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## **MPZL2 is a novel gene associated with autosomal recessive nonsyndromic moderate hearing loss**

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### **Abstract**

While recent studies have revealed a substantial portion of the genes underlying human hearing loss, the extensive genetic landscape has not been completely explored. Here we report a loss of function variant (c.72delA) in *MPZL2* in three unrelated multiplex families from Turkey and Iran

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**Conflict of interest**

The authors declare no conflict of interest.

**Compliance with ethical standards**

As stated in human and animal sections above.

with autosomal recessive nonsyndromic hearing loss. The variant co-segregates with moderate sensorineural hearing loss in all three families. We show a shared haplotype flanking the variant in our families implicating a single founder. While rare in other populations, the allele frequency of the variant is ~0.004 in Ashkenazi Jews, suggesting that it may be an important cause of moderate hearing loss in that population. We show that *Mpzl2* is expressed in mouse inner ear, and the protein localizes in the auditory inner and outer hair cells, with an asymmetric subcellular localization. We thus present *MPZL2* as a novel gene associated with sensorineural hearing loss.

## Keywords

Consanguinity; Deafness; EVA; EVA1; Founder effect; Moderate hearing loss; MPZL2

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## Introduction

Among all sensory deficits, hearing loss (HL) is the most common affecting approximately 1 in 1,000 newborns (Mehl and Thomson 2002). Genetic factors are implicated in the majority of cases with more than 80% of the inherited forms exhibiting autosomal recessive transmission (Morton and Nance 2006). No additional finding is present in over 70% of the cases, which are then classified as nonsyndromic HL (NSHL) (Morton and Nance 2006). Underlying genes are known in two-thirds of the approximately 100 loci reported for autosomal recessive NSHL (ARNSHL; hereditary hearing loss homepage: <http://hereditaryhearingloss.org>). However, mutations in known genes explain only 40–70% of families with ARNSHL (Bademci et al. 2016). A recent study in mice shows that significant portions of the extensive genetic landscape for HL remain unexplored (Bowl et al. 2017). While genetic causes are in place for all degrees of severity, most studies focus on cases with severe or profound HL. Variants in only few genes have been reported to cause mild and/or moderate HL (Iwasaki et al. 2002; Kim et al. 2015; Plevova et al. 2017; Schradars et al. 2012; Tamagawa et al. 1996; Yariz et al. 2012).

To better map the landscape of ARNSHL, we have collected over 800 multiplex families with parental consanguinity. About 10% of our cohort display mild or moderate HL. We performed whole exome sequencing (WES) on a subset of these families and here we report a frameshift variant in *MPZL2* associated with moderate HL in three families. Myelin protein zero like 2 (*MPZL2*; also referred to as epithelial V-like antigen EVA or EVA1; MIM 604873) displays homology to the myelin protein zero (MIM 159440), which is known to cause peripheral neuropathy, sometimes associated with HL, when disrupted by mutations (Seeman et al. 2004). *MPZL1* (MIM 604376) and *MPZL3* (MIM 611707) are other proteins with homology to *MPZL2*, while their dysfunction is not associated with a human phenotype. *MPZL2* is a transmembrane glycoprotein and a member of the immunoglobulin superfamily, likely mediating homophilic cell-cell adhesion. It has been reported to be expressed in epithelial structures during embryogenesis; however there is limited understanding of its function (Guttinger et al. 1998). Our results show that *Mpzl2* is expressed in the inner ear at different times of development and in adult mice, suggesting that this protein plays a fundamental role in sound perception.

## Materials and methods

### Subjects

This study was approved by the University of Miami Institutional Review Board (USA), as well as the Ankara University Medical School (Turkey), Akdeniz University Medical School (Turkey), the University of Würzburg (Germany), and the Sabzevar University of Medical Sciences (Iran) Ethics Committees. A signed informed-consent form was obtained from each participant or, in the case of a minor, from the parents.

We evaluated three unrelated multiplex families with parental consanguinity (Fig. 1a). The diagnosis of sensorineural HL (SNHL) was established via standard audiometry in a soundproofed room according to the current clinical standards. Severity of HL was determined from pure tone average calculated over 0.5–4 kHz: mild = 20–40 dB; moderate = 41–70 dB; severe = 71–95 dB, and profound = >95 (Mazzoli et al. 2003). Clinical evaluation included a thorough physical examination and otoscopy in all cases. DNA was extracted from peripheral blood leukocytes of probands according to the standard procedures.

