB/year (Fig. 2B).

Analysis of PTA data of patients with the TBC1D24 p.Ser178Leu, p.Asp185Asn and p.His487Leu variants revealed variant-dependent differences in the HL degree. In patients with p.Ser178Leu and p.His487Leu, ARTA presents mild HL at the age of 20. Later in life, HL becomes severe to moderate (Fig. 3A). The ATD is significant at all analyzed frequencies (p < 0.001) and ranges from 0.88 (4 kHz) to 1.08 (1 kHz) dB/year for p.Ser178Leu and from 0.59 (4 kHz) to 1.03 (0.5 kHz) dB/year for p.His487Leu (Supplementary Fig. S2). In patients with the p.Asp185Asn variant, ARTA shows a more severe HL over the analyzed time interval. From the age of 50 HL becomes profound at mid and high frequencies (Fig. 3A). For the TBC1D24 p.Asp185Asn pathogenic variant, ATD is statistically significant at all frequencies (p < 0.001) and ranges from 0.83 (0.25 kHz) to 1.72 (2 kHz) dB/year (Supplementary Fig. S2). The observations were confirmed by comparing the threshold feature arrays, which revealed a statistically significant difference between HL pattern found in p.Asp185Asn patients compared to an average TBC1D24 ARTA (Fig. 3B).

Results of additional audiological and neurotological examinations revealed cochlear involvement in the TBC1D24-related ADHL. No TEOAEs were recorded in the tested probands, and no or increased thresholds of stapedial muscle reflexes were observed. The vestibulocochlear nerve function measured by ABR was in line with the PTA results. No vestibular dysfunction or anatomical abnormalities of the cochleovestibular system were found. All probands reported sporadic or chronic tinnitus, one patient had sporadic vertigo (Table 1).
Figure 2. Audiological characteristics of TBC1D24-related ADHL. (A) ARTA for patients with TBC1D24 pathogenic variants. (B) Rate of HL deterioration in PTA.
Identification of \textit{TBC1D24} pathogenic variants. After performing next-generation sequencing (NGS) in four tested families very rare heterozygous variants of the \textit{TBC1D24} gene were selected. In Families 1 and 2 were identified, respectively, a heterozygous c.533C>T, p.Ser178Leu variant and a heterozygous c.553G>A, p.Asp185Asn variant, both located in exon 2 of the \textit{TBC1D24} gene. In Families 3 and 4, a heterozygous c.1460A>T, p.His487Leu variant and a heterozygous c.1461C>G, p.His487Gln variant were found in \textit{TBC1D24} exon 7 (Fig. 4, Supplementary Fig. S3). All selected variants were either very rare or not present in population databases. Most computational algorithms predicted their damaging role (Table 2). No impact of the analyzed variants on \textit{TBC1D24} RNA splicing was predicted. Family studies confirmed variants segregation with HL (Fig. 1A–D). Based on the applicable standards and guidelines, detected \textit{TBC1D24} variants were classified as likely pathogenic\textsuperscript{32,33}. All variants have been submitted to the Global Variome shared LOVD. No other pathogenic or likely pathogenic variants related to isolated or syndromic hereditary HL were found.

The c.533C>T variant identified in Family 1 has already been described in two ADHL families\textsuperscript{1,11}. It was the first, and for a long time, the only known \textit{TBC1D24} variant causally involved in the development of ADHL. The second identified variant (c.553G>A) is located seven amino acids downstream of this known variant; both...
are placed in the TBC domain of the TBC1D24 protein. Variants identified in Families 3 and 4 are nucleotide substitutions in adjacent positions within the same codon of the TBC1D24 sequence, resulting in an amino acid change from histidine respectively to glutamine or leucine. The latter variants are the first ADHL-related genetic alterations located in the TLDc domain of TBC1D24.

**Modeling the functional role of TBC1D24 variants.** The homologs of the human TBC1D24 protein were collected and clustered using the procedure described in the “Materials and methods” section. For the TBC domain, it resulted in 55 proteins that represent the sequence variability of the TBC domain from the TBC1D24 family. Analysis of the multiple sequence alignment (MSA for selected residues and species is presented in Fig. 5C) allowed us to identify that the amino acids crucial for interactions with IP3 in *D. melanogaster*, i.e. Lys75, Arg79, Lys277, Arg281, Arg335, Gly336 and Thr339, are either unchanged or substituted with positively charged residues—Lys36, Arg40, Lys238, Arg242, Arg293, Arg297, Lys298 (residues are numbered according to the human sequence). This might lead to even stronger interactions between the protein and IP3 in humans compared to *D. melanogaster*. Additionally, the MSA analysis revealed that both residues involved in ADHL, i.e., Asp185 and Ser178, have a different conservation level. In only one case Asp185 is replaced with glutamic acid, preserving the negatively charged residue. However, Ser178 is present only in 25 out of 55 sequences and is often replaced by other small residues like alanine or threonine. A similar analysis was independently performed for the TLDc domain, resulting in 118 protein sequences. According to the MSA, His487 is a part of a long loop (between β5 and β6) with variable length across different Chordata (MSA for selected residues and species is presented in Fig. 6C). In the TBC1D24 homolog from *D. melanogaster*, this loop is 18 residues long (from

