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Mouse models to study inner ear development and hereditary hearing loss

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ABSTRACT Hereditary sensorineural hearing loss, derived from inner ear defects, is the most common hereditary disability with a prevalence of 1 in 1000 children, although it can be present in up to 15% of births in isolated communities. The mouse serves as an ideal animal model to identify new deafness-related genes and to study their roles *in vivo*. This review describes mouse models for genes that have been linked with hearing impairment (HI) in humans. Mutations in several groups of genes have been linked with HI in both mice and humans. Mutant mice have been instrumental in elucidating the function and mechanisms of the inner ear. For example, the roles of collagens and tectorins in the tectorial membrane, as well as the necessity of intact links between the hair cell projections, stereocilia and kinocilia, have been discovered in mice. Accurate endolymph composition and the proteins which participate in its production were found to be crucial for inner ear function, as well as several motor proteins such as prestin and myosins. Two systematic projects, KOMP and EUCOMM, which are currently being carried out to create knock-out and conditional mutants for every gene in the mouse genome, promise that many additional deafness-related genes will be identified in the next years, providing models for all forms of human deafness.

KEY WORDS: *mouse, inner ear development, deafness gene, hearing*

Introduction

Hereditary hearing loss (HHL) in humans

Hearing impairment (HI) is traditionally classified as conductive and sensorineural, based on the defective part of the hearing organ. While conductive HI results from defects in the external or middle ear, sensorineural HI results from a defect located along the auditory pathway, from the cochlea to the auditory cerebral cortex. A conductive defect yields a mild to moderate HI and in most cases may be medically solved. In contrast, a sensorineural defect yields a mild to profound HI and, thus far, sensorineurally hearing impaired persons may be aided with cochlear implants or hearing aids, but their problem cannot be completely solved [recently reviewed in (Petit, 2006)]. Therefore, further study is required to enable development of better therapies for sensorineural HI.

At least 60% of persons with early-onset HI have hereditary hearing loss (HHL) due to genetic mutations. In most of these cases, a single mutation in a single gene is responsible for the hearing loss. About 70% of HHL cases in human are isolated or associated with a vestibular dysfunction only (non-syndromic hearing loss; NSHL), but HHL may be also accompanied with other abnormalities (syndromic hearing loss; SHL). The onset of HHL may vary from birth to old age. Different mutations in the same gene may lead to both syndromic and non-syndromic HHL, as well as to different onset times (Van Camp and Smith, 2006). NSHL is inherited mainly (80%) in an autosomal recessive manner. Sixty-three protein-coding chromosomal genes and seven tRNA or rRNA coding mitochondrial genes have been linked to HHL in humans [most of the genes are listed in the Hereditary Hearing Loss Homepage: http://webhost.ua.ac.be/hhh/ (Van Camp and Smith, 2006); additional genes are *FGF3* (Tekin *et al.*, 2007) and *SLC4A11* (Desir *et al.*, 2007)]. The protein-coding genes

Abbreviations used in this paper: ABR, auditory brainstem response; BM, basilar membrane; ENU, N-ethyl-N-nitrosourea; EP, endocochlear potential; HHL, hereditary hearing loss; HI, hearing impairment; IHC, inner hair cells; NSHL, non-syndromic hearing loss; OHC, outer hair cells; PBM, PDZ binding motif; RP, retinitis pigmentosa; SHL, syndromic hearing loss; TM, tectorial membrane; WT, wild type.

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Electronic Supplementary Material (Table S1 - genes that have been linked both with inner ear defects in mice and HHL in humans) is available for this paper at: http://www.ijdb.ehu.es/web/paper.php?doi=072365lf

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include genes that encode for extracellular matrix components, gap junction and adhesion proteins, ion channels and transporters, other cell surface proteins and receptors, as well as myosins (molecular motors), cytoskeletal proteins, transcription factors and other proteins that interact with them to create hearing-related networks.

If mutations in a given gene lead to defective development of the inner ear and to early-onset HHL, the affected gene may be considered as having a role in inner ear development or function. A better understanding of inner ear development is required to understand the mechanisms by which specific mutations lead to HHL.

Mouse models

The study of sensorineural HHL in humans is limited by the inability to follow inner ear development. Genetic linkage analysis of HHL in humans is possible only in large families that contain several hearing impaired members. In addition, the search for the responsible gene in HHL patients may be more complicated than analysis of other inherited characters, since hearing-impaired persons from different families tend to marry each other and marriages between hearing children of hearing-impaired parents are also not rare. As a result, one family may carry two or more deafness-related mutations (Petit, 2006). Moreover, due to the absence of a satisfactory human cell line with similar characteristics to the developing inner ear, only primary cultures or model animals may be used to study the interactions between proteins expressed in the inner ear, their spatial and temporal expression patterns, their functions and for any other biological study.

Mutant mouse models that exhibit HHL due to inner ear defects may help to identify genes that have a role in the development or function of the inner ear. When a gene is suspected as responsible for HHL in humans, similar mutations may be engineered in mice to verify this hypothesis. Gene-targeted mutagenesis, or ‘knockout’ mice, may also be used to uncover the gene’s role by comparison with wild type mice. Knockout mice have also been made for genes suspected as essential for hearing due to known interactions of their products with proteins encoded by other known deafness-related genes, or due to the expression of their products in the inner ear. In addition, mouse models are used to identify new genes that have a role in inner ear development and normal hearing. Many strains of hearing-impaired mice have

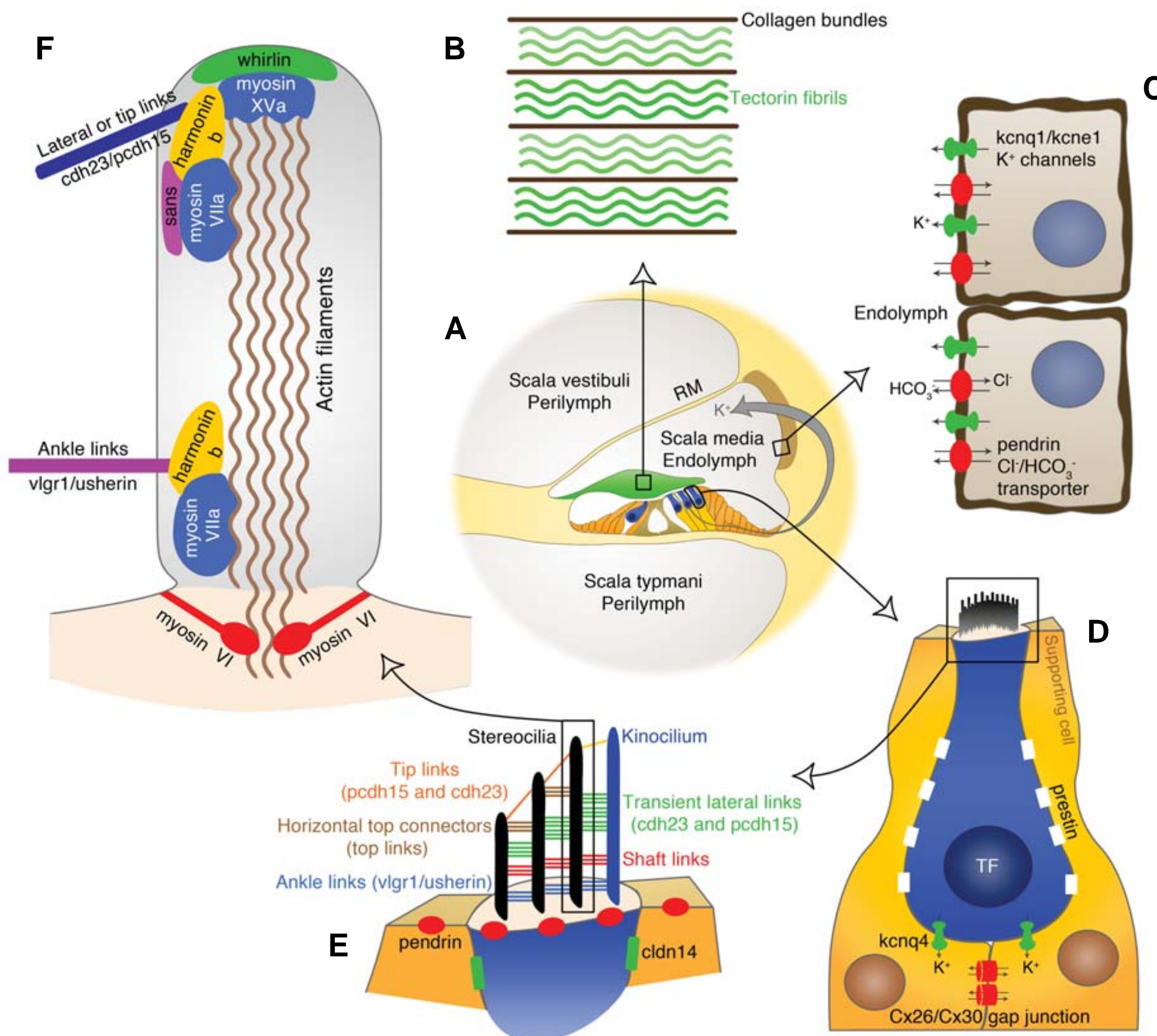
Fig. 1. Schematic illustration of the spatial expression of proteins which have been discovered to play a role in inner ear function both in mice and humans. (A) *A schematic representation of a cross section through one turn of a wild type mammalian cochlea. Reissner’s membrane (RM) separates the scala media from the scala vestibuli. In the organ of Corti, the tectorial membrane (green) covers the neuroepithelium, which contains sensory hair cells (three rows of outer hair cells and a single row of inner hair cells; shown in blue) and supporting cells (orange). The lateral stria vascularis (brown) has a crucial role in endolymph production. A grey arrow represents the main route of potassium cycling.* **(B)** *A schematic illustration of the tectorial membrane that is composed of two types of fibers: thin tectorin fibrils that compose the striated-sheet matrix (green) and heavy collagen bundles (black).* **(C)** *An illustration of the marginal cells of the stria vascularis. These cells laterally cover the lateral wall of the scala media (the endolymphatic space). Their apical plasma membranes contain kcnq1/kcne1 potassium channels (green) and pendrin (red).* **(D)** *An illustration of a single outer hair cell (OHC; blue), surrounded by two Deiters’ supporting cells (yellow). A hair bundle lies on the apical surface of the OHC (box). Many prestin molecules (white rectangles) are expressed along the lateral walls of the OHC, while kcnq4 potassium channels (green) are included in its basal membrane. Gap junctions, composed of connexins 26 and 30 (red), connect the two Deiters’ cells. The OHC nucleus contains many transcription factors (TF) that were linked with HHL.* **(E)** *An enlargement of the hair cell hair bundle. The developing hair bundle contains a microtubules-containing kinocilium (blue projection) and actin-based stereocilia (black). The kinocilium is degenerated in the mature cochlear hair cells (Steyger* et al.*, 1989), but persists in adult vestibular hair cells (Denman-Johnson and Forge, 1999). The stereocilia are connected by a dense network of links to the kinocilium and to adjacent stereocilia, while different links exist in mature and developing hair bundles [reviewed and photographed in (Frolenkov* et al.*, 2004; Goodyear* et al.*, 2005)]. Most of the intra-hair bundle links are illustrated, although not all links are present at the same time. The stereocilia in the mature hair bundle are interconnected by tip links (dark orange) and horizontal top connectors (brown). Tip links (dark orange) are oblique filamentous structures that connect the tops of two adjacent stereocilia with different heights and are thought to gate mechano-electrical transducer channels. Deflections of the stereocilia in the hair bundle toward the tallest row of stereocilia stretch the tip links and increase the open probability of these channels. Deflections of stereocilia in the opposite directions close these ion channels. Thus, tip links are an important component of the mechanotransduction apparatus. Horizontal top connectors (brown), also known as top links, couple adjacent stereocilia just below the tip links. While tip links also exist in the developing hair bundle, horizontal top connectors appear only at a relatively late stage of hair cell development. The developing hair cells also contain lateral (green) and ankle (light blue) links that are lost during development. The fine lateral links (green) are side links that connect adjacent stereocilia at their upper ends below the tip links. Ankle links (light blue) connect the stereocilia at their base and appear only for a short time during the postnatal period. During normal development, their loss is concomitant with the appearance of horizontal top connectors. The developing (and mature vestibular) hair bundles also contain kinocilial links (light orange) that connect the kinocilium to the tallest two or three stereocilia in the same hair bundle and are very similar to the tip links, as well as shaft links (red). Each link type is biochemically different from the others. Prestin molecules, which are expressed along the apical surface of supporting and hair cells (red), as well as cldn14 tight junctions (green) between supporting and hair cells are also shown.* ***(F)*** *An enlargement of a single stereocilium from the hair cell hair bundle. Protein complexes that are expressed at different developmental stages (in mature and developing stereocilia) are illustrated in the same figure, although in reality they are not expressed at the same time. The stereocilium cytoskeleton is based on F-actin filaments (brown). Integral proteins (cdh23, pcdh15, vlgr1, usherin) may dimerize with similar proteins of an adjacent stereocilium to compose lateral or tip links (dark blue rectangle), as well as ankle links (purple rectangle). Multi-protein complexes in the stereocilium, composed of proteins that have been linked with Usher syndrome, contain unconventional myosins (VIIa or XVa; light blue) that can move along the F-actin filaments from the hair cell body to the stereocilium tip, harmonin-b (orange) and whirlin (green) that contain PDZ domains, and sans (fuchsia pink). The complexes contain several additional proteins that are not mentioned in this review, since they have not been linked yet with HI in mice [recently reviewed in (Kremer* et al.*, 2006; Reiners* et al.*, 2006)]. While tip links exist also in mature stereocilia, ankle links exist only in the developing stereocilia. The ankle link complex includes whirlin as well, but in the mature stereocilia whirlin expression is limited to the tip (Adato* et al.*, 2005a; van Wijk* et al.*, 2006). Myosin VI (red) is an additional unconventional myosin, but it moves along actin filaments in the opposite direction, from the plasma membrane into the cell, suggesting that it pulls down the hair cell apical plasma membrane between stereocilia (Cramer, 2000).*

arisen spontaneously during the last century. Moreover, coincidental mutagenesis of mouse chromosomes, by chemicals (mainly by ENU, N-ethyl-N-nitrosourea), by X-ray radiation or by coincidental insertion of an extrinsic sequence («gene trap») has been used to create new hearing-impaired mouse strains. Identification of the responsible gene in such strains is much easier than genetic linkage analysis in humans. In fact, many deafness-related genes were identified in humans only after their identification in hearing-impaired mice (Supplementary Table S1).