### Sequencing and Bioinformatics Analysis

We performed WES in the probands of each family (individuals II:1, II:2, and II:1 in families 1, 2, and 3, respectively). Families 1 and 2 were sequenced and analyzed for single nucleotide, indel, and copy number variants at the Hussman Institute for Human Genomics in Miami, FL (USA) by using our previously published protocol (Bademci et al. 2016). Exome capture of the proband in family 3 was performed at the Institute of Human Genetics at the Julius Maximilians University Würzburg (Germany), following the TruSeq Rapid Exome library preparation protocol (Illumina) using 50 ng of genomic DNA. The 2 × 75 base pair (bp) paired-end library sequencing was performed using a NextSeq 500 desktop sequencer (Illumina) and a v2 reagent kit (Illumina). The generated sequences were demultiplexed and mapped to the human genome reference (NCBI build37/hg 19 version) with Burrows Wheeler Aligner (BWA). Variant calling and analysis was conducted using GensearchNGS software (PhenoSystems SA).

For the comprehensive variant screening of the protein coding as well as noncoding regions, Whole Genome Sequencing (WGS) was performed in the proband (II:1) of family 1 by using a BGISEQ-500 instrument with paired-end 100 bp protocol (Huang et al. 2017). Reads were mapped to the human reference genome (NCBI build37/hg 19 version). BWA was used for the alignment and Genome Analysis Toolkit (GATK) was used for the variant calling (Li and Durbin 2010; McKenna et al. 2010). Copy number variants (CNVs) were called using the CNVnator (Abyzov et al. 2011). Structural variations (SV) were detected with Breakdancer (Chen et al. 2009).

Briefly, for the variant allele frequency filtering EVS (<http://evs.gs.washington.edu/EVS/>), gnomAD (<http://gnomad.broadinstitute.org/>) and dbSNP (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) databases, as well as our internal WES/WGS database that includes > 4,000 exomes from different ethnicities including >1,000 Turkish individuals and >40 genomes, were used. Minor allele frequency thresholds of 0.005 for recessive and 0.0005 for dominant variants were used. We also filtered missense variants by using the combination criteria of

damaging for SIFT (<http://sift.jcvi.org/>), Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>) and FATHMM (<http://fathmm.biocompute.org.uk/>), high impact for MutationAssessor (<http://mutationassessor.org/r3/>), and disease-causing for MutationTaster (<http://www.mutationtaster.org/>). ACMG guidelines were used for variant interpretation (Richards et al. 2015). Sanger sequencing was used to evaluate co-segregation of the variant with HL. Enlis Genome Research software (<https://www.enlis.com/>) was used to identify homozygous regions from WES and WGS data (Supplementary Table S1). After excluding variants in all previously recognized deafness genes, we focused on variants that mapped to runs of homozygosity in each proband.

### TaqMan SNP Genotyping

We used a custom TaqMan SNP assay from Applied Biosystems (Assay ID: AHMSY96; order date: 1/6/2014) specific to *MPZL2* NM\_005797.3:c.72delA variant to screen 704 probands from unrelated families with NSHL. Custom-designed TaqMan SNP Genotyping Assays were designed to identify the presence or absence of the variant (forward primer: 5'-CACCCGGGAGGTATAAATTTCCA-3', reverse primer: 5'-CCCCTGTTAACCCTTCTTTTCTTCT-3', FAM probe: 5'-TTGGCCTATGCAGCTG-3' specific to the mutant allele, VIC probe: 5'-TTGGCCTATAGCAGCTG-3' specific to the wild type allele). Samples were amplified for 40 cycles then the plates were read on the 7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA). Finally, data were analyzed by using the SDS v2.4 software (Cukier et al. 2016).

### Statistics

Single locus two-point LOD scores were calculated using Superlink Online SNP 1.1 with a disease allele frequency of 0.001 under fully penetrant autosomal recessive model (Silberstein et al. 2013).