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![Figure 4](https://example.com/figure4.png)  
*Figure 4.* Schematic representation of TBC1D24 gene and protein organization. Gene and protein structure are depicted based on the canonical transcript NM_001199107.1 and reference protein sequence NP_001186036.1. Previously reported TBC1D24 pathogenic variants involved in the development of nonsyndromic ARHL (DFNB86) are written in black. Variants causative for ADHL (DFNA65) are shown in blue; variants identified in this study are bolded. Domain boundaries (TBC residues 21–314; TLDc residues 339–555) were determined based on the sequence to structure alignment between human TBC1D24 protein and Skywalker/TBC1D24 protein from *D. melanogaster*.

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![Table 2](https://example.com/table2.png)  
*Table 2.* Characteristics of TBC1D24 variants detected in this study. *ACMG classification criteria legend: LP likely pathogenic, PM moderate pathogenicity evidence, PP, Strong strong pathogenicity evidence, D damaging, N/A no data available, PD probably damaging, T tolerated.*
Pro511 to Phe528), while, in humans, it spans 56 residues (from Glu443 to Phe498). Despite high sequence variability observed in the middle of this loop, its termini are conserved across Chordata. His487 is localized near the C-terminus of this loop and is observed in 81 sequences. Since the MSA reflects the sequence variability of TLDc domains, the remaining 37 sequences that lacked H487 were verified manually. It turned out that these sequences originated from 36 organisms. The majority of them were either a partially solved sequence or represented one of many isoforms of the TLDc domain present in that species. Only for ten species we could not find an isoform of TBC1D24 with His at position 487. It can be therefore concluded that His487 is a conserved residue among Chordata.

To analyze the putative role of p.Asp185Asn and p.Ser178Leu mutations, a homology model of the human TBC domain from TBC1D24 was constructed. Asp185 is located outside the active site on the 6th helix and does not interact directly with IP3 (Fig. 5A). The distance to the closest Cα atom of a residue from the binding site (Lys238) is nearly 14 Å. According to our model Asp185 forms a hydrogen bond with Asn307 (Fig. 5B) located in the C-terminal helix of the TBC domain. Asn307, like Asp185, is also conserved among Chordata (with only two exceptions where histidine is present). The C-terminal helix itself is a part of the IP3 binding site, as its N-terminal fragment harbors Ser296, Arg297, and Lys298. Thus, it is plausible that the Asp185-Asn307 bond is a part of a network of interactions responsible for maintaining a proper geometry of the 6th and C-terminal helices, which influences the position of Arg297 and Lys298 within the binding site. Close inspection of our model revealed that two additional polar interactions are part of this network. First, Asn307 forms an additional hydrogen bond with Thr182 residue, also conserved among Chordata. The second interaction in this model involves Ser178 and Glu308 (Fig. 5B). However, as both Ser178 and Glu308 are present only in a subset of chordates’ TBC1D24, this hydrogen bond may perform only an auxiliary role. Intriguingly, p.Ser178Leu was already linked with ADHL11,11 and based on the model proposed by Parzefall et al., Ser178 interacts with Asn30712. Considering the close proximity of both Asn307 and Glu308 residues, Ser178 interaction with either of these two residues is equally probable.

As mentioned above, His487 is part of a long flexible loop that hinders the probability of correctly identifying its position in relation to the rest of the TLDc domain. I-Tasser, employed to predict the possible orientations of that fragment, produces several, equally likely and quite diverse conformations. However, in most cases, residue conservation indicates amino acid importance in maintaining the proper 3D structure of a protein, its function or ability to bind to other protein. We investigated the possible role of His487 in the binding of the TBC domain. This hypothesis was put forward as we observed that the putative position of the loop harboring His487 is close to a patch of several conserved, solvent-exposed residues (Fig. 6A). To obtain a putative conformation of the TBC-TLDc complex, we performed a protein–protein docking using available crystal structures of these domains from D. melanogaster. Analysis of the top ten conformations revealed that the TLDc domain interface involves