Mutations in more than 172 different genes have been reported as responsible for inner ear malformations or dysfunction in mice (most of them are listed in the Jackson Laboratory's Hereditary Hearing Impairment in Mice database: http://www.jax.org/hmr/master_table.html). Only 44 of them have already been linked to human HHL (these genes are listed in Supplementary Table S1). In addition, two genes that were linked with human HHL were found as not crucial for inner ear development and function in knockout mice (Table 1). Figure 1 illustrates the spatial expression of some of the proteins encoded by genes associated with HHL.

Following the identification of the mutated gene, the mutant mice may be used to follow defective inner ear development and to identify specific roles of the gene products. Examples of assays that have been used to evaluate the outcome of deafness-related mutations are shown in Figures 2 and 3. Inner ear development and defects may be followed using bright field light microcopy (Figure 2, A-C), transmission (TEM; Figure 2, D-F) or scanning electron microscopy (SEM; Figure 2, G-J), as well as by paintfill analysis (Figure 2, K-L). Physiological assays may be used to measure ion currents and voltage potentials. The patch clamp assay may be used to measure currents or membrane potentials in a single cell. Length change in individual cells may be used to measure electromotility of outer hair cells (Figure 2, M-N). Temporal and spatial expression patterns of specific mRNAs or proteins in the cells may be observed by *in situ* hybridization (ISH; Figure 2, O-P) and immunofluorescence (Figure 2, Q-V), respectively. Measurement of auditory brainstem response (ABR) to sound signals by scalp electrodes is the most widely used assay for evaluating hearing in mice (Figure 3, A-B). Vestibular defects may be assessed by swimming or other behavioral tests and may induce a characteristic circling behavior (Figure 3, C-E).

This review will describe mutant mouse models for some



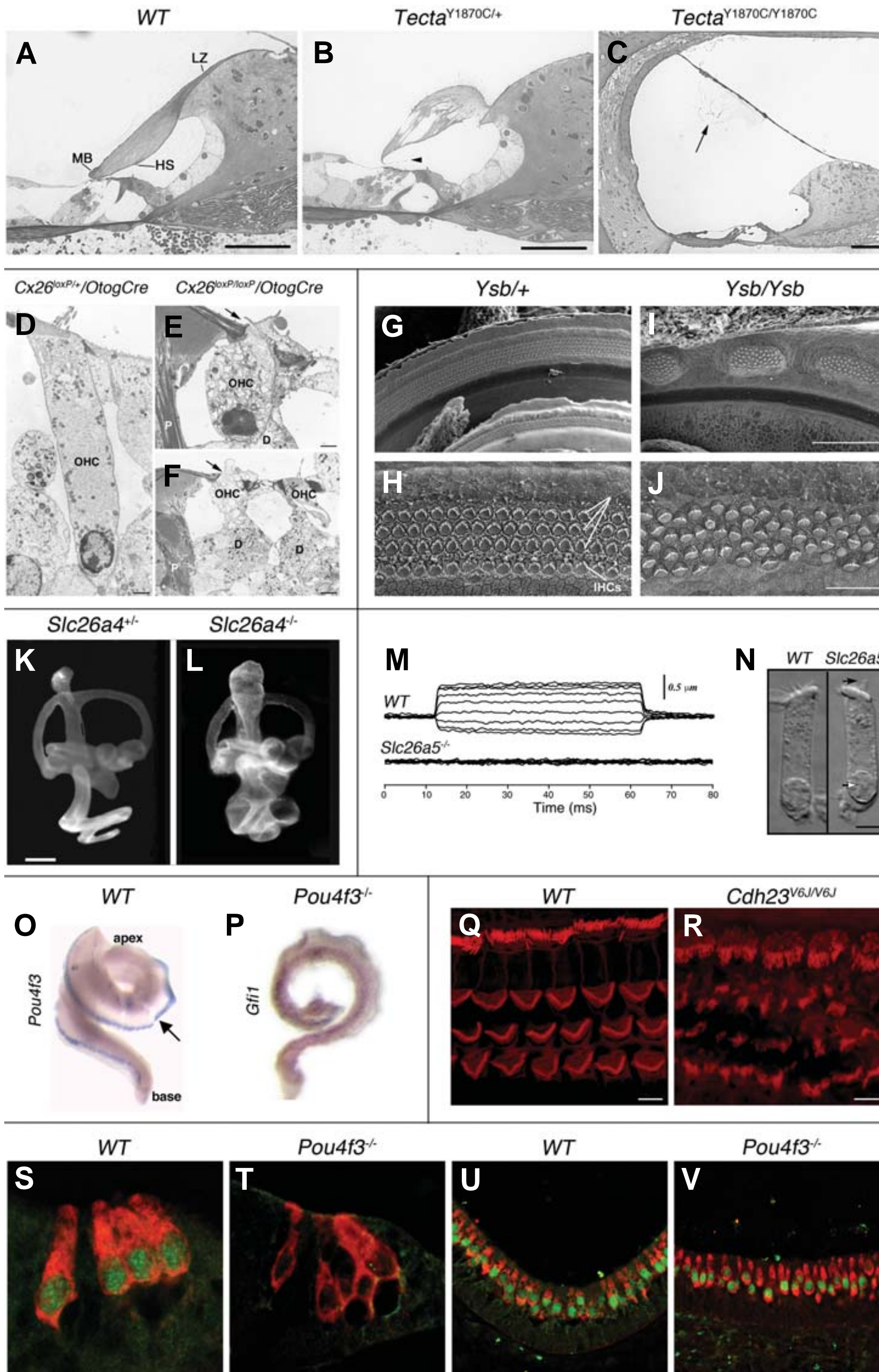


Fig. 2. Examples of assays which have been used to assess roles of specific genes in the mouse inner ear. *(A-C) Light microscopic analysis (toluidine blue staining) of 1 mm thick sections of the cochlear duct from wild type* **(A)**, Tecta$^{Y1870C/+}$ **(B)** *and* Tecta$^{Y1870C/Y1870C}$ **(C)** *mice. Abnormal and detached tectorial membranes are observed when* Tecta *is mutated. Abbreviations: LZ – limbal zone, MB – marginal band, HS – Hensen's stripe. Arrows: an arrowhead in (B) – Kimura's membrane, an arrow in (C) – tectorial membrane. Scale bars, 50 μm. Reprinted with permission from (Legan* et al., *2005).* **(D-F)** *Transmission electron microscopy (TEM) of the organ of Corti at P30 from cochlea that expresses wild type connexin 26* **(D)**, *compared to cochlea in which the* Cx26 *gene was deleted* **(E-F)**. *When Cx26 is absent, damaged Deiters' cells do not stick to OHC, leading to hair cell degeneration. Abbreviations: D – Deiters' cells, P – outer pillar cells. Arrows: disruption of the reticular lamina. Scale bars, 2.3 μm in (D), 1.25 μm in (E) and 0.8 μm in (F). Reprinted with permission from (Cohen-Salmon* et al., *2002).* **(G-J)** *Scanning electron microscopy (SEM) of the organ of Corti basal portions at P0 from yellow submarine (*Ysb*) heterozygous* **(G-H)** *and homozygous* **(I-J)** *mice.* Ysb *is a mutant allele of* Sox2. *Abnormal patches of hair cells are found in* Ysb/Ysb *mice. Three arrows in (H) indicate the OHC. Scale bars, 100 μm in (G, I), 20 μm in (H, J). Reprinted with permission from (Kiernan* et al., *2005).* **(K-L)** *The paint-fill assay is used to present the endolymph labyrinth of the inner ear, in order to identify malformations within the inner ear cavities (Bissonnette and Fekete, 1996). These figures present lateral view of paint-filled P1 inner ears from heterozygous* **(K)** *and homozygous* **(L)** *mice for the knocked-out allele of* Slc26a4/Pds *(encoding pendrin).* Slc26a4$^{-/-}$ *mice exhibit dilated cochleae and endolymphatic ducts and sacs. Scale bar, 500 μm. Reprinted with permission from (Everett* et al., *2001).* **(M-N)** In vitro *analysis of OHC electromotility in wild type and mutant (*Slc26a5/Prestin *knockout) mice.* **(M)** *Length changes of OHC in response to voltage steps (-120-60 mV in 20 mV steps) in whole-cell, voltage-clamp recordings.* **(N)** *Micrographs of OHC isolated from apical turns of cochleae. OHC that do not express prestin are shorter than wild type OHC and do not exhibit electromotility. Arrows in (N): open arrow - nucleus; filled arrow - stereocilia. Scale bar, 5 μm in (N). Reprinted with permission from (Liberman* et al., *2002).* **(O-P)** *Whole mount* in situ *hybridizations (ISH) detect expression of* Pou4f3 **(O)** *and* Gfi1 **(P)** *mRNAs in E18.5 cochleae from wild type (O) and dreidel (P) littermate mice. Dreidel mice, which do not express functional Pou4f3 protein, do not express* Gfi1 *mRNA. Arrow –* Pou4f3 *mRNA expression is detected as a blue band along the lateral wall of the cochlea. Reprinted with permission from (Hertzano* et al., *2004).* **(Q-R)** *Whole mount immunohistochemistry detects spatial expression pattern of F-actin (shown in red, stained with rhodamine phalloidin) in stereocilia in the middle turn of wild type* **(Q)** *and waltzer* V^{6J} **(R)** *organ of Corti at P7. The* V^{6J} *allele was reported to be a functional null allele of* Cdh23 *(Di Palma* et al., *2001a; Di Palma* et al., *2001b). Waltzer mice exhibit disorganized stereocilia (Lagziel* et al., *2005). Scale bars, 5 μm. Figures from Ayala Lagziel and Thomas B. Friedman.* **(S-V)** *Immunohistochemistry of paraffin sections of E18.5 wild-type (S and U) and* Pou4f3$^{-/-}$ *(T and V) mouse inner ears. Expression of Lhx3 (green) and myosin VI (red) was detected in the cochlea* **(S-T)** *and the vestibular system utricle* **(U-V)**. *Whereas Lhx3 is expressed in the nuclei of all hair cells in the wild-type inner ears, Lhx3 expression could be detected only in the vestibular system of the* Pou4f3$^{-/-}$ *mice but not in any of the nuclei of the cochlear hair cells (Hertzano* et al., *2007). Figures from Amiel Dror.*

representative genes that are crucial for normal development and function of the mammalian inner ear. We will focus mainly on genes for extracellular and integral inner ear proteins that were found to be involved both in human HHL and mouse inner ear development. Nonetheless, some examples for genes encoding for intracellular proteins will be also mentioned.

Extracellular matrix components: cartilage and tectorial membrane defects (collagen genes and *Tecta*)

The mammalian hearing organ, the organ of Corti, sits in the snail-shaped cochlea on a strand of connective tissue, the basilar membrane (BM). The collagen-based BM is graded in stiffness along the cochlea and vibrates in response to sound-induced movements of the cochlear fluids. These vibrations are detected by two types of hair cells, included in the sensory epithelium of the organ of Corti, the inner and outer hair cells (IHC and OHC, respectively). The mechanosensory hair bundles of the OHC project up from the reticular lamina, the apical surface of the sensory epithelium and are embedded in the overlying tectorial membrane (TM) [reviewed in (Raphael and Altschuler, 2003)]. A cross section of the organ of Corti is illustrated in Figure 1A. The mammalian TM has a unique and highly organized ultrastructure. It contains two main groups of components: collagen fibrils that are organized in heavy bundles and run radially across the TM and glycoproteins that compose the unusual striated-sheet matrix surrounding the fibrils (schematically illustrated in Figure 1B) (Hasko and Richardson, 1988). Collagens types II, IX and XI compose the radial fibrils (Slepecky *et al.*, 1992; Thalmann, 1993), while two glycoproteins, alpha and beta tectorins (encoded by *Tecta* and *Tectb*), are the major components of the TM matrix (Legan *et al.*, 1997).