### Schwann cell expression

Schwann cell transcriptome sequencing data have been published previously (Monje et al. 2018). Briefly, both rat and human Schwann cells were purified and cultured for three passages on laminin-coated plates as previously described. Total RNA was prepared for sequencing on the Illumina platform using the ScriptSeq Complete Gold Kit (human, mouse, rat; Epicentre, Madison, WI) according to manufacturer's instructions and sequenced on a HiSeq 2000 (Illumina, San Diego, CA). Raw sequencing reads were aligned to the reference genome (*Homo sapiens* or *Rattus norvegicus*, <http://www.ensembl.org/index.html>) using STAR and then quantified using HTSeq (Anders et al. 2015; Dobin et al. 2013). Read counts were normalized to total reads using edgeR and transcript lengths to generate fragments per kilobase per million (FPKM)(Robinson et al. 2010).

### Animals

Wild type C57Bl/6 mice were bred and maintained at the University of Miami. At weaning age, mice were housed 2–4 per cage in a room with a 12 hour light: dark cycle (lights on at 7 AM, off at 7 PM) with access to food and water ad lib. All procedures were approved by the

University of Miami Institutional Animal Care and Use Committee and followed the NIH Guidelines, “Using Animals in Intramural Research”.

### **Mpzl2 mRNA expression**

To check the expression of *Mpzl2* in different tissues, lung, liver, kidney, brain and cochlea were dissected from P15 wild type mice. In addition, the cochlea expression of *Mpzl2* was checked in embryos of 17.5 dpc. Total RNA was isolated with TRIzol Reagent (Invitrogen) according to manufacturer’s instructions. Prior to reverse transcription, RNA samples were treated with rDNAse I (DNA-free kit, Applied Biosystems). cDNA was synthesized using qScript XLT cDNA SuperMix (Quanta Biosciences). The primers used to amplify a 233 bp fragment of the *Mpzl2* transcript were: forward 5’-GCTTGTGCTTCCACTTCTCC-3’ and reverse 5’-TGAAGGGTCCATGTGGTAG-3’. For *Gapdh*, a 129 bp fragment was amplified with 5’-AGGTCGGTGTGAACGGATTTG-3’ forward primer and 5’-TGTAGACCATGTAGTTGAGGTCA-3’ reverse primer.

### **Immunofluorescence**

Tympanic bullae containing the cochleae were dissected from P0 mice under the microscope and locally perfused with 4% PFA through the round and oval windows. Samples were kept in 4% PFA at 4 °C overnight and rinsed in 1X PBS. Cochlea whole mounts were permeabilized with 0.5% Triton X-100 and blocked in 5% BSA for 1 hour at room temperature, followed by overnight incubation at 4 °C with primary antibodies. Images were captured with a Zeiss LSM710 confocal microscope. Anti-*Mpzl2* (Proteintech 11787-1-AP), anti- Occludin (Thermofisher OC-3F10) antibodies were used as primary antibodies for immunostaining and Alexa Fluor 647-Phalloidin (Thermofisher A22287) was used to detect F-actin. The specificity of Anti-*Mpzl2* was validated via immunocytochemistry (Supplementary Fig.S1).

## **Results**

### **Clinical findings show nonsyndromic moderate sensorineural HL in affected individuals**

All probands were the result of consanguineous matings. Families 1 and 2 originate from Turkey, while family 3 is of Turkmen ethnicity from northeastern Iran. The parents are first cousins in all three families. Eight affected individuals with HL in three families were available for clinical evaluation (Fig. 1a). Audiograms from seven individuals show bilateral symmetrical and moderate SNHL (Fig. 1b). Individual II:3 (5 y.o.) of family 3 has an asymmetrical HL with moderate (borderline severe) HL in the right and profound HL in the left ear. In this family there are other relatives with HL; their clinical details are not available. Congenital or prelingual-onset of HL is suspected in all affected individuals; the age of onset could not be precisely determined because previous audiograms were not available. Families verbally stated that they did not notice progression to HL; serial audiograms were not available. The remainder of the examination was normal. There was no history of delays in gross motor development, balance problems, vertigo, dizziness, or nystagmus. Tandem walking was normal and Romberg test was negative.

