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## Research Paper

## Hereditary hearing loss: From human mutation to mechanism

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## ABSTRACT

The genetic heterogeneity of hereditary hearing loss is thus far represented by hundreds of genes encoding a large variety of proteins. Mutations in these genes have been discovered for patients with different modes of inheritance and types of hearing loss, ranging from syndromic to non-syndromic and mild to profound. In many cases, the mechanisms whereby the mutations lead to hearing loss have been partly elucidated using cell culture systems and mouse and other animal models. The discovery of the genes has completely changed the practice of genetic counseling in this area, providing potential diagnosis in many cases that can be coupled with clinical phenotypes and offer predictive information for families. In this review we provide three examples of gene discovery in families with hereditary hearing loss, all associated with elucidation of some of the mechanisms leading to hair cell degeneration and pathology of deafness.

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## 1. Introduction

The genetic basis of hearing loss (HL) has undergone a dramatic transformation in the past 15 years, since the discovery of mutations in a gene, *GJB2*, encoding connexin 26, responsible for a large portion of recessive deafness (Kelsell et al., 1997; Rabionet et al., 2000). However, even before the identification of mutations in this gene accounting for 30–50% of childhood deafness, it was clear that a significant proportion of HL is due to genetic mutations (Gorlin et al., 1995). Genetic HL is present in the form of syndromic HL (SHL), where deafness is associated with other phenotypes such as blindness (Usher Syndrome), goiter (Pendred Syndrome) or nephritis (Alport Syndrome), to name a few. Alternatively, deafness may be an isolated disorder, in the form of non-syndromic HL (NSHL), although frequently associated with vestibular dysfunction as well.

Families with hereditary HL had been observed for many years with different modes of inheritance. The most prominent group, particularly in countries with high rates of consanguinity, is recessive inheritance. This form is usually responsible for congenital, severe to profound HL, although this is a general trend, and not the rule. Dominant inheritance usually involves later onset of HL. Two smaller groups are mutations on the X chromosome and in mitochondrial genes, with X-linked and maternal inheritance, respectively (Gorlin et al., 1995).

Mutations in the *POU3F4* gene were found in 1995 (de Kok et al., 1995), following its chromosomal localization to the X-chromosome in 1988 (Wallis et al., 1988). In 1992, the first dominant locus was mapped to chromosome 5q31 (Leon et al., 1992), with the mutation identified in the *DIAPH1* gene in 1997 (Lynch et al., 1997). In 1994, the first locus for recessive deafness was found on 13q12 (Guilford et al., 1994), which subsequently led to the identification of the *GJB2* gene.

Today, the chromosomal locations for NSHL are known for 50 dominant loci, with 24 of the genes identified (Hereditary Hearing Loss Homepage, <http://hereditaryhearingloss.org/>, Fig. 1); for 79 recessive loci, with 40 of the genes found; and more loci for SHL, X- and Y-linked HL, modifiers, and mitochondrial mutations.

The identification of the molecular bases of many of these forms of HL has played a major role in advancing genetic counseling for the deaf (Rehm, 2005). Early diagnosis can facilitate the type of rehabilitation a child receives, for example, with Usher syndrome (Kimberling et al., 2010), and may allow predictions to be made about the progression of HL in the future (Brownstein and Avraham, 2009). Gene discovery has also provided compelling evidence for the mechanisms leading to deafness (Richardson et al., 2011).

Given the genetic heterogeneity of HL, it is no surprise that the genes found so far encode a large variety of proteins with many functions in the inner ear. These have included proteins that are responsible for, but not limited to, gene regulation, fluid homeostasis, synaptic transmission and hair cell bundle morphology and development (Dror and Avraham, 2010; Richardson et al., 2011).

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**List of Abbreviations**

HL	Hearing loss
SHL	Syndromic hearing loss
NSHL	Non-syndromic hearing loss
TEM	Transmission electron microscopy
KO	Knock-out
P	Post natal
MET	Mechanoelectrical transduction
ArrayCGH	Array comparative genomic hybridization
TJP2	Tight-junction protein 2
ARHL	Age related hearing loss
GSK-3 $\beta$	Glycogen synthase kinase-3 $\beta$
NIHL	Noise induced hearing loss
miRNA	MicroRNA, MIR
Dmdo	Diminuendo
ACh	Acetyl-Choline
NGS	Next-generation sequencing
SEM	Scanning electron micrograph
IHC	Inner hair cells
OHC	Outer hair cells
TC	Tunnel of Corti

In this review, we highlight three cases of gene identification, with particular emphasis on mechanisms elucidated with the discovery of the molecular basis of these forms of HL. In the first, mutations in large, mostly consanguineous families led to the