Seven collagen proteins were linked with human HHL: COL2A1, COL4A3, COL4A4, COL4A5, COL9A1, COL11A1 and COL11A2 (Van Camp and Smith, 2006). Only five of these have mouse models (Supplementary Table S1). A mutation in *COL11A2* was linked with autosomal dominant NSHL in humans (*DFNA13* locus), but also with Stickler syndrome. The other collagen genes were only linked with SHL in humans, mainly Stickler (*COL2A1*, *COL9A1* and *COL11A1*) and Alport (*COL4A3-5*) syndromes.

Alport syndrome-related collagens (chains alpha-3, 4 and 5 of collagen type IV) are included in basement membranes of the inner ear and the kidney's glomeruli. In the cochlea, they are expressed in the BM, parts of the spiral ligament and stria vascularis. As a result, Alport syndrome (Alport, 1927) combines sensorineural HHL and progressive nephritis, often progressing up to renal failure [reviewed in (Hudson *et al.*, 2003)]. Following the identification of mutations in the human *COL4A3* gene as responsible for Alport syndrome (Mochizuki *et al.*, 1994), *Col4a3* was knocked out in mice (Cosgrove *et al.*, 1996). Homozygotes died at about 14 weeks of age due to renal failure. Defective basement membranes were found in the renal glomeruli and cochlear membranous labyrinth, similar to the human disease. The renal phenotype included progressive glomerulonephritis with proteinuria and microhematuria, focal multilaminated thickening and thinning of the glomerular basement membranes, as well as fibrotic glomeruli with collapsed capillaries. In the cochlear membranous labyrinth, both Col4a3 and Col4a4 chains were completely absent. Basement membranes of specific parts of the membranous labyrinth were significant thinner, thicker or undetectable compared to wild type cochleae and nearby capillaries were collapsed. Both renal and cochlear defects were progressive and HI was detected only after 6 weeks of age (Cosgrove *et al.*, 1996; Cosgrove *et al.*, 1998).

Stickler syndrome (Stickler *et al.*, 1965) includes, in addition to a progressive sensorineural HHL, premature degenerative changes in various joints with abnormal epiphyseal development, vertebral abnormalities, osteoarthritis and sometimes also unusual face and cleft palate. There are three types of Stickler syndrome: type 1 includes also progressive myopathy and blindness due to vitreoretinal degeneration and retinal detachment, while type 2 displays different vitreous defects with no retinal detachment [reviewed in (Snead and Yates, 1999)]. Type 3 is milder, with neither myopathy nor eye involvement (Vikkula *et al.*, 1995). The Stickler syndrome-related collagens Col2a1, Col11a1 and Col11a2 are important components not only of the cochlear TM but also of the cartilage (Col2a1 is expressed also in the eye's vitreous). Since the inner ear has a cartilage cover, which has an important role in its embryogenesis, mutated collagens types II and XI affect the inner ear size, structure and development.

COL2A1 was found to be involved in sensorineural deafness that accompanies several similar hereditary syndromes in humans, such as Stickler syndrome, spondyloepiphyseal dysplasia congenita (SEDC) and chondrodysplasia. Dmm (autosomal semi-dominant disproportionate micromelia), a mouse with a mutated *Col2a1* gene produced in 1966, is an offspring of a male whose spermatogonia had been irradiated. The *Dmm* mutation is a three-nucleotide deletion in the region encoding the C-propeptide globular domain of Col2a1. The deletion leads to the replacement of two amino acids, Lys and Thr, by a single amino acid, Asn, in the mutated protein (Pace *et al.*, 1997). *Dmm* mice expressed a reduced level of collagen II and suffered from cartilage defects

TABLE 1

HEREDITARY HEARING LOSS (HHL)- LINKED GENES IN HUMANS, WHICH ARE NOT AS CRUCIAL FOR INNER EAR DEVELOPMENT AND FUNCTION IN MICE

Gene name	Full name	Main role of gene product	Human reference*	Human syndrome	Mouse reference&	Mouse strain (mutagenesis method§)
Coch	Coagulation factor C homolog, cochlin	Unknown. Secreted protein, most abundant protein in cochlea.	(Robertson *et al.*, 1998)	NS	(Makishima *et al.*, 2005)	*Coch*$^{-/-}$ (KO)
Myh9	non-muscle myosin heavy polypeptide 9	Actin-binding motor protein	(Lalwani *et al.*, 2000)	NS (autosomal dominant)	(Parker *et al.*, 2006)	*Myh9*$^{+/-}$ (GT) Homozygotes died during gestation

(*) First reference that linked the gene to HHL in humans; NS, non syndromic hearing loss; (&) Reference for hearing and inner ear examination of knockout mice; (§) KO, knockout; GT, gene trapping.

that affect inner ear development as well. The homozygotes were dwarf with disproportionate short limbs (micromelia), had a cleft palate (Brown *et al.*, 1981; Seegmiller *et al.*, 1988) and died at birth due to lung hypoplasia (Foster *et al.*, 1994). Inner ears of homozygous Dmm embryos had less collagen fibrils and presented irregular cytodifferentiation of chondrocytes in the extracellular matrix, compared to wild type embryos (Berggren *et al.*, 1997). As a result, dysmorphogenesis of the otic capsule and perilymphatic spaces during embryogenesis led to the development of malformed inner ears with a bulky cartilaginous capsule and a lack or reduction of defined perilymphatic spaces (Van de Water and Galinovic-Schwartz, 1987). More recently, a missense mutation in the mouse *Col2a1* gene was produced spontaneously (R1417C). These mice were named sedc, since their phenotype was similar to human spondyloepiphyseal dysplasia congenita. Homozygous *sedc* adult mice had shortened noses, dysplastic vertebrae, femora and tibias, retinoschisis and hearing loss (Donahue *et al.*, 2003). Gene targeted mutagenesis was used to create *Col2a1* G574S mice, developed as a model for chondrodysplasia, following a parallel mutation that was found in humans. In addition to skeletal malformations, the mice were hearing impaired due to the development of a misshapen otic capsule. While the normal otic capsule is rounded, the transgenic otic capsule was flattened and elongated. The authors suggested that the weaker cartilage of the optic capsule could not resist the mechanical pressures from the developing brain and face and was squashed (Maddox *et al.*, 1998). Heterozygote *Col2a1* mutated mice displayed a milder but not normal phenotype.

Col9a1 is an example to a gene that was linked to HHL in mice before its mapping to a deafness-related locus in humans. *Col9a1*-knockout mice were raised as soon as 1994, but their inner ears were not studied and the observed phenotype was mainly non-inflammatory joint disease resembling human osteoarthritis (Fassler *et al.*, 1994). Only 11 years later, following the re-finding that *Col9a1* is highly expressed in the human inner ear (Abe *et al.*, 2003) [collagens IX were found to be a major component of the TM also previously (Richardson *et al.*, 1987)], the inner ears and hearing of *Col9a1* knockout mice were studied (Asamura *et al.*, 2005). Indeed, these mice displayed a progressive hearing loss, most probably due to a disturbed organization of collagen fibrils in the TM, leading to an abnormal shape of this membrane. TM of *Col9a1* knockout mice contained neither collagens IX nor collagens II. Therefore, it was suggested that collagens IX and II may interact in the TM to determine its three-dimensional structure (Asamura *et al.*, 2005; Suzuki *et al.*, 2005). A year later, a mutation in COL9A1 was linked to an autosomal recessive Stickler syndrome in humans (Van Camp *et al.*, 2006).

Cho mice arose spontaneously in 1971 (Seegmiller *et al.*, 1971). Homozygotes had a cleft palate and died soon after birth due to lethal chondrodysplasia. The *cho* mutation is a 1-nt deletion in the *Col11a1* gene that causes a frameshift and a premature termination codon, resulting in a truncated gene product that cannot assemble with other collagen molecules. Thus, *cho* is actually a functional null allele of *Col11a1* (Li *et al.*, 1995). Homozygotes were severely hearing impaired at birth due to underdevelopment of the organ of Corti in the lower turn of the cochlea, with no hair cells, supporting cells, nerve endings and pillar cells (Cho *et al.*, 1991). Since heterozygous *cho* mice, which expressed both wild type and *cho* alleles of *Col11a1*, suffered from age-dependent osteoarthritis, it was suggested that the *cho* allele may have a destructive effect on connective tissues. However, heterozygous mice were well hearing during their first two months of life and developed a moderate and progressive hearing loss later (age-related) that was not significantly differ from wild type mice (Szymko-Bennett *et al.*, 2003). In contrast to findings in mice, human *COL11A1*-linked SHL is expressed also in heterozygotes: a point mutation in *COL11A1* (G97V) was linked with an autosomal dominant Stickler syndrome (Richards *et al.*, 1996) and a splice-donor-site mutation in this gene was linked with the similar autosomal dominant Marshall syndrome (Griffith *et al.*, 1998).

Col11a2 was knocked out in mice by insertion of a neomycin-resistance cassette in the reverse orientation in place of exons 27 and 28. The inserted sequence included a premature termination codon. Thus, the full length protein was not expressed. The phenotype was much milder compared to *cho* (functional null

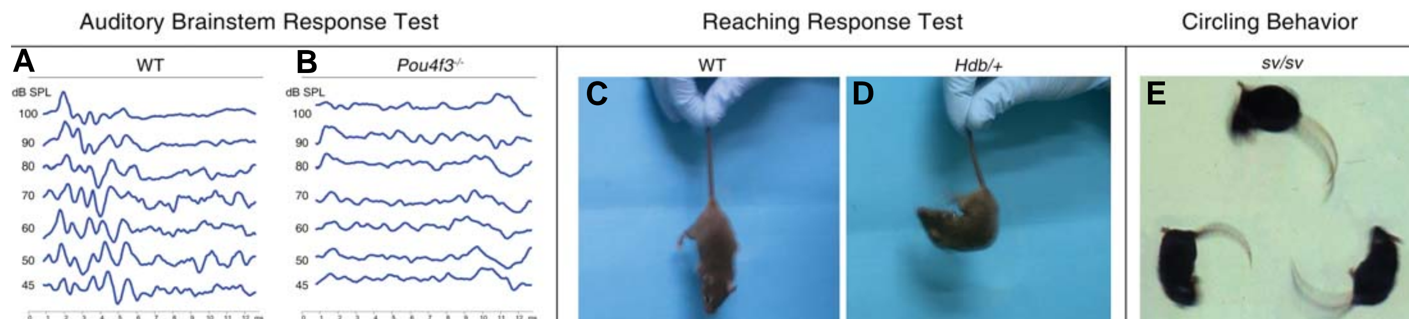


Fig. 3. Common behavioral tests for measuring and observing auditory and vestibular dysfunctions in mouse models. (A-B) *Comparison between wild type and* Pou4f3$^{-/-}$ *auditory brainstem response (ABR) tests.* WT *mice show typical graphs with peaks in response to various intensities (45-100 dB) of sound stimulations* **(A)**. *Flattened graphs are observed in* Pou4f3$^{-/-}$ *mice even at the highest sound level (100 dB), indicating the profound hearing loss of* Pou4f3$^{-/-}$ *mice at the measured frequency of 16 KHz* **(B)**. **(C-D)** *Vestibular apparatus defects can be determined by reaching response. By holding their tails, normal mice will stretch out their legs to make contact with the underneath surface* **(C)**. Hdb *(headbanger) is a dominant mutated allele of* Myo7a, *induced by ENU mutagenesis (Rhodes* et al., *2004). Mice heterozygous for this mutation (*Hdb/+*) failed in recapitulate their three dimensional position, curling up towards their tails* **(D)**. *Figures from Amiel Dror.* **(E)** *A Snell's waltzer mouse, which is homozygous for the* sv *allele of* Myo6 *(spontaneous mutation), exhibits circling behavior, which is another strong indicator for an abnormal balance system.*

Col11a1) mice. Homozygous mice had a smaller body size due to shorter long bones, receding snouts due to shorter nasal bones and hearing loss. The only morphologic abnormality observed in the inner ear was a larger and less compact TM with disorganized collagen fibrils (McGuirt *et al.*, 1999; Li *et al.*, 2001). The mild phenotype in homozygous mice correlates to the mild phenotype in humans (compared to mutations in other collagens): mutations in *COL11A2* are responsible for a milder type of Stickler syndrome (type 3) with no eye abnormalities (Vikkula *et al.*, 1995) and point missense mutations in this gene are responsible for NSHL (McGuirt *et al.*, 1999).