## Exome sequencing reveals a frameshift variant in *MPZL2*

On average, exomes had 99.2 and 93.2% of mappable bases of the target coverage of 1X, and 10X reads, respectively. Average read depth was 55X, 56.6X and 73X for the probands of the families 1, 2 and 3, respectively. Whole genome sequencing of the proband (II:1) in family 1 achieved an average sequencing read depth of 44X and coverage at least 4X was ascertained for 99.5% of the genome.

We did not identify a single nucleotide, indel, or copy number variant in any gene previously reported to cause SNHL. In each proband, there were multiple runs of homozygosity with a size of >2 MB (Supplementary Table S2). *STRC* (MIM 606440), *OTOG* (MIM 604487), and *OTOGL* (MIM 614925), genes that typically cause moderate HL when mutated, were not within homozygous runs in any proband. *TECTA* (MIM 602574; hg19 chr11:120,973,375–121,061,515), a gene that is sometimes associated with moderate HL maps to a homozygous region of the probands. Both WES as well as WGS fully covered exons and intron-exon boundaries of *TECTA* and did not reveal any variant. Within the homozygous regions, we identified the *MPZL2* NM\_005797.3:c.72delA (p.I24Mfs\*22) (rs752672077) variant in each of the three probands. Sanger sequencing confirmed the co-segregation of the variant with ARNSHL in families (Fig. 1a, c). The variant leads to a frameshift in the signal peptide domain of *MPZL2* (Fig. 1d), which may trigger nonsense mediated decay or produces a truncated protein missing all functional domains. A combined two-point LOD score assuming autozygosity for rs752672077 in three families is 6. Evaluation of genotypes flanking *MPZL2* demonstrates a shared haplotype, suggesting a single founder for the variant (Supplementary Table S3; Fig. 1e).

Genotyping the variant rs752672077 in 704 probands from unrelated Turkish families with severe or profound SNHL identified two heterozygotes. Sanger sequencing of all exons of *MPZL2* in these samples did not reveal a second variant, suggesting that the *MPZL2* variant is not the cause of their HL. To understand the frequency of this variant, in-house exome data that included 1,300 Iranian patients with various phenotypes were analyzed and disclosed one c.72delA allele in an individual of Azeri (Turkish) ethnicity. Similarly, Iranome (<http://www.iranome.ir/>) reported this variant in one individual of Azeri ethnicity and provided an allele frequency of 0.00006258. This variant is absent from the Greater Middle East Variome Project (<http://igm.ucsd.edu/gme/index.php>) database.

## *MPZL2* is not expressed in human or rat Schwann cells

To investigate transcription of *MPZ* and related genes in the nervous system, we turned to whole transcriptome sequencing (RNA-Seq) data in primary Schwann cell cultures collected from humans and rats. The expression of *MPZ* was high in both human and rat samples, and the transcription of *MPZL1* was lower, but still clearly expressed. *MPZL2* and *MPZL3* were both below levels that we have been able to detect in the lab, and *MPZL2* had read counts that were negligible in both human and rat Schwann cells (0.12 and 0.07 FPKM in human and rat Schwann cells, respectively. Supplementary Tables S4 and S5). The relative levels of expression of *MPZL1–3* with respect to *MPZ* were confirmed in at least 2 independent rounds of sequencing using cells from different organ donors. Contrary to *MPZ*, *MPZL2* transcription was insensitive to stimulation with cAMP-stimulating agents, which is a strong

inductive signal for myelin gene expression in Schwann cells (not shown) (Bacallao and Monje 2015).

### **Mpzl2 is conserved between human and mouse and expressed in mouse inner ear**

Human MPZL2 protein (NP\_005788.1) has its counterpart in mouse (NP\_001344656.1). Both proteins are composed of 215 amino acids, with 81% identity in protein level between the species. We evaluated the expression of Mpzl2 in different mouse tissues, including the inner ear, and specifically, the cochlea. Total RNA was isolated from wild type at E17.5 and P15. RT-PCR with a forward primer located in exon 1 and a reverse primer in exon 3 of the Mpzl2 gene produced a unique band of 233 bp corresponding to the wild type mRNA. The amplification product was present in cDNA derived from wild type mice from all the analyzed tissues. In addition we found that Mpzl2 is expressed in the cochlea at E17.5 and P15 (Fig. 2a).