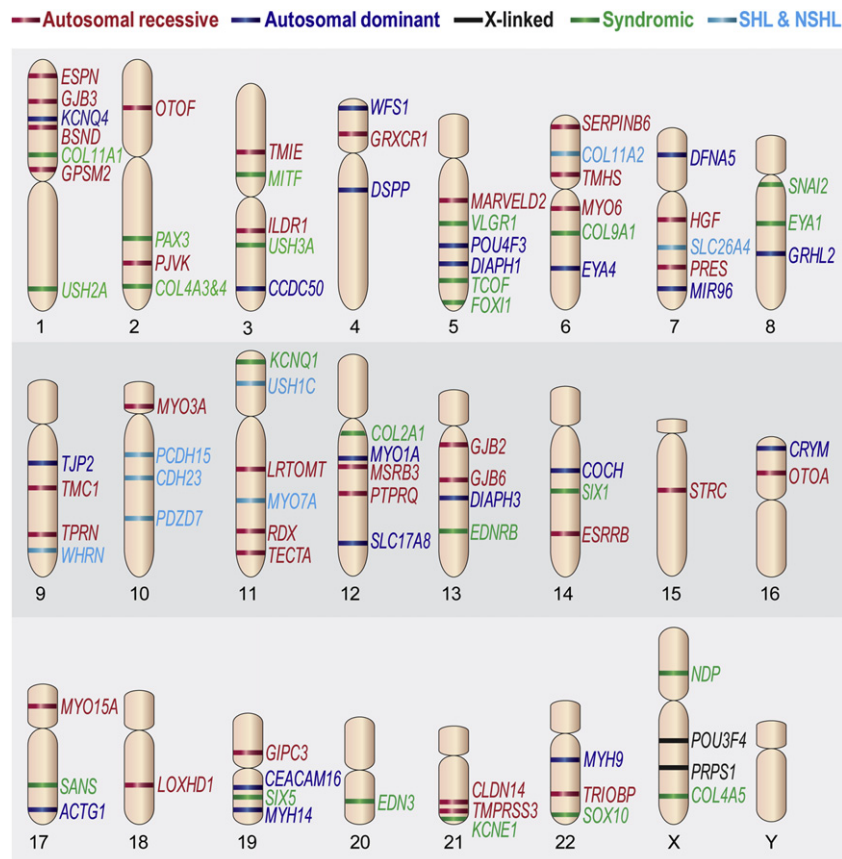
discovery of an essential protein for stereocilia function; in the second, the discovery of a duplication of a gene led to identification of the apoptotic pathway associated with HL; and in the third, mutations in a microRNA revealed its role in hair cell and neuronal maturation. In all cases, the mouse has been instrumental for revealing inner ear expression and function, which has been the case in many of the gene discoveries for HL (Friedman et al., 2007; Leibovici et al., 2008).

## 2. DFNB28 is caused by mutations in the actin-bundling protein *TRIOBP*

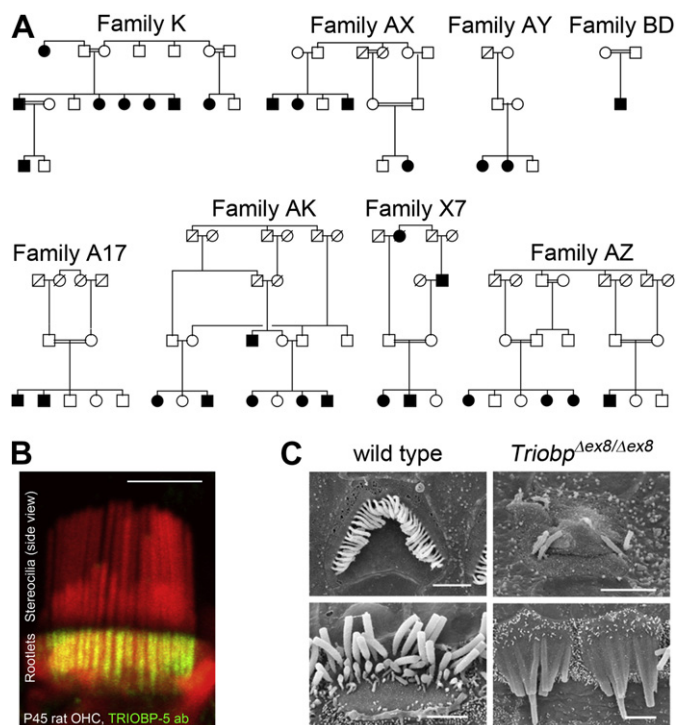
### 2.1. Various mutations in *TRIOBP* are associated with hearing loss

*TRIOBP* (previously referred to as TARA) was first associated with HL through the identification of nine different mutations, causing prelingual severe to profound non-syndromic deafness, of recessive inheritance, in ten Pakistani, Indian and Palestinian families (Riazuddin et al., 2006; Shahin et al., 2006) (Fig. 2A). *TRIOBP*-1 was described as a stabilizer of F-actin that is widely expressed in many tissues (Seipel et al., 2001). Further analysis of the *TRIOBP* gene structure identified additional essential isoforms (*TRIOBP*-4 and *TRIOBP*-5) that are necessary for hearing.