Two mouse models with targeted mutations in *Tecta* (alpha tectorin) have been developed by the same group. Both mutations induced defective TM and HI. The first mutation was a targeted deletion in *Tecta* (named *Tecta*$^{\Delta ENT}$). The only defect in the homozygous mice, which did not express alpha tectorin (null mutation), was observed in the TM, which lacked all non-collageneous matrix and was completely detached from the organ of Corti and spiral limbus. Their inner ears were less sensitive to sound stimulation, supporting the hypothesis that the TM amplifies the hair cell response to low level signals (Legan *et al.*, 2000; Lukashkin *et al.*, 2004). Examination of homozygous *Tecta*$^{\Delta ENT/\Delta ENT}$ mice, together with studying of the motion of the TM and BM of the organ of Corti [e.g. (Hemmert *et al.*, 2000)], helped to uncover the roles of these membranes [For details, see (Legan *et al.*, 2005)].

The second mouse model carried a missense mutation in *Tecta* (Legan *et al.*, 2005), identical to the Y1870C mutation that had been found in hearing impaired humans (Verhoeven *et al.*, 1998). Homozygous *Tecta*$^{Y1870C/Y1870C}$ mice presented a detached TM with no tectorins, similar to the *Tecta*$^{\Delta ENT/\Delta ENT}$ mice. Heterozygous *Tecta*$^{Y1870C/+}$ mice displayed a disrupted and partially thinner TM that expressed tectorins and was still partially attached to the organ of Corti (Figure 2, A-C). Although the interactions between the heterozygote TM and OHC seemed normal, with an almost normal transport of feedback from OHC to BM, the sensitivity for sound signals was reduced due to an elevation in the neural activation thresholds. The space between the TM and the IHC was enlarged in heterozygotes and IHC and reticular lamina movements were specifically reduced at the characteristic frequency. Thus, the heterozygous *Tecta*$^{Y1870C/+}$ mice helped to suggest a second role for the TM: although IHC hair bundles are not imbedded directly in the TM, the TM has still a role in transmitting the BM vibrations to the IHC in the characteristic frequencies. In other words, the TM adjusts the BM vibrations to optimally stimulate IHC at their best frequencies (Legan *et al.*, 2005). This hypothesis was supported recently by a physiological study, suggesting that the hair bundles of the IHC are moved in response to fluid movements in the narrow space between the IHC and the TM. These fluid movements result from the TM vibration and movements of the OHC hair bundles (Nowotny and Gummer, 2006).

While mutations in *TECTA* have already been linked to NSHL in humans (Hughes *et al.*, 1998; Verhoeven *et al.*, 1998; Mustapha *et al.*, 1999), *TECTB* mutations have not been found yet in hearing impaired persons. However, knockout mice for beta-tectorin were recently reported. Although the TM matrix in homozygous mice was disrupted, their inner ears were less sensitive only for low frequency tones, while in high frequencies the frequency resolution was sharpened with little or no loss of sensitivity (sharpness cochlear tuning). These results suggest a third role for the TM: to affect cochlear frequency resolution (Russell *et al.*, 2007).

Intra-hair bundle link proteins: Cdh23, Pcdh15, Vlgr1 and Ush2a

Usher syndrome is the most common etiology for a combination of hereditary deafness and blindness. This disease combines congenital sensorineural hearing loss and a progressive loss of the visual field due to retinitis pigmentosa (RP) that leads to a progressive retinal degeneration. Three clinical subtypes of Usher syndrome have been described. These types differ by the onset time and character of the hearing loss, onset time of the RP and involvement of vestibular dysfunction [recently reviewed in (Nikolopoulos *et al.*, 2006)]. Thus far, mutations in nine genes have been linked to Usher syndrome in humans. Five of these genes have been also linked to NSHL in humans: *MYOVIIA*, *USH1C/Harmonin*, *CDH23, PCDH15* and *VLGR1/MASS1* (Van Camp and Smith, 2006). Mouse mutants are currently available for eight of the Usher-linked genes. The proteins encoded by Usher genes belong to different classes and have different functions. However, all these proteins have a role in the molecular function, development and/or maintenance of the hair cell hair bundle. Recently it was established that all the Usher-related proteins are bound (directly or indirectly) to each other through the harmonin's PDZ sites and form a multi-protein unit that may be shuttled (via the motor myosins myosin VIIa and/or myosin XVa) along the hair cell's actin filaments to their site of action within the stereocilia (Figure 1F) [recently reviewed in (Kremer *et al.*, 2006; Reiners *et al.*, 2006)]. Four Usher-related genes encode for adhesion proteins (cadherin 23, *CDH23*; protocadherin 15, *PCDH15;* Very Large G-protein coupled Receptor-1, *VLGR1/MASS1;* and usherin, *USH2A*). The other Usher-related genes encode for intracellular hair cell proteins (Supplementary Table S1). The roles of Usher-related proteins in the eye have recently been reviewed (Reiners *et al.*, 2006).

Stereocilia are highly specialized microvilli with an actin core that project from hair cells to the endolympahtic space. In the hair bundle, the stereocilia are arranged in rows with a special staircase pattern (illustrated in Figure 1, D-F). In mice, hair bundle development extends from embryogenesis into the first two weeks after birth. The Usher-related adhesion proteins participate in inter-stereociliar links essential for mechanotransduction, a process where the cochlear and vestibular hair cells translate mechanic movements of their hair bundles to electrochemical signals. In the mammalian inner ear, developing and mature hair bundles are considerably different (for details, Figure 1E legend).

Cadherin 23 (also known as otocadherin, *Cdh23*) and protocadherin 15 (*Pcdh15*) are transmembrane proteins with a short intracellular and a long extracellular domain, which are atypical members of the cadherin superfamily. All cadherin molecules contain cadherin domains ('EC' domains) along their extracellular portion, which mediate Ca^{2+}-dependent dimerization of cadherin molecules. Dimerization of cadherin proteins from two neighboring cells links the cells [reviewed in (Reiners *et al.*, 2006)]. In their cytoplasmic tail, cadherin 23 and protocadherin 15 contain class I-PDZ binding (PBM) motifs that can bind PDZ-containing proteins. Therefore, they can bind harmonin. Through

harmonin, the Usher-related cadherins are linked to cytoskeletal actin filaments and are part of the Usher-related multi-protein unit (Siemens *et al.*, 2002; Adato *et al.*, 2005b)].

In wild type mouse inner ears, cadherin 23 was localized to the hair cell stereocilia and Reissner's membrane (Wilson *et al.*, 2001; Boeda *et al.*, 2002; Lagziel *et al.*, 2005). In the mouse hair bundle, cadherin 23 was localized along the length of growing stereocilia and to the tips of mature stereocilia. More precisely, cadherin 23 was localized to links between stereocilia in the hair bundle (Boeda *et al.*, 2002; Siemens *et al.*, 2002; Siemens *et al.*, 2004; Lagziel *et al.*, 2005; Michel *et al.*, 2005; Rzadzinska *et al.*, 2005). Two splice variants of *Cdh23* were found in the mouse inner ear. Both have PDZ-binding motifs that can bind harmonin. A truncated cadherin 23 that lacks the extracellular domain was also reported [reviewed in (Reiners *et al.*, 2006)]. Protocadherin 15 is widely expressed in many tissues in mice (Alagramam *et al.*, 2001a; Murcia and Woychik, 2001) and humans (Alagramam *et al.*, 2001b), including the brain, cochlea and vestibule, from early development through adulthood. In the developing cochlea, protocadherin 15 was localized to the apical surface of hair cells, supporting cells, outer sulcus cells and spiral ganglion cells, while mature cochleae express protocadherin 15 only in hair cell stereocilia (Alagramam *et al.*, 2001b).

Many mutant mice for *Cdh23* are available. Four different mutations in *Cdh23* arose spontaneously in mice: *waltzer* (Deol, 1956; Di Palma *et al.*, 2001a; Wilson *et al.*, 2001; Lagziel *et al.*, 2005), *waltzer niigata* (Wada *et al.*, 2001), modifier of deafwaddler – *mdfw* (Bryda *et al.*, 2001) and age-related hearing loss – *Ahl* (Noben-Trauth *et al.*, 2003). Injections of chemicals to male mice were also used to generate *Cdh23* mutated offspring. Both chlorambucil, that induces deletion mutations [*Albany-waltzer* (Bryda *et al.*, 1997)] and ENU, that induces point mutations (three types of *waltzer-Jackson* alleles; reported only in the Mouse Genome Database: http://www.informatics.jax.org) gave rise to *Cdh23* mutated mice. Seven of these *Cdh23*-mutated mouse strains (except *Ahl*) displayed a similar phenotype: NSHL with circling behavior, head tossing and erratic movements that appear in homozygotes from birth. Heterozygotes appeared normal at birth, but had a tendency to develop a progressive hearing loss at older ages and had a higher sensitivity for noise-induced hearing loss (Holme and Steel, 2004). The *Ahl* allele is a naturally-occurred *Cdh23*^{G753A} dimorphism that appears in many common laboratory inbred mouse strains. The replacement of guanosine 753 by adenosine causes in-frame skipping of exon 7, resulting in the tendency to develop a progressive hearing loss during aging and a higher sensitivity for noise-induced hearing loss (Davis *et al.*, 2001; Noben-Trauth *et al.*, 2003).

Waltzer mouse mutants exhibit a progressive disorganization of the hair bundle, which is first observed at the beginning of the bundle formation at embryonic day 18.5 (E18.5) and becomes more pronounced as the hair cells mature (Figure 2, Q-R). In addition, the kinocilium is misplaced. At older age, stereocilia seem thicker and fused, leading to hair cell degeneration (Di Palma *et al.*, 2001a; Wada *et al.*, 2001; Holme and Steel, 2002). C57BL/6J mice, which are homozygous for the *Ahl* allele, display hair cell degeneration in old age, more pronounced in the apical part of the cochlea. OHC are affected more than the IHC. Degeneration of the efferent nerve fibers was also observed (Mizuta *et al.*, 1993). In the developing mouse inner ear hair cell, cadherin 23 was located both in kinocilial and transient lateral links (Boeda *et al.*, 2002; Lagziel *et al.*, 2005; Michel *et al.*, 2005), but *waltzer* mutated cadherin 23 was absent only from lateral links. Cadherin 23 was observed along kinocilia of mature vestibular hair cells as well (Lagziel *et al.*, 2005). Hair cells of *Cdh23*-deficient zebrafish mutants lacked tip links and these fish had balance and hearing defects (Sollner *et al.*, 2004). Two groups reported that cadherin 23 in mice is also a component of the tip links between stereocilia of the cochlear and vestibule hair bundles. Moreover, cadherin 23 has biochemical properties similar to those of the tip link. Therefore, it was suggested that cadherin 23 composes the tip link that regulates the mechanically gated ion channels in hair cells stereocilia (Goodyear and Richardson, 2003; Siemens *et al.*, 2004).

The first mouse model for a mutated *Pcdh15* was Ames-waltzer (*av*). Originally, Ames-waltzer mice were reported in 1956 as carrying a recessive spontaneous mutation causing deafness, circling behavior, head-tossing and hyperactivity, similar to the waltzer (*v*) phenotype (Schaible, 1956). In the following years, several mutations in the same locus arose independently, resulting in similar phenotypes. The mutated gene was found to be *Pcdh15* in an Ames-waltzer allele that was raised in transgenic mice following insertional mutagenesis (Alagramam *et al.*, 1999). Circling behavior and a reduced AM1-43 dye uptake, that had been shown to correlate with normal transduction function in hair cells, preceded structural defects in the vestibule that could be observed by light or scanning electron microscopy. The functional defect led to disorganization of stereocilia in the cochlea and saccule, which resulted in hair cell dysfunction and progressive degeneration. While inner ears of P10 homozygotes displayed only abnormal stereocilia in the cochlea, saccular stereocilia began to be disorganized only at P30 and inner ears of adult homozygous mice (P50 or older) presented an almost complete degeneration of the cochlea's organ of Corti and vestibular saccular macula (both supporting and hair cells were absent). In the cochlea, a secondary degeneration of the spiral ganglion neurons was also observed. The neuroepithelia of the utricle and the semicircular canals cristae appeared normal, but the utricular otoconia were large and malformed (Alagramam *et al.*, 1999; Alagramam *et al.*, 2001a; Alagramam *et al.*, 2005). In another spontaneous *Pcdh15* mutant, resulting from an insertion of a cytosine residue which led to a frame-shift and premature stop codon, the phenotype was very similar, although the mice were not completely deaf but only hearing impaired. Disorganization of cochlear stereocilia was observed in newborns (P0) (Hampton *et al.*, 2003). An ENU-induced *Pcdh15* mutated mouse presented a similar phenotype as well, with cochlear stereocilia disorganization not before the age of P2. In the cochlea, IHC were less affected compared to OHC (Washington *et al.*, 2005). The three models described above are homozygous for functional null alleles. Milder phenotypes were reported in mice homozygous to less severe mutations in *Pcdh15* (Pawlowski *et al.*, 2006).