To study the localization of Mpzl2, we performed immunofluorescence staining in whole mount cochlea derived from P0 wild type mice. Mpzl2 is detected in both outer and inner auditory hair cells (Fig. 2b, c). The subcellular localization is asymmetric consisting in a “vesicle like” structure towards the apical portion of the hair cells and at the cell membrane towards the basolateral portion.

### **Discussion**

In this study, we present a loss of function variant in *MPZL2* that co-segregates with moderate SNHL in three families as a fully penetrant autosomal recessive trait. The presence of the same variant in three unrelated families in Turkey and Iran suggests that this variant may have a high frequency in this part of the world. In our cohort of 704 Turkish probands with severe to profound HL, we found two heterozygotes, for an allele frequency of 0.00142. To understand the frequency of this variant in the Iranian population, we calculated an allele frequency of 0.000384 in 1,300 Iranian individuals. The allele frequency of this variant is 0.00375 in Ashkenazi Jews and 0.00127 in Europeans in gnomAD (<http://gnomad.broadinstitute.org/>). It may thus explain a significant portion of moderate SNHL, especially in Ashkenazi Jews. It is noteworthy that in one affected person (family 3; II:3), HL was asymmetrical and more severe compared to other affected individuals. In this individual an environmental factor, a genetic modifier, or an unrelated causative gene variant might be responsible for the difference in the audiological phenotype.

Little is known about the function of MPZL2. MPZL2 shows a 29% identity with MPZ, which is involved in compaction of peripheral nervous system myelin (Lemke and Axel 1985). Similar to MPZ, MPZL2 mediates homophilic cell–cell adhesion through its extracellular domain, and likely with a support from the interaction of its cytoplasmic tail with the cytoskeleton (Guttinger et al. 1998). In this study we show that unlike MPZ, MPZL2 is not expressed in Schwann cells. Differential expression of MPZ and structurally similar MPZL1, MPZL2, and MPZL3 in Schwann cells suggests that they have specialized functions in different tissues. MPZL2 has been reported to be expressed during mouse embryonic development in various differentiating epithelia (Teesalu et al. 1998); it is expressed in the thymus epithelium and then downregulated with the development of adult