Subsequent to linkage analysis on the families, all mutations were linked to the 22q13 region defined as DFNB28 spanning 34 genes, including *TRIOBP*-1 (Walsh et al., 2000). Since *TRIOBP* was a relevant candidate gene due to its actin binding properties, a search for mutations was undertaken. Nevertheless, no pathogenic



**Fig. 1.** Genes causing hearing loss. A schematic representation of all the genes cloned thus far that are associated with non-syndromic autosomal recessive (red), autosomal dominant (blue) and X-linked (black) hearing loss, syndromic hearing loss (green) and that are known to be involved both in syndromic and non-syndromic hearing loss (light blue). Adapted with permission from (Dror and Avraham, 2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** *TRIOBP* is associated with hearing loss in humans and mice. (A) Pedigrees of the eight families presenting hearing loss, mapped to DFNB28, in which mutations in *TRIOBP* have been identified. Adapted with permission from (Shahin et al., 2006). (B) *TRIOBP-5* (green) is localized to the rootlet region of the stereocilia bundle (phalloidin, red) in a P45 mouse inner ear using immunofluorescence. Adapted with permission from (Kitajiri et al., 2010). (C) Scanning electron micrograph (SEM) at P49 demonstrating fused and degraded stereocilia in a mouse model lacking exon 8 of *Triobp-4/5*. Adapted with permission from (Kitajiri et al., 2010). Scale bar: (B) 5  $\mu\text{m}$ . (C) 2  $\mu\text{m}$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutations could be identified in any of the known exons encoding the *TRIOBP-1* isoform, leading to the search for additional upstream exons of *TRIOBP*, since there were several highly conserved regions upstream to the ORF of *TRIOBP-1*. Three additional isoforms were identified, varying in length, beginning at the same upstream ORF and translated in the same reading frame as *TRIOBP-1* (Riazuddin et al., 2006; Shahin et al., 2006). The additional isoforms bear several copies of two repeated motifs in exon 6 that is not shared by *TRIOBP-1*. Surprisingly, all nine mutations reside in exon 6, affecting only the newly identified isoforms. Out of the nine mutations, six were nonsense (Q297X, R347X, Q581X, R788X, R1068X, and R117X), one was a missense (G1019R) and two were frameshift (D1069fsX1082 and R1078fsX1083) alleles also generating a stop codon. Both nonsense and frameshift mutations result in predicted null alleles, while the missense mutation is presumed to affect ligand binding and thus damage the function of the protein (Shahin et al., 2006). While *TRIOBP-1* is widely expressed, the other isoforms are restricted mainly to the retina and inner ear, possibly accounting for the absence of additional abnormalities in the patients (Riazuddin et al., 2006; Shahin et al., 2006).

## 2.2. *TRIOBP* is essential for stereocilia function

The mouse serves as an essential tool in order to examine the influence of a human mutation on inner ear structure and function. Mouse *Triobp* was discovered to bear a similar structure to the human *TRIOBP*, including a long isoform and two short non-overlapping isoforms referred to as *Triobp-5*, *Triobp-4* and *Triobp-*

1, respectively (Riazuddin et al., 2006; Shahin et al., 2006). Also in a similar manner, *Triobp-5* and *Triobp-4* include the repeat motifs of human exon 6 (mouse exon 8), which contains all the known human mutations; while *Triobp-1* is transcribed from a downstream promoter, excludes exon 6, but shares the carboxy domain with *Triobp-5*.

Using isoform-specific antibodies enabled the distinction between *TRIOBP-5*, localizing mainly to the stereocilia rootlets, and *TRIOBP-4* that is expressed along the rootlets and the entire stereocilia length (Kitajiri et al., 2010) (Fig. 2B). The taper of the stereocilia is defined as the bottom region, where the bundle becomes thinner and penetrates the cuticular plate at the pivot point (Karavitaki and Corey, 2006). Condensed actin filaments in the center of stereocilia core, which extend into the cuticular plate, is referred to as the rootlet. The rootlet begins above the taper but continues, for a similar length, into the cuticular plate to allow anchoring of each stereocilium to the hair cell intercellular actin mesh, retaining its ability to deflect (Flock and Cheung, 1977). The rootlets develop after birth, so that in the newborn inner ear, *Triobp-5* expression is at the base of the stereocilia (Kitajiri et al., 2010). With the maturation of the sensory epithelia, *TRIOBP-5* expression becomes restricted to the rootlets. In contrast, *TRIOBP-4* is maintained along the entire length of the stereocilia and rootlets. Specifically, *TRIOBP-5* expression, as assessed by transmission electron microscopy (TEM) and immunogold labeling, appears at rootlets periphery, adjacent to the actin filaments. Both isoforms are also expressed in the processes of pillar and Deiter supporting cells that are composed of actin filaments.