In mouse inner ear hair cells, several isoforms of protocadherin 15 are expressed and two of them were suggested to be part of the tip and kinocilial link complexes in the hair bundles. Another isoform may be associated with transient lateral links between developing stereocilia and to kinocilial links, since the expression pattern of this isoform was similar to that of cadherin 23 (Ahmed *et al.*, 2006).

The *Vlgr1/Mass1* gene in mice is transcribed to several splicing variants that encode integral and secreted proteins. The longest isoform, *Vlgr1b*, which is approximately 19 kb is size, is translated to the largest known cell surface protein (approximately 6300 amino acids), containing a large extracellular domain. Its intracellular domain contains a PBM motif that may interact with harmonin's PDZ domain. Although the Vlgr1b protein has a typical structure of a G-protein coupled receptor with seven transmembrane domains, its function is unknown (McMillan *et al.*, 2002; Yagi *et al.*, 2005). *Mass1* is a smaller (approximately 9400 bases) splice variant of *Vlgr1*. Vlgr1 receptors are expressed predominantly in the neuroepithelium of the mouse developing brain (Yagi *et al.*, 2005) and *Vlgr1* mutations, in particular mutated *Vlgr1b* and *Mass1* transcripts, have been associated with audiogenic seizures in mice (Skradski *et al.*, 2001; McMillan and White, 2004; Yagi *et al.*, 2005) and seizures in humans (Nakayama *et al.*, 2002). The extracellular domains of Vlgr1 receptors contain multiple repeated units of CalX-β modules that bind Ca^{2+} cations and may have a role in Ca^{2+}-dependent intercellular adhesion. It was also proposed that these modules may monitor the extracellular Ca^{2+} level and participate in intra- and extra-cellular Ca^{2+} trafficking (Nikkila *et al.*, 2000; Weston *et al.*, 2004). Additional motifs in the extracellular domains of Vlgr1 proteins were suggested to interact with other Usher-related proteins [reviewed in (Reiners *et al.*, 2006)].

The first mouse model for mutant *Vlgr1* was *Mass1^Frings^*, which arose spontaneously in 1951 (Frings *et al.*, 1951), serves as a mouse model for epilepsy due to its susceptibility to loud noise-induced seizures. The BUB/BnJ inbred mouse strain is homozygous for the $Mass1^{Frings}$ mutation and displays both audiogenic seizures and progressive hearing loss that begins postnatally and progresses to complete deafness (Zheng *et al.*, 1999; Skradski *et al.*, 2001). BUB/BnJ mice are also homozygous for the *Ahl* allele of *Cdh23*, but this fact does not explain the deafness of all these mice, since in other strains homozygous to *Ahl* the probability and severity of hearing loss are much lower. The association of *VLGR1* mutations with HHL included in Usher type II syndrome in humans (Weston *et al.*, 2004) raised the possibility that the $Mass1^{Frings}$ mutation underlies hearing loss in BUB/BnJ mice. Indeed, it was shown that the co-mutation of *Cdh23* and *Vlgr1* is responsible for most of the severe hearing loss in BUB/BnJ mice. In young BUB/BnJ mice, the cochlear stereocilia developed abnormally and remained immature. Stereocilia were disconnected and detached, sometimes found outside their unit and the most severely affected bundles lost their polarity and graded height. At older ages, hair cells and spiral ganglion cells were degenerated (Johnson *et al.*, 2005).

Wild type Vlgr1 receptors expression in the inner ear was found to be limited to the synapse region and the hair cell stereocilia, both in the vestibule and cochlea. In hair cells, Vlgr1 receptors were expressed only at the base of developing stereocilia in the same location and timing as ankle links: their expression is maximal at the perinatal period and diminished during hair cell development. A monoclonal antibody that is used to identify ankle links in chickens was found to bind the avian ortholog of Vlgr1b. Two mouse models with mutant *Vlgr1* were developed: (a) knockout mice that express no Vlgr1 proteins (Yagi *et al.*, 2005) and (b) Vlgr1/del7TM mice, in which a targeted deletion was used to delete the transmembrane domain of Vlgr1 (McGee *et al.*, 2006). In both models, Vlgr1 receptors deficiency resulted in similar cochlear abnormalities. Homozygous mice did not display ankle links between the hair cell stereocilia. Although the hair bundles seemed normal at birth, they became disorganized thereafter. Mice homozygous for mutant *Vlgr1* developed profound deafness by the third week of life and from this age displayed disorganized hair bundles, including displaced kinocilia, resulting in distorted stereocilia development. Thus, the Vlgr1 receptor is proposed to be a crucial member in the ankle link complex. Surprisingly, although developing vestibular hair bundles have ankle links and express Vlgr1 as well, only cochlear hair cells were damaged in homozygous mice. Vestibular cells were not degenerated and a vestibular phenotype was not observed, corresponding with a lack of vestibular symptoms in Usher II patients (McGee *et al.*, 2006; Yagi *et al.*, 2007).

Another integral Usher-related protein, usherin (encoded by the long transcript of *Ush2a*), was also suggested to be a component of the ankle links in developing stereocilia (Adato *et al.*, 2005a). In humans, *USH2A* mutations are responsible for the most common genetic form of Usher syndrome (Eudy *et al.*, 1998). Similarly, while knockout $Ush2a^{-/-}$ mice exhibited a progressive degeneration of photoreceptor cells, their hearing was only moderately affected, presenting moderate and non-progressive HI at higher frequencies. Although usherin was predicted as part of the ankle protein and was detected mainly in the base of developing stereocilia in both inner and outer hair cells (from E20) along the entire cochlea, $Ush2a^{-/-}$ mice presented normal hair bundles and lost only OHC in the basal turn of the cochlea (Adato *et al.*, 2005a; Liu *et al.*, 2007).

The studies reviewed above suggest that the expression of link molecules early in the development of the hair bundle is essential for its correct formation and maturation. Correct maturation of the hair bundle is crucial for hair cell survival.

Genes responsible for endolymph production

The cochlea contains two segregated fluid-filled compartments with different ion concentrations (Figure 1A). The perilymphatic space contains the perilymph, a high Na^+ and low K^+ solution, similar to other body extracellular fluids. The apices of the hair cells face the endolymph, which has an opposite cationic composition of high K^+ and low Na^+, while their basolateral surface is bathed in perilymph. Circulation of potassium cations in the cochlea from perilymph to endolymph through the cochlear lateral wall and maintenance of the unique ion composition of the endolymph, are essential for auditory function. Studies in mouse models for mutated proteins that are involved in K^+ recycling in the cochlea helped to establish the recycling mechanism. Acoustically evoked receptor potentials are generated by the influx of K^+ ions from the endolymph into hair cells. These K^+ ions are then secreted basolaterally to the extracellular space of the organ of Corti and picked up by supporting cells. Thereafter, K^+ ions are transported laterally toward the spiral ligament through gap junctions between supporting cells and from supporting cells to root cells, released to the extracellular space of the spiral ligament and then, by a second network of gap junctions between connective tissue fibrocytes, the cations are transferred toward the stria vascularis. K^+ ions pass the basement membrane between the connective tissue and stria vascularis epithelial cells through tight

junctions and are released from the epithelial basal cells to the extracellular space of the stria vascularis. Then, stria vascularis marginal cells take up the K^+ ions and release them back into the endolymph. Stria vascularis marginal cells and Deiters' cells, as an example for supporting cells, are illustrated in Figures 1C and 1D, respectively. A similar recycling pathway exists in the vestibule. This description is somewhat simplistic, since some of the K^+ leakage from the endolymph is through outer sulcus cells and Reissner's membrane [reviewed and illustrated in (Kikuchi *et al.*, 2000; Wangemann, 2002)].

Several genes that account for HHL in humans encode proteins that participate in K^+ circulation in the cochlea. Mutated mouse models were developed for the following genes: (a) *Gjb2/Cx26, Gjb6/Cx30* and *Cldn14* that encode intercellular adhesion proteins: *Gjb2* and *Gjb6* genes encode the gap junction proteins connexin 26 (Cx26) and connexin 30 (Cx30), while *Cldn14* encodes a tight junction protein; (b) *Kcne1, Kcnq1* and *Kcnq4* that encode potassium ion channels; and (c) *Slc26a4* that encodes an anion transporter.

Gap junctions are channels interconnecting two cells and allow a rapid transport of wide variety of ions and small molecules (including nucleotides, siRNAs and inositol phosphates) between the connected cells. Gap junctions are composed of closely aggregated intramembranous channel particles (connexons), which in turn are hexameric assemblies of connexin proteins. The inner ear hair cells do not contain gap junctions. Two distinct networks of gap junctions exist in the cochlea: between connective tissue cells and between non-sensory epithelial cells. Cx26 and Cx30 are part of both cochlear gap junction systems and can co-assemble to form hybrid (heteromeric) gap junctions. However, the predominant connexin isoform expressed in cochlear supporting cells is Cx26 (Ahmad *et al.*, 2003; Forge *et al.*, 2003; Buniello *et al.*, 2004).

In the human genome, *GJB2* and *GJB6* genes are located in the same chromosomal locus (*DFNB1*, 13q11-12). Mutations in this locus account for a high proportion of congenital hereditary NSHL with variability depending on the population [approximately 30-60%; e.g. (Zelante *et al.*, 1997)]. *GJB2* mutations are the most prevalent inherited source of deafness in humans (30-50% of prelingual hereditary NSHL cases). In most of these cases, the responsible mutations are small deletions in the *GJB2* gene and the inheritance type is autosomal recessive. However, few cases of dominant inherited SHL due to *GJB2* mutations were also reported. Thus far, more than a hundred deafness-related different mutations in *GJB2* have been identified in humans. Large deletions in the *GJB6* gene can also cause deafness in homozygotes. In addition, a combination of a large deletion in *GJB6* and a point mutation in *GJB2* can induce NSHL in heterozygotes [Connexins and Deafness Homepage; http://davinci.crg.es/deafness/ (Ballana *et al.*, 2007)].

Two different approaches, targeted mutagenesis (Gabriel *et al.*, 1998) and ENU-induced mutagenesis (Coghill *et al.*, 2002), were used to knock out the *Gjb2/Cx26* gene in mice. Both approaches led to birth of well hearing heterozygous offspring only, while homozygous embryos died in utero due to placental defects. Two additional strategies were taken to generate mutant *Gjb2* mouse models that will be both hearing impaired and viable. *Gjb2* was specifically knocked out in the cochlear epithelial network (supporting and flanking epithelial cells), using the conditional *cre-loxP* system to generate mice that are homozygous for *Gjb2-loxP* and carry *Cre* after an *Otog* promoter, which is expressed only in cochlear epithelial cells (Figure 2, D-F) (Cohen-Salmon *et al.*, 2002). In a second approach, targeted point mutagenesis was used to replicate the Cx26 R75W mutation (Kudo *et al.*, 2003) that is responsible for autosomal dominant SHL (HHL and skin disease) in human heterozygotes (Richard *et al.*, 1998). The dominant inheritance was explained by the ability of the mutant Cx26 to inhibit the function of gap junctions that co-assemble wild type and mutant Cx26 molecules (Richard *et al.*, 1998).

Both *Gjb2* knockout homozygotes and *Cx26*^{R75W} heterozygotes exhibited similar HI in adults and histological phenotypes, although the second model displayed a more severe phenotype. In both models, the inner ear development was normal until postnatal day 14 (P14). Only after onset of hearing, at P15-P16, epithelial cells began to die due to apoptosis. The IHC-neighboring supporting cells were first damaged. Thereafter, OHC and their supporting cells began to die. The tunnel of Corti was collapsed. *Cx26*^{R75W} heterozygotes displayed degeneration of all organ of Corti that began at P14 and led to a complete degeneration of both hair cells and supporting cells by seven weeks of age. In *Gjb2* knockout mice, IHC died only in the more profoundly hearing impaired mice (but displayed immature synapses even when they survived) and some of the intradental cells of the spiral limbus were degenerated at older age (P60). The reticular lamina at the apical surface of the sensory epithelium, which is composed of tight junctions between hair cells and their supporting cells, was disrupted from an early stage in *Gjb2* knockout mice (Figure 2, E-F). Therefore, Cx26 seems to be essential for survival and function of the organ of Corti, but is not required for its normal development. Differences between the models were observed in the maintenance of electric potential difference between the endolymphatic and perilymphatic compartments of the cochlea, represented by the endocochlear potential (EP). In *Gjb2* knockout mice, endolymphatic K^+ concentration and EP were much lower in homozygous mice, as expected, supporting the hypothesis that Cx26-based gap junctions are required for K^+ recycling in the cochlea. Surprisingly, EPs of *Cx26*^{R75W} heterozygotes were normal, suggesting that the reason for apoptosis of organ of Corti cells in the presence of a mutant Cx26 is an impaired K^+ transport by supporting cells rather than affecting endolymph homeostasis, as originally hypothesized. Since *Cx26* was not knocked out in the vestibule in the conditional model and its vestibular expression was normal in homozygous mice, these mice did not exhibit vestibular defects. However, no vestibular or other abnormalities were found in the second model as well. In addition, although the dominant mutant Cx26 R75W was expressed also in the cochlear connective tissue cell system, no obvious structural change was observed in the stria vascularis or spiral ligament (Cohen-Salmon *et al.*, 2002; Kudo *et al.*, 2003).