thymocytes with a potential role in thymic stromal organization and early lymphocyte development (DeMonte et al. 2007). The expression pattern of *Mpzl2* during mouse placenta formation suggests that the protein product may play a role in the processes of trophoblast invasion, decidual response, and trophoblast–decidual interaction (Teesalu et al. 1998). Our study shows for the first time that MPZL2 plays a necessary role in hearing. We found that *Mpzl2* is expressed in the cochlea of developing and adult mice and that the protein product specifically localizes to the inner and outer auditory hair cells. This is consistent with the expression pattern found in the gEAR database for *Mpzl2* (<https://gear.igs.umaryland.edu>). Interestingly the subcellular distribution pattern is asymmetrical within the hair cells. Further studies are needed to determine the nature of the apical localization. *Mpzl2* may function in cell-cell adhesion processes in the basolateral portion of hair cells (Guttinger et al. 1998). We conclude that MPZL2 plays a role for hearing physiology and/or maintenance of hair cells and its disruption by a mutation leads to SNHL. As the HL in affected subjects is moderate, it is possible that some of the functions of MPZL2 are compensated by other proteins. It will be interesting to identify these proteins and their roles in hearing.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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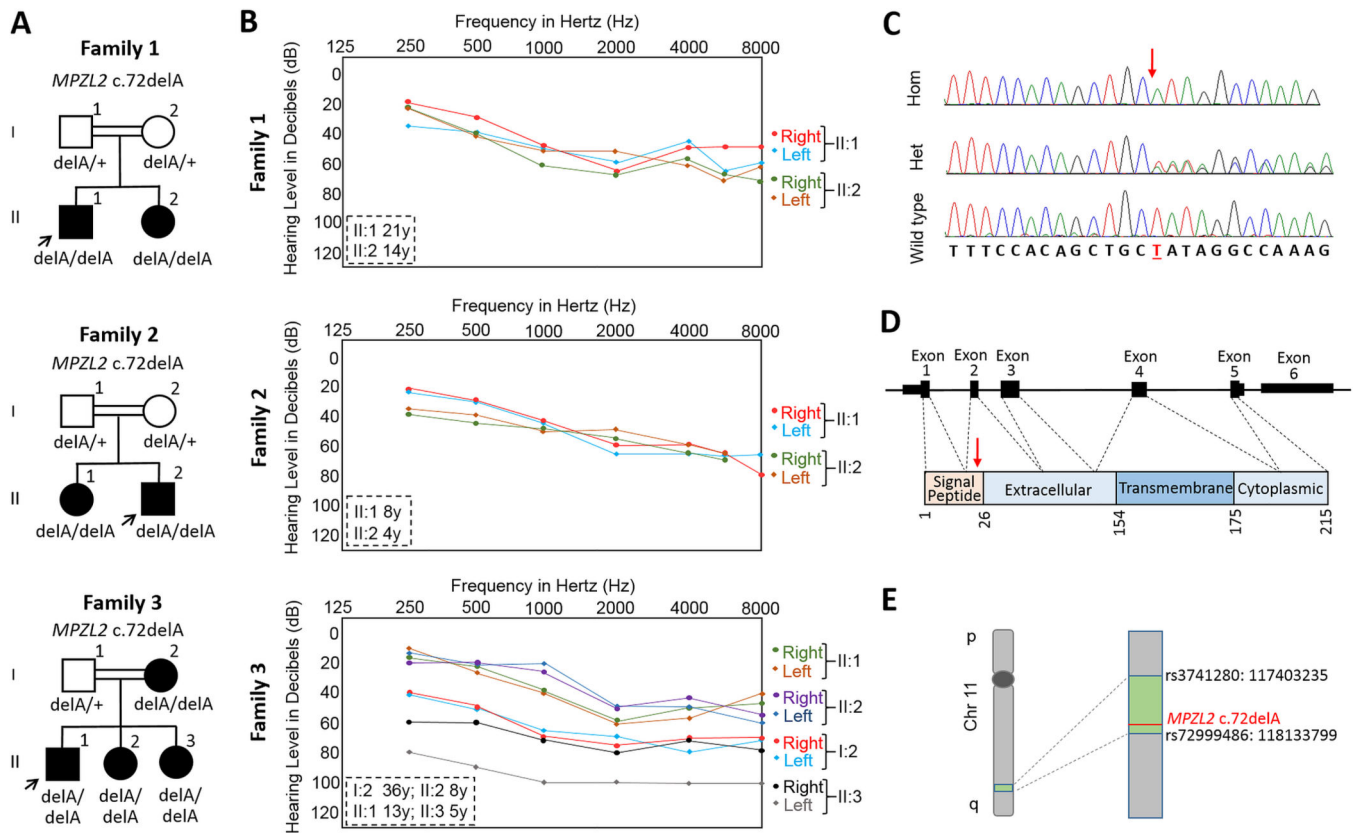
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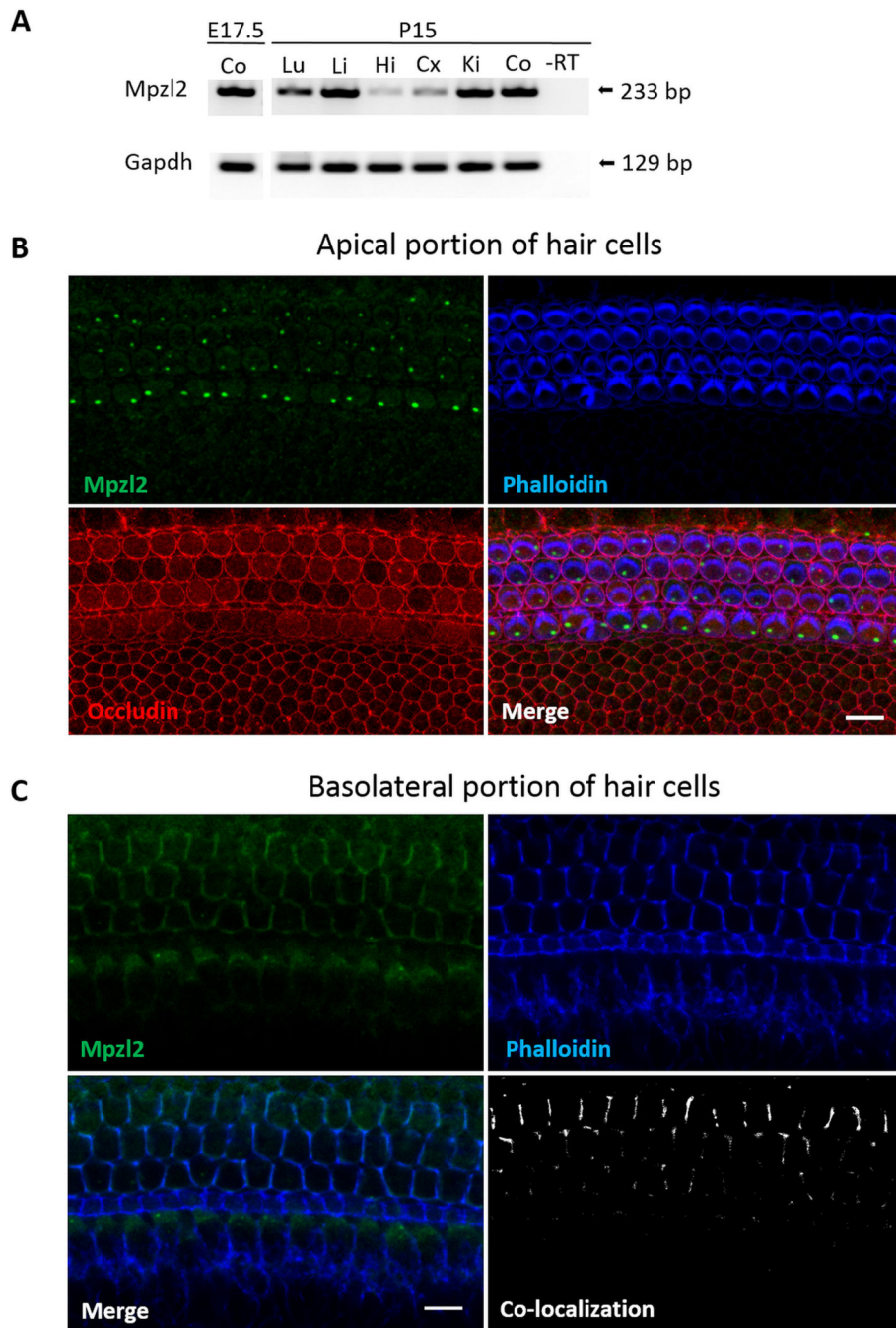
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**Fig. 1. Characteristics of families and the *MPZL2* c.72delA variant.**