Figure 5. Structure of the human TBC domain from TBC1D24. (A) The homology model of TBC1D24 protein was superimposed over the IP3-Skywalker complex to obtain the structure of the human protein—IP3 complex. The binding sites constitute of several, conserved, mostly positively charged amino acids. (B) The C-terminal helix is stabilized by polar interactions between N307, E308 with T182, D185 and S178. The latter two amino acids are changed to N and L, respectively, in patients with ADHL (this study). (C) Multiple sequence alignment of the human TBC domain from TBC1D24 and its homologs from selected organisms. Only regions comprising the binding site residues and two helices harboring S178, T182, D185, N307 and E308 are shown. D185 is conserved among Chordata, while S178 is present only in a subset of organisms, suggesting its auxiliary role in stabilizing the C-terminal helix. Residues highlighted in grey are involved in recognizing the membrane by the TBC domain, while residues highlighted in cyan are involved in the stabilization of conformation of the C-terminal helix of the TBC domain.
residues from this conserved patch (Fig. 6B). No such consistency was observed for the TBC domain. Considering the His487 proximity to this region, p.His487Gln and p.His487Leu might be responsible for weakening the interface between both domains of the TBC1D24 protein.

Discussion

Since the first association of TBC1D24 with isolated HL in 2014, the number of its identified variants causing a recessive form of HL has been steadily growing, currently reaching as many as ten. The case has been different for TBC1D24 variants involved in ADHL development. The first ADHL-related TBC1D24 pathogenic variant (p.Ser178Leu) was also identified in 2014, found in parallel in a European and a Chinese family. Before 2020, no other TBC1D24 alteration has been associated with this condition. The second TBC1D24-related ADHL variant (p.Asn307His) has been reported recently in a study of two unrelated HL families from Austria and the UK (Table 3). Altogether, the data suggested that the involvement of TBC1D24 in ADHL might be considered relatively rare and exceptional.

Our study shows that the role of TBC1D24 in ADHL may be underestimated. After testing 102 multigenerational families fulfilling strict criteria for the autosomal dominant inheritance of HL, the pathogenic TBC1D24 variant was detected in almost 4% (4/102). The involvement of TBC1D24 in ADHL can be even more significant than that. There are two additional ADHL families in our cohort whose probable underlying cause of HL is a defective TBC1D24 (data not shown). As further investigations are required to unequivocally confirm the TBC1D24 pathogenic potential, the families have not been presented here. We can also speculate that there were other patients with TBC1D24-related ADHL who have been excluded from our analysis because (i) their pattern of HL inheritance was mimicking a mitochondrial mode of HL inheritance, or (ii) the variants arose de novo, and the family history of HL was negative.

It is important to note that the contribution of one particular gene to ADHL development does not exceed a dozen percent. Among the currently known 50 genes implicated in ADHL development (https://hereditaryhearingloss.org/dominant-genes; accessed 03/2021), there is no single major gene responsible for ADHL. It varies among populations, but pathogenic variants in TECTA, KCNQ4, WFS1, or MYO6 are more frequent than other ADHL genes. Pathogenic variants in MYO6, KCNQ4, WFS1, GSDME (DFNA5) or TECTA were also repeatedly found in our ADHL cohort (unpublished data). While MYO6 pathogenic variants explained 11% of
Table 3. Comparison of demographic, molecular, and clinical findings in patients with isolated HL due to TBC1D24 pathogenic variants. HL hearing loss, N no, N/A no data available, Y yes.

| Family # and origin | Consanguinity | Tested patients with HL (No.) | Type of HL, onset | Reference SNP ID | Variant cDNA level | Variant Protein level | Protein domain | Ref. |
|---------------------|---------------|-------------------------------|-------------------|-----------------|-------------------|---------------------|---------------|------|
| 1 Pakistani         | Y             | 11                            | Profound, congenital | rs87777147      | c.208G>T          | p.Asp70Tyr          | TBC           |     |
| 2 Pakistani         | Y             | 9                             | Profound, congenital | rs87777147      | c.208G>T          | p.Asp70Tyr          | TBC           |     |
| 3 Pakistani         | Y             | 4                             | Profound, congenital | rs87777147      | c.208G>T          | p.Asp70Tyr          | TBC           |     |
| 4 Pakistani         | Y             | 7                             | Profound, congenital | rs199700840     | c.878G>C          | p.Arg293Pro         | Other         |     |
| 5 Moroccan          | Y             | 3                             | Severe to profound congenital | N/A            | 1.333GdupG        | p.Val144Glyfs*33    | TLDc          |     |
| 6 Moroccan          | Y             | 2                             | Severe to profound congenital | rs376712059    | c.457G>A          | p.Glu153lys         | TBC           |     |
| 7 Israeli           | Y             | 3                             | Profound, congenital | rs878853232     | c.194G>T          | p.Arg65Leu          | TBC           |     |
| 8 Czech             | N             | 1                             | Profound, congenital | N/A            | 1.526G>0A         | p.Gly509Glu        | TLDc          |     |
| 9 Chinese           | N             | 1                             | Profound, congenital | rs367966267     | c.877C>T          | p.Arg293Cys         | Other         |     |
| 10 USA              | N             | 10                            | Progressive, 3rd decade | rs483552866    | c.553C>T          | p.Ser178Leu         | TBC           |     |
| 11 Chinese          | N             | 9                             | Progressive, 3rd decade | rs483552866    | c.553C>T          | p.Ser178Leu         | TBC           |     |
| 12 Austrian         | N             | 7                             | Progressive, 2nd decade | rs919A>C       | c.919A>C          | p.Asn307His         | TBC           |     |
| 13 British          | N             | 3                             | Progressive, 3rd decade | N/A            | 9.19A>C           | p.Asn307His         | TBC           |     |
| 14 Polish           | N             | 6                             | Progressive, 2nd decade | rs483552866    | c.553C>T          | p.Ser178Leu         | TBC           |     |
| 15 Polish           | N             | 12                            | Progressive, 2nd decade | N/A            | 5.53G>A           | p.Asp185Asn         | TBC           |     |
| 16 Polish           | N             | 6                             | Progressive, 2nd decade | N/A            | c.1461C>G          | p.His487Gln         | TLDc          |     |
| 17 Polish           | N             | 5                             | Progressive, 3rd decade | N/A            | c.1460A>T          | p.His487Gln         | TLDc          |     |