The possible involvement of *Triobp* in the formation and maintenance of the rootlet structure is evident by *in vitro* examination of actin polymerization and bundling in the presence of *TRIOBP-4* (Kitajiri et al., 2010). A purified GFP-*Triobp-4* protein was demonstrated to bind F-actin, spread along the length of actin filaments and possibly have an actin-bundling activity as well. Each *Triobp-4* molecule appears to bind 3–4 actin molecules and promote organization into extremely condensed bundles, more condensed than what was previously determined for *espin 3A* that cross-links actin (Purdy et al., 2007), so that no interfilament space is detectable (Kitajiri et al., 2010). The bundled structures, formed *in vitro* in the presence of *Triobp-4*, significantly resemble those of the *in vivo* rootlets. Both the condensation level of the actin bundle, formed in the presence of *Triobp-4 in vitro*, and the distribution of *Triobp-5* in the periphery of actin bundles *in vivo*, result in the assumption that unlike *espin 3A*, both *Triobp* proteins bind actin filaments from outside the bundle and are not intercalated in between the filaments.

Knock-out (KO) mice for *Triobp-1* and *Triobp-5* die before birth; however, a joint *Triobp-5* and *Triobp-4* knock-out allele is not an embryonic lethal, indicating that ubiquitously expressed *Triobp-1* is essential during development. Nevertheless, the *Triobp-4/5* KO mice present profound hearing loss throughout the spectrum at post-natal (P)35, without additional abnormalities, similar to the phenotype observed in human patients. While the structure of the hair cells and the mechano-electrical transduction (MET) measurements at P4–P9 appear normal both from inner and outer hair cells, slight changes in the position of the stereocilia can be detected already at P1 and gross degeneration is evident by P16 (Fig. 2C), which could account for the profound hearing loss observed.

The rootlet structure develops between P1 and P16 (Kitajiri et al., 2010). Since no rootlets are detected in the mutant mice, despite the remaining expression of *Triobp-1*, it appears that *Triobp-4/5* are essential for the proper assembly of the rootlet and that *Triobp-1* cannot compensate for their absence. It should be mentioned, that despite the lack of rootlet formation, stereocilia

length appears normal throughout their development and only deteriorates at the onset of hearing. Together with the rootlets, tip links and side links may account for the stiffness and rigidity of the stereocilia at rest and during deflection. When the latter are ablated using  $\text{Ca}^{2+}$ -free medium and BAPTA treatment, the significance of the rootlets alone can be evaluated. Indeed, in the absence of rootlets in the KO mice, the stereocilia are more flexible, less stiff and more fragile, both at resting state and when applying increasing deflection intensities, compared to stereocilia of wild-type mice omitting tip links and side links alone. Thus, the rootlets are indeed essential both for the stiffness of the hair bundle and the resilience to deflection. Despite normal MET values at P4–P9, the hair cells of mice lacking *Triobp-4/5* are most likely non-functioning after maturation due to the absence of rootlets, which in turn leads to degeneration or fusion of the stereocilia and profound hearing loss. *Triobp-4* and *Triobp-5* are therefore necessary for the bundling of actin, and the formation of rootlets and thus for proper hearing.

### 3. DFNA51 is caused by an inverted-duplication of the tight-junction protein TJP2

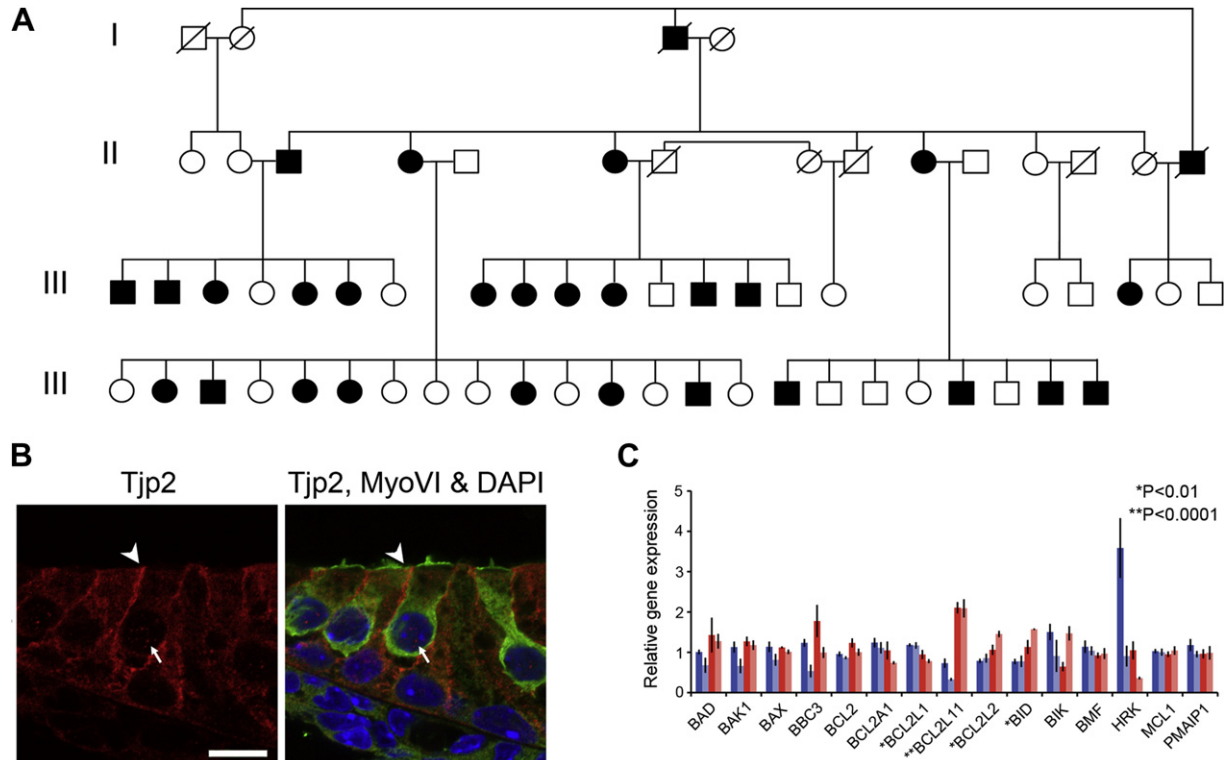
#### 3.1. Overexpression of TJP2 is associated with hearing loss in a human family