A *Gjb6* knockout mouse model was also developed by insertion of a missense mutation. Homozygous mice were viable and fertile, but hearing impaired and lacked EP. Degeneration of the organ of Corti, due to apoptosis, was observed from the age of P18, similar to *Gjb2* mutant mice (Teubner *et al.*, 2003).

Cx26 and Cx30 co-assemble in the same gap junctions (Ahmad *et al.*, 2003; Forge *et al.*, 2003). Although Cx30 did not fail to form homomeric gap junctions in Cx26-deficient cells, Cx30 could not

compensate for the lack of Cx26 in the conditional knockout model (Cohen-Salmon *et al.*, 2002). Different connexins differ in size and ionic selectivity and have distinct voltage-gating sensitivities. As a result, connexons assembled from different connexins have different permeation and gating functions (Bruzzone and Cohen-Salmon, 2005; Zhao *et al.*, 2006)]. Thus, characteristics of homomeric connexons, assembled from Cx30 only, may be different from those of heteromeric connexons assembled from both Cx26 and Cx30. Even if the permeation of small ions (like K^+) is similar in different connexon types, the delivery of bigger secondary messenger molecules may be different, affecting K^+ influxes indirectly. A recent paper offered that some *Gjb2* mutations affect the gap junction permeability for inositol triphosphate rather than for K^+. The failure to recycle K^+ from the supporting cells back to the endolymph was suggested to be secondary to inositol triphosphate transport (Beltramello *et al.*, 2005). Nonetheless, the failure of Cx30 to compensate for Cx26 lack may result from its low expression. In the opposite case, over-expression of Cx26 in *Gjb6* knockout mice completely restored hearing sensitivity and prevented hair cell degeneration. Thus, at least Cx26 can compensate for the absence of Cx30, suggesting that heteromeric gap junctions that contain both Cx26 and Cx30 are not essential for normal hearing and for organ of Corti survival in mice. Interestingly, *Gjb6* knockout mice under-expressed Cx26 protein in the cochlea, suggesting an accelerated degradation of the homomeric gap junctions. *Gjb6* knockout mice that also carried the gene for over-expression of Cx26, over-expressed Cx26 in the liver, but in the cochlea Cx26 levels were normal, suggesting that homomeric Cx26 gap junctions are less stable than heteromeric Cx26-Cx30 assemblies, but have a similar function (Ahmad *et al.*, 2007).

Although connexin 29 (Cx29) is not involved in K^+ ions recycling in the cochlea, it is worth mentioning, since mutations in the *GJE1*/*Cx29* gene were found in NSHL patients recently (Yang *et al.*, 2007). The cochlear distribution of Cx29 is very different from that of Cx26 and Cx30. Unlike Cx26 and Cx30, which are mostly expressed in cochlear supporting cells and fibrocytes, Cx29 is expressed mainly in Schwann cells of the spiral ganglion and at lower abundance in the stria vascularis (Eiberger *et al.*, 2006; Tang *et al.*, 2006b). The expression of Cx29 in brain and other organs is also mainly in myelinating cells. Two groups created knockout *Gje1* mice. While one group reported no abnormalities in Cx29-deficient C57BL/6 mice, including normal myelin sheets (Eiberger *et al.*, 2006), the other group reported hearing loss due to severe demyelination at the soma of spiral ganglion neurons (neuropathy), with a penetrance of ~50% and no damage to the inner ear neuroepithelium in BALB/c mice (Tang *et al.*, 2006b).

Tight junctions, the most apical junctions in epithelial cells, serve as the major ion-selective barrier against paracellular transfer of fluids. In addition, they contribute to the maintenance of cellular polarity by forming an intramembrane barrier that restricts the lateral diffusion of apical and basolateral membrane components. Tight junctions are composed of at least three types of transmembrane proteins: occludin, claudins and members of the junction adhesion molecule (JAM) family. More than 20 claudins are known, each with a distinct permeability [recently reviewed in (Kondoh *et al.*, 2006)]. In the cochlea, the essential separation of perilymph from endolymph is achieved by tight junctions that seal the spaces between the cells bordering the fluid compartments. Following the identification of recessive mutations of human *CLDN14* as responsible for profound NSHL in humans (Wilcox *et al.*, 2001), *Cldn14*-null mice were created to explore the role of claudin 14 in the inner ear. Claudin 14 was detected in tight junctions of the cochlea's reticular lamina (tight junctions between hair cells and supporting cells and between neighboring supporting cells). *Cldn14*-null mice had a normal EP, but were deaf. No vestibular phenotype was observed. Although the reticular lamina tight junctions seemed normal microscopically in *Cldn14*-null mice, the hair cell stereocilia were lost or disorganized during the first 3 weeks of life, rapidly followed by hair cell degeneration. OHC were degenerated before IHC. Since claudin 14 has a higher permeability to K^+ than Na^+, it may be required to maintain the proper ionic composition of the perilymphatic fluid surrounding the basolateral surface of OHC. The accurate ionic composition of this fluid may be essential for OHC survival (Ben-Yosef *et al.*, 2003).

The genes *Kcne1, Kcnq1* and *Kcnq4* encode for subunits of slow voltage activated potassium channels, which are the major determinants of cellular repolarization in excitable cells. They open in response to depolarization and facilitate selective efflux of K^+ across the plasma membrane. Each channel is composed of four alpha and some beta subunits. While the pore-forming alpha subunits are sufficient to form functional channels, beta subunits determine the channel's unique properties, including its single-channel conductance, overall channel activity, voltage dependence, activation time dependence, temperature and pH sensitivity, as well as drug sensitivity [reviewed in (Wangemann, 2002)].

Stria vascularis marginal cells and vestibular dark cells secrete K^+ into the endolymph only by K^+ channels composed of Kcnq1 (alpha) and Kcne1 (beta) subunits. Therefore, Kcnq1/Kcne1 channels are responsible for endolymph formation (Marcus *et al.*, 1997; Neyroud *et al.*, 1997; Marcus *et al.*, 1998; Nicolas *et al.*, 2001). In cardiac myocytes, Kcnq1/Kcne1 K^+ channels carry the slowly activating rectifier K^+ current that plays a major role in the repolarization phase of the cardiac action potential. Therefore, mutations in *KCNE1* or *KCNQ1* in humans induce indistinguishable SHL phenotypes (Jervell and Lange-Nielsen Syndrome) of HHL and cardiac symptoms, including prolonged QT intervals and arrhythmias followed by syncope or sudden death (Neyroud *et al.*, 1997; Schulze-Bahr *et al.*, 1997; Tyson *et al.*, 1997).

Kcne1 (Vetter *et al.*, 1996; Nicolas *et al.*, 2001) or *Kcnq1* (Lee *et al.*, 2000; Casimiro *et al.*, 2001; Rivas and Francis, 2005) knockout mice exhibited a classic waltzer-like phenotype with severe hearing loss and vestibular symptoms, up to complete deafness in adult mice. Although the inner ear histology was normal at birth, changes developed later. The strial marginal cells and the vestibular dark cells were unable to secrete K^+ ions, leading to a secondary degeneration of the neuroepithelium including the hair cells and to collapse of the endolymphatic space. Similarly, the endolymphatic space is also collapsed in Jervell and Lange-Nielsen syndrome patients (Friedmann *et al.*, 1966). At birth, the wild type mouse EP is very low, with high Na^+ and low K^+ concentrations in the endolymph. After birth, the EP is increased gradually (in particular from P7), reaching the adult value at P14 (Yamasaki *et al.*, 2000). Accordingly, *Kcne1* (Vetter *et al.*, 1996) or *Kcnq1* (Casimiro *et al.*, 2001) knockout mice displayed normal endolymphatic spaces at birth. Only 3 days after

birth, a collapse of the Reissner's membrane and a decrease in the endolymphatic space volume began to be detected. A spontaneous point mutation in *Kcne1* also arose in mice (punk rocker mice; *Kcne1^{pkr}*). Homozygous mice expressed a severely truncated Kcne1 protein and a similar phenotype to that of *Kcne1* knockout mice (Letts *et al.*, 2000). *Kcnq1* knockout mice also exhibited cardiac repolarization defects (Casimiro *et al.*, 2001; Casimiro *et al.*, 2004). While Kcnq1 is the channel core, it appears that Kcne1 is required for its trafficking to the plasma membrane, since vestibular dark cells in *Kcne1* knockout mice expressed Kcnq1 in their cytoplasm rather than in their apical membranes (Nicolas *et al.*, 2001). Thus, Kcne1 seems to be essential for Kcnq1 membrane targeting and/or stability of Kcnq1 in the membrane.

Kcnq4 is an alpha subunit of an M-type K^+ channel. M-type channels are very slow voltage-dependent K^+ channels. In neurons, M-channels can oppose sustained membrane depolarization and repetitive firing of action potentials following a strong excitatory input, but they also can transiently elevate the neuron excitability following its exposure to modulatory neurotransmitters (Cooper and Jan, 2003). Accordingly, Kcnq4 channels were found in neurons of several nuclei of the central auditory pathway. However, Kcnq4 was also detected in the basolateral membrane of cochlear (Beisel *et al.*, 2000) and vestibular (Rocha-Sanchez *et al.*, 2007) mouse hair cells (both OHC and IHC). After the onset of hearing (P12–14), it localized exclusively to the basal pole. Therefore, it was suggested that Kcnq4 channels are responsible for the secretion of surplus K^+ ions from the hair cell to the perilymph surrounding its basolateral membrane and for setting the hair cell resting membrane potential (Kharkovets *et al.*, 2000; Boettger *et al.*, 2002; Beisel *et al.*, 2005; Rocha-Sanchez *et al.*, 2007). In humans, *KCNQ4* mutations induce autosomal dominant NSHL, suggesting that the mutated gene has a dominant negative effect when it is co-expressed with the wild type allele (Kubisch *et al.*, 1999). Two mouse models with mutated *Kcnq4* were developed: a homozygous knockout mouse and a knock-in mouse with a point mutation that imitates the dominant negative mutation in humans. No vestibular symptoms were observed in both mouse models, although Kcnq4 is strongly expressed in WT vestibular hair cells. The mice had normal hearing at postnatal stages, but displayed a progressive hearing loss that was accompanied with a progressive degeneration of OHC. The progression of both deafness and OHC loss was faster in homozygous knockout and knock-in mice (weeks) compared to heterozygous knock-in mice (months). Using a selective inhibitor of Kcnq channels to isolate Kcnq-dependent K^+ currents, no Kcnq-dependent K^+ currents were detected in OHC from homozygous or dominant negative heterozygous mice, resulting in depolarized resting membrane potentials of the OHC. IHC were not significantly affected. Therefore, it was proposed that *Kcnq4* mutations induce a progressive HHL due to chronic depolarization of OHC, leading to their degeneration (Kharkovets *et al.*, 2006). Recently, Kcnq4 expression in OHC was found to be regulated by thyroid hormones. The thyroid hormone receptor TRα directly affected Kcnq4 expression during OHC final differentiation. In *TRα1* knockout mice, Kcnq4 was expressed but abnormally distributed along both the basal and lateral membranes of the OHC (Winter *et al.*, 2006).

The SLC26 (solute carrier protein 26) family of anion exchangers includes integral proteins with 10-12 transmembrane domains that can transport several anions, including chloride, iodide, sulfate, nitrate, bicarbonate, hydroxyl, oxalate and formate. Each member in this family has different affinity and specificity per each anion. Two members of the SLC26 have been linked with HHL in humans: SLC26A4/pendrin and SLC26A5/prestin. *SLC26A4* mutations were associated with both SHL (Pendred syndrome) (Everett *et al.*, 1997) and NSHL (Li *et al.*, 1998; Usami *et al.*, 1999), while *SLC26A5* was associated only with NSHL (Liu *et al.*, 2003).

Pendred Syndrome, first described in 1896 (Pendred, 1896), is characterized by sensorineural deafness and enlarged thyroid goiter with elevated iodine discharge after perchlorate administration. Most of the patients also display radiologically detectable structural malformations of the inner ear, the most common feature of which is an enlarged vestibular endolymphatic duct [reviewed in (Glaser, 2003)]. Enlarged endolymphatic ducts were also observed in some patients with NSHL due to mutations in *SLC26A4* (Li *et al.*, 1998; Usami *et al.*, 1999). In heterologous expression systems, pendrin has been shown to transport iodide, chloride, formate and nitrate (Scott *et al.*, 1999; Scott and Karniski, 2000). Using mice and rats, pendrin was found to be expressed on apical membranes of thyroid, kidney and inner ear cells. The absence of pendrin was proposed as directly responsible for the defective organification of iodide in Pendred patients. However, *Slc26a4* knockout mice lack thyroid symptoms (Everett *et al.*, 2001) and the exact role of pendrin in the thyroid is still not clear. In the mouse inner ear, pendrin was detected on apical membranes of cells covered the endolymphatic cavities, which are considered to have a role in endolymph homeostasis (Everett *et al.*, 1999; Royaux *et al.*, 2003; Yoshino *et al.*, 2004). In addition, the cochlear expression of pendrin included also supporting cells of the organ of Corti (Claudius and Deiters' cells), as well as the spiral ligament and the spiral ganglion. Recently, a more sensitive approach (postembedding immunogold analysis under an electron microscope) revealed some pendrin expression also in OHC and IHC, in particular in their apical membranes and stereocilia (Yoshino *et al.*, 2006).