**a** Pedigrees and segregation of the *MPZL2* variant in families. Filled symbols denote affected individuals and double lines indicate first cousin consanguinity. **b** Hearing thresholds obtained from pure tone audiograms of the affected individuals showing moderate HL. **c** Electropherograms showing the identified variant. The wild type traces are from an unrelated individual. Hom: Homozygous mutant, Het: Heterozygous mutant. **d** The variant c.72delA located in exon 2 of *MPZL2* (NM\_005797.3) causing a frameshift in the signal peptide of *MPZL2*. **e** A haplotype is shared in all three probands suggesting single origin.



**Fig. 2. Mpz12 expression in P0 mouse cochlea.**

**a** Expression of *Mpz12* mRNA in different tissues in E17.5, P15 wild type mice. Co: cochlea, Lu: lung, Li: liver, Hi: hippocampus, Cx: brain cortex, Ki: kidney. **b,c** Representative images of P0 wild type whole mount cochlea. Images are showing *Mpz12* expression in green at the apical portion and the basolateral portion transversal planes of the auditory hair cells. In addition, immunostaining with anti- Occludin to reveal the junctions (red) and phalloidin to see the stereocilia and the cytoskeleton (blue) are depicted. B&W

image from the co-localization highlighter plugin (ImageJ) of Mpzl2 and phalloidin. Scale bars: 10µm.

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