A large Israeli family of Tunisian origin presented dominant inheritance of high tone hearing loss, which begins during the fourth decade and progresses with age, resulting in severe to profound deafness at all frequencies (Walsh et al., 2010) (Fig. 3A). Linkage analysis refined the locus to a 41.7 MB area on chromosome

9 (9p13.3–q21.13), defined as DFNA51, but despite repeated sequencing of 21 residing genes, no mutation could be detected. For this reason, array comparative genomic hybridization (arrayCGH), a technique that enables the examination of duplications and deletions in the genome, was used. Indeed, an inverted-duplication was found at 9q21.11, spanning an area of  $\sim 270$  kb, containing the entire Tight-junction protein 2 (*TJP2*) locus, along with a small fraction of the gene *FAM1802*. The latter was shown to retain normal levels of expression; however, *TJP2* was demonstrated to be overexpressed both at the mRNA and protein levels. Both the duplication and the subsequent overexpression were shown to segregate with the mutation in 58 affected family members. Overall, this study identifies *TJP2* expression in the context of the inner ear and its involvement in hearing and deafness. Specifically, due to the late onset nature of the hearing loss, *TJP2* has been implicated in age related hearing loss (ARHL).

#### 3.2. TJP2 overexpression is associated with induction of apoptosis

*TJP2* is a part of the MAGUK family of proteins, known for their dual role: at the membrane boundaries and in the nucleus (Traweger et al., 2008). On one hand, it is a scaffold protein, connecting tight junctions to the actin cytoskeleton. As such, it is expressed in the mouse inner ear at the apical region of hair cells and supporting cells, aiding the maintenance of the barrier between endolymph and perilymph (Walsh et al., 2010) (Fig. 3B). In contrast, under various conditions it translocates to the nucleus, where it is presumed to take part in signal transduction pathways. Among these, it was shown that *Tjp2* has anti-proliferating activity during progression of the cell cycle. Toward the end of G1, *Tjp2*



**Fig. 3.** *TJP2* overexpression induces apoptosis. (A) Schematic representation of Family T, bearing an inverted-duplication of *TJP2*. (B) *Tjp2* (red) is expressed in the mouse inner ear at P0, most prominently in the apical tight junctions between hair cells and supporting cells, but also in the side-junctions and in the cytoplasm and nuclei. Myosin VI (green) stains the hair cells and DAPI (blue) marks the nuclei. Scale bar: 10  $\mu\text{m}$ . (C) Quantitative real-time PCR analysis demonstrating altered expression of genes from the BCL-2 family in lymphoblasts from affected members of Family T (red), compared to unaffected members (blue). The pro-apoptotic genes *BCL2L11* (BIM) and *BID* are up-regulated in the affected individuals, while the anti-apoptotic gene *BCL2L1* is down-regulated, indicating an overall shift toward apoptosis in cells from affected members. A, B, C adapted with permission from (Walsh et al., 2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

translocates to the nucleus, causes dephosphorylation and activation of Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), which in turn phosphorylates CD1, causing it to be exported out of the nucleus and degraded by the proteasome (Tapia et al., 2009). Despite the fact that cells in the inner ear in general, and hair cells specifically are post-mitotic, a reduced yet clear expression of *Tjp2* is also detected in the cytoplasm and nuclei of various cells in the sensory epithelia.

Lymphoblastoid cell lines are cultured immortal cell lines, generated from patients' blood samples in order to compare DNA, RNA and proteins between affected and unaffected individuals, and can thus provide evidence regarding the mechanism underlying the pathology. These cell lines were used to validate TJP2 overexpression at mRNA and protein levels (Walsh et al., 2010). In concordance with the data from Tapia et al. described above, subsequent analysis of GSK-3 $\beta$  levels, both at the native form, and phosphorylated on Ser9, showed a decrease in the phosphorylated form, which indicates induction of the active GSK-3 $\beta$ . While cells of the inner ear do not proliferate after maturation, GSK-3 $\beta$  involvement in apoptosis and survival-related pathways suggested an alternative mechanism. GSK-3 $\beta$  has an essential role in apoptosis through the intrinsic apoptosis pathway and activation of pro-apoptotic members of the *Bcl-2* family (Beurel and Jope, 2006). GSK-3 $\beta$  was also shown to activate BAX in apoptotic neurons (Linseman et al., 2004) and to positively regulate the pro-apoptotic effect of BIM (BCL2L11) in cancer (Nuutinen et al., 2009). Further studies demonstrated a delay in apoptosis in cisplatin-exposed hair cells once they were treated with GSK-3 inhibitors, which were the first implication of GSK-3 apoptosis induction in the inner ear (Park et al., 2009). These findings strengthened the association between TJP2 overexpression and induction of apoptosis and led to the further inspection of additional apoptosis-related genes, namely from the *BCL2* family.