HL causes in the studied ADHL families (data not shown), the remaining genes did not exceed 4%, as observed for TBC1D24.

The TBC1D24 gene (OMIM *613577) has five transcripts (www.ensembl.org; accessed 02/2021) encoding different isoforms found in multiple human tissues. The highest expression of the TBC1D24 gene was observed in the brain, but it was also found in testes, skeletal muscle, heart, kidneys, lung, and liver. In the human auditory system, TBC1D24 mRNA and protein were detected in hair cells and spiral ganglion neurons. Clinically, our patients presented typical features of isolated sensorineural HL with cochlea involvement, impaired hair cell function, normal function of the auditory nerve and normal anatomical ear structures. Their HL was progressing slowly and was accompanied by tinnitus. Some differences in the HL progression rate were mutation-specific and the highest progression rate was observed for p.Asp185Asn. It was significantly higher than the average TBC1D24 progression rate calculated based on all available data.

The majority of our patients benefit from using hearing aids, even throughout their lifetime. An exception in this regard was the proband from Family 3. Unlike other patients with TBC1D24-related ADHL, including her family members, she was diagnosed with HL at 8 and received a cochlear implant at 22. In this patient we could not identify any additional genetic or environmental factors that could aggravate her HL. Generally, the age of HL onset in our patients ranged between the second and the fourth decade of life, which was in line with the data reported previously.

Both Ser178 and Asp185 appear to serve a similar structural role in establishing the proper conformation of the TBC1D24 C-terminal helix and, consequently, the IP3 binding site. Considering their structural proximity, both mutations identified in this work, i.e., p.Ser178Leu and p.Asp185Asn should have a common molecular mechanism leading to HL. In our opinion, both mutations would most likely influence the conformation of the
C-terminal helix. That would affect the orientation of binding site residues, i.e., Arg293, Arg297 and Lys298, which could result in the disruption of some of the interactions between IP₃ and the TBC1D24 protein. Consequently, the decreased binding affinity would be responsible for impaired vesicular traffic that could lead to HL. The mutations affecting the TLDc domain, target conserved His487 amino acid. This residue is located in the long loop near the putative interface between TBC and TLDc domains. Thus, these mutations can destabilize the formation of this complex.

In summary, our data revealed that TBC1D24 should be considered an important contributor to ADHL. As ADHL shows similarities with presbycusis, TBC1D24 can be proposed as a candidate gene for the age-related HL. Similar to other TBC1D24-associated disorders, variants causative for ADHL can be found in different parts of the gene, which makes the studies of its functional role even more challenging. Based on the in silico analysis of homology models, we assume that the positions of amino acids involved in ADHL development are parts of the gene, which makes the studies of its functional role even more challenging. Based on the in silico analysis of homology models, we assume that the positions of amino acids involved in ADHL development are parts of the gene, which makes the studies of its functional role even more challenging.

**Data availability**

Results of the bioinformatic analysis can be found in a GitHub repository: https://github.com/SFGLab/TBC1D24.

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Author contributions

D.O. and M.L.L. performed genotyping; D.O., A.S., K.K., H.S. and M.O. participated in phenotyping and clinical data collection; D.O., M.L. and D.P. performed computational analysis; D.O., M.O. and M.L. analyzed the data and wrote the manuscript; All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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