Slc26a4 knockout mice (*Pds$^{-/-}$*) exhibited waltzer-like vestibular dysfunction and complete deafness. Their inner ears developed normally only until E15, two days after the beginning of pendrin expression in wild type mice. Thereafter, a severe dilatation of endolymphatic cavities was developed, both in cochlea and vestibule (Figure 2, K-L). This dilatation was proposed to be secondary to an altered osmotic condition and an increased volume of the endolymphatic fluid. During the second postnatal week, hair cells began to degenerate. In the vestibule, the otoconia and otoconial membranes were also destructed (Everett *et al.*, 2001). After weaning, the strial vascularis marginal cells of *Pds$^{-/-}$* mice displayed irregular shapes and sizes, resulting in a thinner stria vascularis. In adult *Pds$^{-/-}$* mice, hyperpigmentation of strial vascularis cells preceded their degeneration, suggesting free radical damage. Functional experiments revealed that *Pds$^{-/-}$* mice gradually loss the EP, beginning at P12, before the normal onset of hearing. Nevertheless, the endolymphatic K^+ concentration and the expression of Kcnq1/Kcne1 channels were normal. Pendrin deficiency also abolished the expression of Kcnj10 K^+ channels in strial intermediate cells, although the *Kcnj10* mRNA was normally expressed (Royaux *et al.*, 2003; Wangemann *et al.*, 2004). Kcnj10 channels have a role in recycling K^+ ions across the

basal cell barrier of the stria vascularis. Thus, pendrin may serve a role in maintaining the EP without affecting K^+ secretion from the stria vascularis marginal cells, but rather by affecting K^+ fluxes in intermediate cells. *Kcnj10* knockout mice did not generate an EP, but had a reduced endolymphatic volume and K^+ concentration (Marcus *et al.*, 2002). Therefore, pendrin deficiency may have additional outcomes. Another role of pendrin was recently revealed both in cochlea (Wangemann *et al.*, 2007) and vestibule (Nakaya *et al.*, 2007). Ca^{2+} channels (Trpv5 and Trpv6) in vestibular and cochlear epithelial cells reabsorb calcium ions from the endolymph and are inhibited by a low pH. In the cochlea, Trpv5 and Trpv6 are expressed in the strial vascularis marginal cells and sulcus epithelial cells, respectively. These channels maintain the low Ca^{2+} concentration of the normal endolymph. *Pendrin*-knockout mice displayed lower pH and higher Ca^{2+} concentration in the endolymph, resulting in a reduced transepithelial potential in the utricle. The higher Ca^{2+} level in the endolymph may inhibit sensory transduction necessary for hearing and promote hair cell degeneration. Thus, in the inner ear, pendrin was proposed to function as a Cl^-/HCO_3^- that mediates secretion of alkaline HCO_3^- ions to the endolymphatic space and one of its important roles may be to maintain the endolymph pH (Nakaya *et al.*, 2007; Wangemann *et al.*, 2007). The hyperpigmentation of stria vascularis in adult *Pds*$^{-/-}$ mice raised the hypothesis that an inflammation process is involved in their degeneration. Indeed, this hyperpigmentation and marginal cell reorganization occurred concurrently with invasion of macrophages specifically to the stria vascularis and expression of macrophage and complement markers (Jabba *et al.*, 2006). The winged helix/forkhead gene *Foxi1* (also known as *Fkh10*) was proposed to induce pendrin expression, since *Foxi1*-null mice do not express pendrin and exhibit a similar phenotype to pendrin knockout mice (Hulander *et al.*, 2003).

SLC26A5/prestin - the motor protein of outer hair cell electromotility

The mammalian cochlea presents two mechanisms for amplification of sound signals: (a) amplification of stereocilia motions by mechano-electric transducer channels (exists in all known auditory organs); and (b) OHC somatic electromotility – a voltage-dependent rapid alteration of the length and stiffness of OHC (exists only in mammalian inner ears), termed also as the cochlear amplifier. Electromotility includes shortening of depolarized OHC and lengthening of hyperpolarized cells, independently on ATP or OHC Ca^{2+} level. Amplification by OHC electromotility is believed to amplify cochlear vibrations and enable the acute hearing sensitivity and frequency selectivity of the mammalian cochlea. This mechanism enables the cochlear response to low (<1 KHz) frequency signals [recently reviewed in (Frolenkov, 2006)].

Prestin is an integral protein that is expressed only in the cochlear OHC (an OHC is illustrated in Figure 1D). Prestin molecules, both as monomers and tetramers, are abundantly expressed along the OHC lateral membrane and for a lesser extent – in the basal membrane. Developmental expression of prestin coincides with the appearance of electromotility (Belyantseva *et al.*, 2000; Zheng *et al.*, 2000; Yu *et al.*, 2006). Although prestin belongs to the SLC26 family of anion exchangers and has a similar structure to other members of this family, clear evidence indicating that it functions as ion transporter has not reported yet. Moreover, a knock-out of prestin (*Slc26a5*) in mice did not affect whole-cell currents of OHC (Liberman *et al.*, 2002). Instead, prestin is considered as the voltage-dependent motor protein responsible for OHC electromotility (Zheng *et al.*, 2000), as *Slc26a5* knockout mice displayed no OHC electromotility (Figure 2M) and frequency selectivity. These mice support the hypothesis that OHC electromotility enhances the inner ear sensitivity, since they exhibited 40-60 dB loss of cochlear sensitivity with no disruption of OHC hair bundles and mechano-electrical transduction. In addition, *Slc26a5*-null mice displayed shorter OHC (Figure 2N), which is not surprising, as prestin is very abundant in the lateral walls of these cells. At 4-9 weeks of age, a secondary apoptosis of OHC was observed in the cochlea's basal quarter in *Slc26a5*-null mice, followed by IHC degeneration, although IHC do not express prestin. However, the HI preceded hair cells degeneration by at least two weeks, implying that lack of electromotility was the primary reason for hearing loss (Liberman *et al.*, 2002; Cheatham *et al.*, 2004; Wu *et al.*, 2004). Recently, the typical distribution of prestin along the OHC lateral membrane was found to depend on the thyroid hormone receptor TRβ (Winter *et al.*, 2006). Although the absolute magnitude of OHC electromotility in heterozygous mice was about half of normal (Liberman *et al.*, 2002), cochlear function and appearance in mice with only one copy of the *Slc26a5* gene were normal (Cheatham *et al.*, 2005). It was suggested that prestin senses voltage by binding an intracellular Cl^- ion in depolarized cells. As a result, its conformational is altered. Thus, prestin is a very efficient direct voltage-to-force converter. Its function is associated with a typical nonlinear capacitance, which may be measured [recently reviewed in (Dallos *et al.*, 2006)].

Unconventional myosins

Unconventional myosins are motor molecules that contain an actin-binding domain in their N-terminal motor or head domain. Using ATP as an energy source, they can move along actin filaments. Unconventional myosins also have binding sites for proteins on their C-terminal tails and thus, they may serve as «cars» that drag cargo proteins to their target sites in the cell. The mammalian inner ear expresses several unconventional myosins, each of which has a unique expression pattern and function in the inner ear. Mutations in five myosin genes (*Myo1a, Myo3a*, *Myo6*, *Myo7a* and *Myo15a*) have been associated with HHL in humans. The expression pattern of myosin 1A in the mouse inner ear has not been studied yet. *Myo3a*, *Myo6*, *Myo7a* and *Myo15a* are expressed within the mouse inner ear only in hair cells, and have a role in hair bundle organization [recently reviewed in (Hertzano and Avraham, 2005)]. Two of them, myosins VIIa and XV, can bind the PDZ sites on harmonin or whirlin and are part of the Usher-related network that is illustrated in Figure 1F [reviewed in (Reiners *et al.*, 2006)]. Thus, myosin VIIa (Boeda *et al.*, 2002; Senften *et al.*, 2006) and myosin XVa (Belyantseva *et al.*, 2005) actively transport harmonin and whirlin, together with attached proteins, to the proper sites in the stereocilia. Recently, myosin IIIa was also shown to be localized at stereocilia tips and required for their proper maintenance (Schneider *et al.*, 2006).

Mouse models are available only with mutations in *Myo6*,

Myo7a and *Myo15a*. Null mutations of *Myo6* [Snell's waltzer (Avraham *et al.*, 1995)], *Myo7a* [shaker1 (Self *et al.*, 1998)] and *Myo15a* [shaker2 (Probst *et al.*, 1998)] induced similar waltzer-like phenotypes in homozygotes (deafness and vestibular dysfunction), resulting from stereocilia fusion (*Myo6*), disorganization (*Myo7a*) or shortening (*Myo15a*). *Myo6* and *Myo7a* null mice also displayed subsequent degeneration of the hair cell. While mice homozygous for null mutations in *Myo6*, *Myo7a* or *Myo15a* were deaf, heterozygotes displayed a normal phenotype. Moreover, doubly heterozygous mice for both *Myo15a* and another (*Myo6* or *Myo7a*) null allele were also normal (Karolyi *et al.*, 2003). However, a missense *Myo7a* mutation (headbanger mice; *Hdb*) induced vestibular phenotype and mild HI also in heterozygotes (Figure 3D), resulted from elongation and fusion of hair cell stereocilia. Homozygotes presented a more severe phenotype (Rhodes *et al.*, 2004). *Myo6* will be reviewed in more details as an example.

A spontaneous mutation, *Snell's waltzer* (*sv*), arose in 1966 (Deol and Green, 1966). The circling behavior of Snell's waltzer mice is presented in Figure 3E. A radiation-induced mutation in the same locus is also available (se^{sv}/se^{sv}) (Russell, 1971), as well as an ENU-generated mutant (ENU89) (personal communication, Colin Fletcher and Karen Avraham). A mutation in the *Myo6* gene was found in the *sv* allele (Avraham *et al.*, 1995). In mice and zebrafish inner ears, myosin VI is expressed specifically in apical plasma membrane of hair cells, near the stereocilia base (Self *et al.*, 1999; Kappler *et al.*, 2004). Homozygous *sv* mice exhibited a progressive degeneration of inner ear hair cells from P12, leading to degeneration of the entire neuroepithelium of the inner ear. Early stages of hair cells and stereocilia development were normal, since at birth only part of the hair bundles were disorganized. However, during the first postnatal week, hair bundles were progressively disorganized and the hair cell apical plasma membrane was raised. Thereafter, during the following two weeks, stereocilia were abnormally fused together to form giant non-functional stereocilia (Self *et al.*, 1999; Kappler *et al.*, 2004). A similar phenotype was observed in zebrafish with mutations in the *Myo6b* gene. In zebrafish, the *Myo6* gene is duplicated (*Myo6a* and *Myo6b*) and only *Myo6b* is predominantly expressed in the inner ear and lateral line neuroepithelium. Similar to *sv* mice, mutations in the zebrafish *Myo6b* are responsible for auditory and vestibular defects (*satellite* mutants) due to disorganized hair bundles in which the stereocilia are eventually fused. Structural defects at the apical plasma membrane were observed as well and large vesicles were accumulated near the cuticular plate (Seiler *et al.*, 2004). Based on zebrafish *satellite* and mouse *sv* mutants, it was suggested that myosin VI anchors the apical plasma membrane of the stereocilium to the core actin filaments (Figure 1F). In the absence of myosin VI, the apical plasma membrane pulled up above the epithelium and between the stereocilia, leading to stereocilia fusion. Mutations in the human *MYO6* gene were linked with HHL in humans only six years after the identification of *Myo6* mutations in *sv* mice. While in mice *Myo6* mutations were associated only with recessive NSHL, human *MYO6* mutations were linked both with dominant (Melchionda *et al.*, 2001) and recessive NSHL (Ahmed *et al.*, 2003), as well as with dominant SHL that includes cardiac hypertrophy and prolonged QT in addition to sensorineural HHL (Mohiddin *et al.*, 2004).