The *BCL2* family of proteins is composed of pro-apoptotic and anti-apoptotic genes that interact according to a combination of signals, to activate or repress the intrinsic apoptosis pathway (Tadros et al., 2008). Different forms of regulation, from the transcriptional level to post-translational modifications, monitor the ability of the factors to produce the overall pro- or anti-apoptotic effect that is required in the cell. In lymphoblasts from affected members of Family T, both *BIM* and *BID*, two pro-apoptotic factors, are significantly up-regulated, while *BCL-xL* (*BCL2L1*), an anti-apoptotic factor, is down-regulated (Walsh et al., 2010) (Fig. 3C). Despite the additional up-regulation of the anti-apoptotic factor *BCL-w* (*BCL2L2*), an overall shift toward apoptosis is most likely generated. Since all of the above mentioned genes are known to be expressed in the inner ear (Tadros et al., 2008), a mechanism, by which the overexpression of TJP2 leads to activation of GSK-3 $\beta$  and subsequent induction of apoptosis through pro-apoptotic members of the *BCL2* family, is therefore proposed. Both ARHL and noise induced hearing loss (NIHL) are suggested to evolve primarily from hair cell and neuronal death through apoptosis in general, and the intrinsic apoptosis pathway specifically (Someya and Prolla, 2010). Several genes have been demonstrated to take part in these processes, among them the above mentioned *BIM* and additional members of the *BCL2* family such as *BAX* and *BAK* (Someya et al., 2009; Tadros et al., 2008). These findings implicate TJP2 in deafness due to induction of apoptosis as a primary mechanism, and thus possibly mark this form of late-onset progressive genetic deafness as a model for ARHL.

### 3.3. Other mutations in TJP2 and future directions

Missense mutations in TJP2 have been associated with NSHL (Hilgert et al., 2008) and oligogenic inheritance of hypercholesterolemia

(MIM 607748) (along with mutations in BAAT, MIM 602938) (Carlton et al., 2003). No reports of syndromic HL have been described thus far with TJP2, despite its early embryonic and ubiquitous expression. We suggest that mutations in this gene are more likely to be embryonic lethal, and in fact, this is corroborated by the *Tjp2* knock-out, which is not viable (Xu et al., 2008). Only a missense or more complex gain-of-function mutation may be viable and therefore identifiable in human patients. In DFNA51 patients, the inner ear may be more susceptible to TJP2 mutations due to different regenerative or compensatory mechanisms in other tissues with TJP2 expression. A comprehensive analysis of the findings made in the human patients in relevant mouse models will be able to lead to a more thorough understanding of the mechanisms involved, as well as further specify the components that are involved in the pathway leading from a TJP2 duplication to hearing loss.

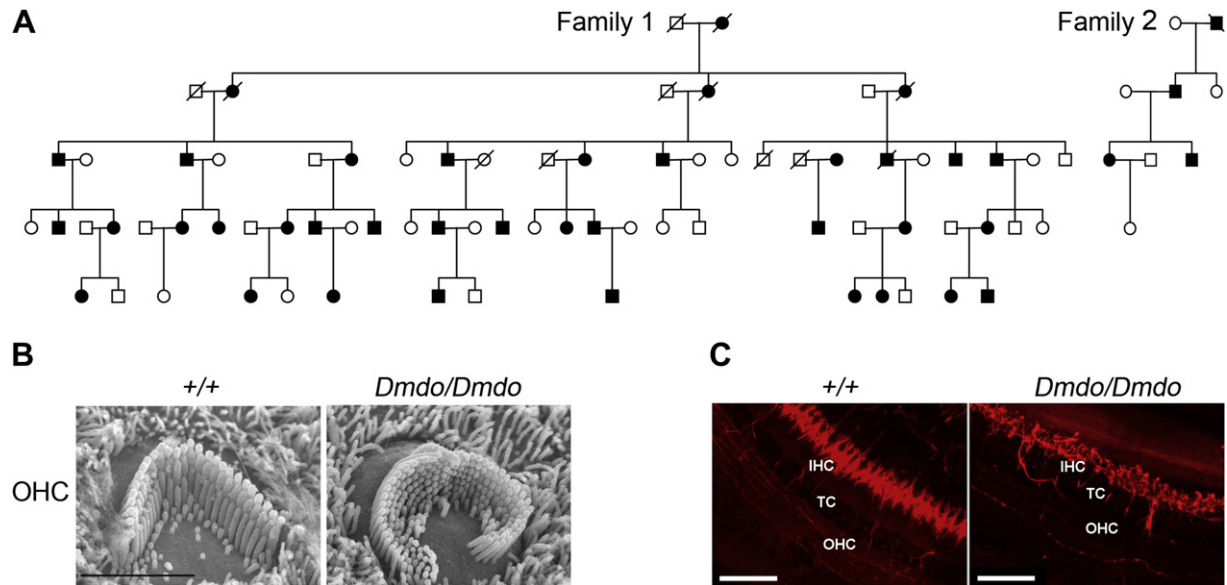
## 4. DFNA50 is caused by mutations in the seed region of miR-96

### 4.1. Alterations in the seed region of MIR-96 are associated with hearing loss in two families

MicroRNAs (miRNAs) are small RNA molecules that repress mRNAs of target genes upon binding to their 3'UTR (Guo et al., 2010). Each miRNA inhibits many targets, defined by the compatibility between the seed region of the miRNA and the 3'UTR of the target. The seed is defined by 7–8 nucleotides at the 5' end of the mature miRNA, and it is essential for the miRNA-target recognition (Grimson et al., 2007). MiRNAs are transcribed from the genome as a pri-miRNA, cleaved to create a pre-miRNA that is exported from the nucleus, and processed further to generate the mature functional miRNA (Bartel, 2004). A number of miRNAs have been shown to play a crucial role in the development and function of the inner ear (Weston et al., 2006). The association of miRNA96 (*MIR-96*) with HL was the first evidence of a miRNA directly involved in a human, Mendelian-inherited pathology in general, and in deafness specifically (Lewis et al., 2009; Mencia et al., 2009). HL in two Spanish families, presenting postlingual, progressive, non-syndromic deafness with dominant inheritance was linked to the deafness locus 7q32, referred to as DFNA50 (Fig. 4A). Several genes that reside in this region were sequenced, but no mutation was identified. However, inspection of *MIR-96* enabled the discovery of two causative mutations in the seed region of the MIR, a G > A in position +13 and a C > A in position +14. Neither mutation was detected in 462 hearing controls. Mutations in the seed region may affect proper processing, required for the generation of the mature MIR, as well as damage binding of the MIR to its targets. However, despite the obvious importance of the MIRs and MIR-target binding, no additional disrupting mutations have been discovered. Thus far, it appears that this form of deafness is relatively rare (Hildebrand et al., 2010).

### 4.2. MiR-96 has a role in hair cell and neuronal maturation

*MIR-96* belongs to a triad, together with *MIR-182* and *MIR-183*, which is known to be expressed in the inner ear sensory epithelia and ganglia neurons and in neurosensory cells of the eye and nose (Pierce et al., 2008; Wienholds et al., 2005). The two human mutations described above result in a reduction of the mature *MIR-96*, probably due to improper processing of the pre-miRNA, and reduced binding of the MIR to at least several of its targets, as was demonstrated using a luciferase assay (Mencia et al., 2009). In parallel to the identification of the human mutations, an ENU-induced mouse mutant was discovered, bearing a similar



**Fig. 4.** MiR-96 underlies hearing loss in humans and mice. (A) Pedigrees of the two families with hearing loss linked to DFNA50. Mutations in the seed region of miR-96, located in this locus, are associated with the deafness (Mencia et al., 2009). The ENU-induced mouse mutant *Diminuendo* (*Dmdo/Dmdo*) bears a point mutation in the seed region of miR-96 as well and therefore serves as a model for the human hearing loss. Adapted with permission from (Lewis et al., 2009). (B) SEM reveals an immature structure of the hair bundle at P4. (C) Immunofluorescent staining using neurofilament (red) demonstrates irregular wiring of the nerves to the inner hair cells. Adapted with permission from (Kuhn et al., 2011). Inner hair cells (IHC); outer hair cells (OHC); tunnel of Corti (TC). Scale bar: (B) 3  $\mu\text{m}$ , (C) 50  $\mu\text{m}$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutation in *Mir-96*, an A > T transversion at the seed region (Lewis et al., 2009). This mouse serves as a model for human DFNA50, enabling the study of the mechanism underlying this form of hearing loss. It should be noted that no additional abnormality has been reported in the human families or in the mouse.

The *diminuendo* mutant mouse (*Dmdo*) presents a semi-dominant pattern of inheritance, in which homozygous mice suffer from profound hearing loss, already at P15, while heterozygous mice present decreased thresholds and become deaf only at a later stage. In concordance with the hearing phenotype, homozygote's stereocilia bundles are slightly misshaped as early as P4–P5 (Fig. 4B) and complete degeneration of the hair cells is detected by 4–6 weeks. In heterozygotes, however, the bundles appear normal at P4–P5, yet many outer hair cells are lost by 4–6 weeks, while the inner hair cells are preserved (Kuhn et al., 2011; Lewis et al., 2009).