Hair cell genes for transcription factors

Sensory hair cells and non-sensory supporting cells in the inner ear neuroepithelium arise from a common progenitor. The

TABLE 2

GENES THAT WERE LINKED WITH HUMAN HHL AND CLONED, BUT HAVE NO MUTANT MOUSE MODEL THUS FAR

	Gene name	Full name	Year of first linkage of the gene to HHL in humans	Gene cloning in humans – first reference	Human syndrome
1	*COL4A4*	collagen type IV, alpha 4 chain	1994	(Mochizuki *et al.*, 1994)	Alport
2	*COL4A5*	collagen type IV, alpha 5 chain	1994	(Barker *et al.*, 1990)	Alport
3	*TIMM8A*	translocase of inner mitochondrial membrane 8 homolog A (yeast)	1995	(Jin *et al.*, 1996)	NS
4	*USH3A*	Usher syndrome 2A / clarin-1	1995	(Joensuu *et al.*, 2001)	Usher
5	*TCOF1* (*)	Treacher Collins-Franceschetti syndrome 1	1996	(Dixon *et al.*, 1996)	Treacher Collins
6	*DIAPH1*	diaphanous 1	1997	(Lynch *et al.*, 1997)	NS
7	*DSPP*	dentin sialophosphoprotein	2001	(Xiao *et al.*, 2001)	NS
8	*EYA4*	eyes absent homolog 4 *(Drosophila)*	2001	(Wayne *et al.*, 2001)	NS
9	*STRC*	stereocilin	2001	(Verpy *et al.*, 2001)	NS
10	*TMPRSS3*	transmembrane protease, serine 3	2001	(Scott *et al.*, 2001)	NS
11	*WFS1*	Wolfram syndrome 1/wolframin	2001	(Bespalova *et al.*, 2001; Young *et al.*, 2001)	Wolfram and NS
12	*MYO3A*	myosin IIIA	2002	(Walsh *et al.*, 2002)	NS
13	*OTOA*	otoancorin	2002	(Zwaenepoel *et al.*, 2002)	NS
14	*TFCP2L3 / GRHL2*	grainyhead-like 2 *(Drosophila)*	2002	(Peters *et al.*, 2002)	NS
15	*ACTG1*	actin, gamma 1	2003	(van Wijk *et al.*, 2003; Zhu *et al.*, 2003)	NS
16	*MYO1A*	myosin 1A	2003	(Donaudy *et al.*, 2003)	NS
17	*MYH14*	non-muscle myosin, heavy chain 14	2004	(Donaudy *et al.*, 2004)	NS
18	*TRIOBP*	TRIO and F-actin binding protein	2006	(Riazuddin *et al.*, 2006; Shahin *et al.*, 2006)	NS

NS, only non syndromic hearing loss; (*) There is a *Tcof1*-knockout mouse model. Heterozygous mice exhibited severe craniofacial malformations, including malformations of external and internal ear, and died at birth (Dixon *et al.*, 2000; Dixon *et al.*, 2006). However, the effect *Tcof1* haploinsufficiency on the mice inner ears has not been reported.

prosensory progenitor cells differentiate to hair cells by default, but this differentiation decision is generally inhibited by Notch signaling (Yamamoto *et al.*, 2006). Notch activation laterally represses expression of the Math1/Atoh1 transcription factor, which is required, together with Sox2 (described below), to induce differentiation of prosensory progenitor cells to hair cells. Indeed, *Atoh1* knockout mice have no hair cells in their vestibules and cochleae (Bermingham *et al.*, 1999; Yamamoto *et al.*, 2006).

Many additional transcription factors are crucial for inner ear development. Some of them are expressed specifically in the inner ear hair cells (e.g. Pou4f3) and others are crucial for the development of other organs as well. Several transcription factors have been correlated with deafness both in humans and mice (*Eya1, Pou3f4, Pou4f3, Mitf, Pax3, Snai2/Slug, Sox2, Sox10, Six1*; see Supplementary Table S1). *Sox2* and *Pou4f3* will be described here as examples.

SOX2 mutations in humans are correlated mainly with bilateral anophthalmia (eye malformations) in heterozygotes (Fantes *et al.*, 2003). However, two *de novo SOX2* mutations have been correlated with SHL in heterozygotes. A nonsense mutation (Q155X) was suggested to be responsible for HI, in addition to anophthalmia, absence of all optic pathways and other neurological abnormalities (Hagstrom *et al.*, 2005); and a missense mutation (479delA) was suggested to be responsible for a syndrome combining congenital hypothalamo-pituitary disorder and HI (Kelberman *et al.*, 2006). In mouse embryos, *Sox2* is expressed mainly in the developing CNS and sensory placodes, where it plays critical roles in embryogenesis. At E9.5, *Sox2* is expressed not only in the neural tube but also in the otocyst, from which the inner ear neuroepithelium will be developed. In the developing cochlea, *Sox2* is normally expressed only in prosensory progenitor cells, as well as in differentiated hair cells and supporting cells in the developing organ of Corti (Wood and Episkopou, 1999; Kiernan *et al.*, 2005). Two mutated *Sox2* alleles, *Lcc* (light coat and circling) and *Ysb* (yellow submarine), were generated in mice by coincidental mutagenesis, using X-ray radiation or transgene insertion, respectively. Mice carrying the mutated alleles could be easily identified, due to a semi-dominant yellow coat color. The *Lcc* and *Ysb* alleles contained intact coding and nearby sequences of *Sox2*, but regulatory elements that affect *Sox2* expression were mutated (the inserted sequence used to raise the *Ysb* allele contained a regulatory sequence from the *Col2a1* gene). As a result, *Lcc* and *Ysb* homozygous E9.5 mouse embryos expressed normal *Sox2* in the neural tube but no (*Lcc*) or less (*Ysb*) *Sox2* in the otocyst. Thus, the mutations did not disturb brain development, inducing milder phenotypes compared to *SOX2* mutations that have been reported in humans. Homozygous mice exhibited a severe HI (*Ysb* mice) or complete deafness (*Lcc* mice), as well as circling behavior, due to malformation of the inner ear and its neuroepithelium. The vestibule was more severely affected. At birth, *Lcc* mice, which did not express *Sox2* in their inner ears, displayed more severely malformed inner ears and the neuroepithelium was completely absent, since both hair cells and supporting cells failed to differentiate. *Ysb* homozygotes, which expressed a low level of *Sox2* in the inner ear, exhibited almost no hair cells in their vestibule. In the basal region of the cochlea, *Ysb* homozygotes displayed abnormal patches of disorganized hair cells, with regions containing no hair cells between them (Figure 2, G-J). The apical region of their cochleae included disorganized hair cells with no clear delineation of IHC and OHC. The unique pattern of hair cells development in *Ysb* mice may result from the inserted *Col2a1* regulatory motif to the regulatory sequence of *Sox2*. *Lcc* homozygotes, that exhibited no *Sox2* expression in the inner ear, did not express *Atoh1* either, while *Ysb* homozygotes that expressed some *Sox2* also expressed *Atoh1*. Therefore, *Sox2* was suggested to act upstream to *Atoh1* (Dong *et al.*, 2002; Kiernan *et al.*, 2005).

POU-domain transcription factor genes are known as controlling terminal stages of central nervous system (CNS) development [reviewed in (Ryan and Rosenfeld, 1997)]. In mice, *Pou4f3* (also known as *Brn-3c* or *Brn3.1*) is expressed quite specifically in cochlear (Figure 2O) and vestibular hair cells. Its expression may be detected in inner ear hair cells from E12.5, after *Atoh1* expression and is gradually increased until birth (Xiang *et al.*, 1998; Hertzano *et al.*, 2004). A *POU4F3* mutation has been linked to autosomal dominant progressive NSHL in humans (Vahava *et al.*, 1998). *Pou4f3*-knockout mice (Erkman *et al.*, 1996; Xiang *et al.*, 1997), as well as dreidel (*ddl*) mice that do not express a functional Pou4f3 (Hertzano *et al.*, 2004), displayed a similar waltzer-like phenotype of profound deafness (Figure 3, A-B) and vestibular dysfunction, including head tossing, circling behavior and hyperactivity. *Pou4f3*-knockout mice exhibited a progressive loss of inner ear hair cells both in the vestibule and cochlea that led to a secondary degeneration of supporting cells, as well as degeneration of spiral and vestibular ganglion neurons. Pou4f3 is expressed in postmitotic prosensory progenitor cells that are committed to develop to hair cells, but not in the pre-commitment mitotic cells. Hair cells in the developing inner ears of *Pou4f3* knockout mice underwent initial differentiation, but failed to form mature stereocilia, some of the hair cells were mislocalized to the supporting cell layer and all or most of them were degenerated by apoptosis during late gestation and early postnatal days. Thus, Pou4f3 is crucial for normal terminal differentiation, migration and survival of the inner ear hair cells (Erkman *et al.*, 1996; Xiang *et al.*, 1997; Xiang *et al.*, 1998). The *Gfi1* and *Lhx3* transcription factor genes were suggested as targets of Pou4f3. Homozygous dreidel mice expressed a minimal level of *Gfi1* mRNA in both cochlear and vestibular hair cells (Figure 2P) and did not express Lhx3 in cochlear hair cells (Figure 2, S-V) (Hertzano *et al.*, 2007).

Deaf mouse mutants not correlated with human hereditary hearing loss

Mutant mouse models have not been developed yet for all the genes that had been linked with human HHL. From 61 cloned genes that have been associated with human HHL (Van Camp and Smith, 2006), 18 have no mouse model thus far (Table 2). As mentioned in the introduction, 75% of the genes that have been linked with inner ear malformations or dysfunction in mice (Jackson_Laboratory, 2007) have not been linked with HHL in humans yet (Van Camp and Smith, 2006). Products of some of these genes may interact with already known deafness-related networks. For example, the lysosomal membrane protein Scarb2/LIMP-2, which regulates membrane transport of some proteins, was found to be essential for localization of Kcnq1/Kcne1 potassium channels in the apical membrane of stria vascularis marginal cells and vestibular dark cells of adult mice. As a result, *Scarb2*-deficient mice displayed a progressive hearing loss due to degen-

eration of the stria vascularis (Knipper *et al.*, 2006).

Other genes that have been linked with HI in knockout mice represent product classes that have not been related yet to HHL in humans and yet are crucial for inner ear function. For example, creatine kinase (Ckb) may have a role in ATP transfer to stereociliar ATPases. *Ckb* knockout mice display HI and vestibular dysfunction. The cytosolic brain isoform of creatine kinase is the most abundant protein after β-actin in the avian utricle hair bundle, as discovered using mass spectrometry (Shin *et al.*, 2007).

Another recent example is a spontaneous mutation (*jbg*) that led to HI and vestibular dysfunction in the jitterbug mice and was mapped to the *Clic5* gene. Clic5 belongs to the chloride intracellular channels family. In the mouse inner ear, it was detected specifically in the basal region of stereocilia in both cochlear and vestibular hair cells. Jitterbug mice exhibited aberrant stereocilia and progressive hair cell degeneration, suggesting that Clic5 may have a role in assembly or maintenance of stereocilia. Clic5 appeared to associate with radixin in stereocilia bases and was suggested to participate in formation or stabilization of connections between the stereociliar plasma membrane and its actin core (Gagnon *et al.*, 2006).

The same mutation may induce different phenotypes in different inbred mouse strains that express different genetic modifiers. Such modifiers were also reported in humans [the first was the *DFNM1* locus (Riazuddin *et al.*, 2000)]. In mice, this phenomenon in known as the strain background effect. For example, *mdfw* and *Ahl* alleles of the *Cdh23* gene can induce age-related and noise-induced hearing loss in homozygotes in several mouse strains (with different onset times in different strains), but other strains are relatively resistance for these mutations (Zheng and Johnson, 2001; Noben-Trauth *et al.*, 2003). Mutated alleles of *Cdh23* may act as genetic modifiers in heterozygotes. Thus, the *Ahl* allele may modify hearing loss in *Mass1^{frings}* mutant mice (Johnson *et al.*, 2005). At least an additional seven loci may induce age-related hearing loss in mice. In digenic mouse mutants, who carry two mutated genes and display a different phenotype compared to mice homozygous for a mutation in one of these genes, one of the mutated genes may be considered as a genetic modifier [e.g. (Adato *et al.*, 1999; Johnson *et al.*, 2005; Zheng *et al.*, 2005)]. In order to identify such modifiers, some groups mated mice carrying deafness-related mutations with mice from different strains [e.g. (Asher *et al.*, 1996; Niu *et al.*, 2006)]. This subject was recently reviewed (Johnson *et al.*, 2006).

Summary

Efforts are now underway to create knock-outs and conditional mutants for every gene in the mouse genome [NIH knockout mouse project, (KOMP): http://www.nih.gov/science/models/mouse/knockout/; and European conditional mouse mutagenesis program (EUCOMM): http://www.eucomm.org/]. This endeavor will undoubtedly create many more mouse models for human HHL. As discussed above, there have been many cases where the mouse gene has led to the discovery of the human HI gene (and vice versa), emphasizing the complementarity of mouse and human studies in the auditory and vestibular systems. Complex hearing impairment, which includes both noise-induced hearing loss and age-related hearing loss (presbyacusis), as well as the identification of modifiers, will require additional mouse models. The identity of a human mutation is critical for human diagnostics and genetic counseling, and early identification and intervention is beneficial for hearing impaired patients (White, 2004; Hyde, 2005). The information acquired from mouse morphological and physiological studies, as exemplified from the various techniques in Figure 2, demonstrates that the study of mouse models for deafness will undoubtedly provide a key to understand auditory function and help develop critical elements for therapeutics (Atar and Avraham, 2005; Tang *et al.*, 2006a).

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