At the molecular level, five targets of miR-96 were validated in the inner ear: *Aqp5*, *Celsr2*, *Myrip*, *Odf2* and *Ryk*. In addition, thirteen genes were validated to be up-regulated or down-regulated in the mutant mice, among them genes that are direct targets of miR-96 and genes that alter their expression as a downstream effect of the mutation. Specifically, the most interesting set of down-regulated genes, *Slc26a5*, *Ocm*, *Pitpnm1*, *Gfi1* and *Ptprq*, do not contain a target site for the wild-type nor the mutant miR-96. Nevertheless, all these genes were implicated in deafness, thus their down-regulation could account for at least a portion of the observed phenotype. Since each miRNA is known to regulate the expression of many target genes (Bartel, 2004), the observed phenotype is probably a result of a complex mechanism, in which a combination of down-regulated and up-regulated genes are involved.

HL may occur as a result of various abnormalities and controlled by different genes. As such, hair cell loss can be generated as a primary process or arise secondarily to prior dysfunction of the hair cells. In the *Dmdo* mutant mice, the inner ear in general, and the organ of Corti specifically, appear to develop normally until birth, creating one row of inner hair cells and three rows of outer hair cells with stereocilia projections (Lewis et al., 2009). For this

reason, miR-96 is not implicated in the primary development of the sensory epithelia. Around the time of birth, outer and inner hair cells differentiate apart in a basal-to-apical gradient, expressing particular ion channels, attracting specific neuronal processes and ultimately maturing to functional hair cells that can transduce sound (Housley et al., 2006). This maturation route is what fails to occur properly in *Dmdo* mutants (Kuhn et al., 2011).

The stereocilia of inner hair cells do not widen appropriately, the microvilli in outer hair cells remain present and bundles create a round shape instead of the normal double-u shape (Kuhn et al., 2011). The down-regulation of *Ptprq* described above may explain some of the observed hair bundle malformations. Both outer and inner hair cells cease to grow, remaining shorter than the wild-type hair cells. This abnormality might be caused by the down-regulation of *Slc26a5* mentioned above, since in its KO mouse, the hair cells are shorter as well. Several biophysical attributes also remained underdeveloped in mutant hair cells, namely  $\text{K}^+$  currents of inner hair cells, which failed to mature, remained very low and lacked adult qualities. *Tmc1* is also down-regulated in the mutant inner ear, which could be associated with alterations in biophysical properties. Synapses of adult inner hair cells of *Dmdo* mice preserve a smaller change in membrane capacitance ( $\Delta C_m$ ) and non-linear dependence on exocytotic  $\text{Ca}^{2+}$  ( $I_{\text{Ca}}$ ), strengthened by the down-regulation of *Cacna1d*, which encode a  $\text{Ca}^{2+}$  channel. These characteristics are similar to the observations made before birth, demonstrating abnormal synaptic development. In addition, the ribbon synapses appear spherical and afferent neurons are disorganized (Fig. 4C), advocating that the neurons fail to mature as well, either due to the immaturity of the hair cells or to miR-96 expression in the neurons themselves. In contrast, efferent neurons appear functional in adult mutant mice, but their innervations were misplaced, possibly in association with the continued response to the efferent neurotransmitter Acetyl-Choline (ACh) in inner hair cells, which should be restricted to immature cells (Marcotti et al., 2004). The up-regulation of *Chrna10* ( $\alpha 10$ -nAChR) and the down-regulation of *Chrna9* ( $\alpha 9$ -nAChR), both of which encode ACh receptors expressed in hair cells (Kong et al., 2008),

may partially account for the improper late response to ACh. The wide range of abnormalities all point to a failure of hair cells to mature and associate miR-96 with progression of differentiation and maturation of the hair cells, rather than with their development (Kuhn et al., 2011). In addition, the semi-dominant inheritance and intermediate phenotype observed in the heterozygotes for all properties emphasizes the importance of tight regulation of miR-96 quantity in the cells and the inability of miR-182 and miR-183 to compensate for the absence of miR-96.

## 5. Concluding remarks

Discovery of human genetic mutations is undergoing a revolution with the advent of next-generation sequencing (NGS) (Metzker, 2010). While the majority of mutations for HL thus far have been discovered by linkage analysis and Sanger sequencing, the next phase of discovery will be by methods that will allow for an even greater number of mutations to be found (for example in HL, see (Shearer et al., 2010)). The challenge remaining will be to prove causality of each variant found. In the process, new paradigms for pathogenic mutations may be found for deafness. The remaining patients with undiagnosed genetic deafness will be able to have their mutations resolved, leading to improved genetic counseling. Gene discovery will undeniably continue to provide essential data regarding the mechanisms leading to deafness. In the future, genetic diagnosis may be integrated with therapy, if, for example, the mechanism of pathogenesis appears dependent on the mutation, as is the case for DFNA51 described above. In addition, general mechanisms defined by genetic pathways leading to hair cell death may provide leads for therapy that include regeneration and stem cells (Oshima et al., 2010; Shibata and Raphael, 2010).

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