Molecular Etiology of Deafness and Cochlear Consequences

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1 Introduction

Hearing loss (HL) is caused by environmental and/or genetic factors, including exposure to ototoxic drugs, rubella during pregnancy, trauma, excessive noise, and/or mutations in one of the approximately 20,000 genes that define the human genome. Genetic factors are now regarded as the main cause of HL, as many of the environmental causes have been recognized by modern medicine and eliminated by regulations and lifestyle. Remaining environmentally caused HL most likely have a genetic component as well, as the genetic background of the individual might influence susceptibility to, onset, or severity of acquired hearing impairment. HL is classified according to cause (genetic or non-genetic), association with other symptoms (syndromic or nonsyndromic), onset (before or after language acquisition—prelingual or postlingual, respectively), type (sensorineural, conductive, or mixed), severity (mild, 21–40 dB; moderate, 41–70 dB; severe, 71–90 dB, and profound, >90 dB) and frequencies (low, <500 Hz; middle, 500–2000 Hz, and high, >2000 Hz) (Petit, 2006). Approximately 30% of genetic HL is in the form of syndromic HL (SHL), wherein HL is only one of several symptoms. Approximately 70% of all genetic HL is nonsyndromic (NSHL), wherein HL is the only symptom

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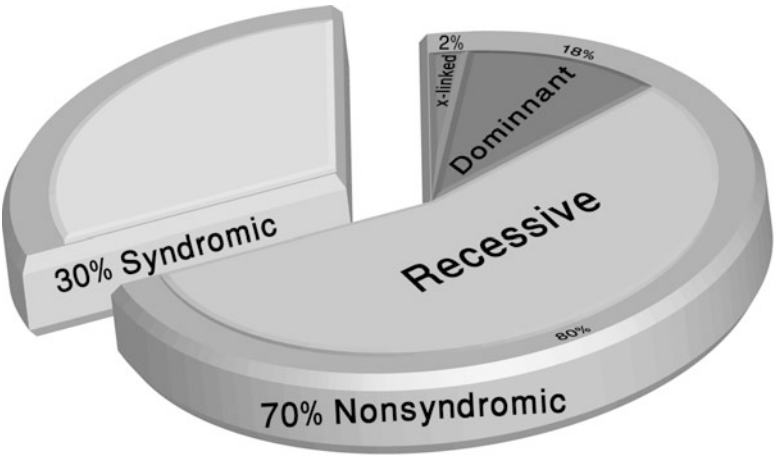

Fig. 1 Heterogeneity of hereditary HL. A pie diagram demonstrates the distribution of hereditary HL. (Source: S. Shivatski, Tel Aviv University, Israel)

observed. Half of prelingual NSHL is considered to be monogenic, wherein HL is due to mutations in one gene. NSHL is inherited in a recessive mode in approximately 80% of cases, in a dominant mode in approximately 20%, and is either X-linked or mitochondrial in 2%–3% of this group (Fig. 1). Further, it is estimated that approximately 36% of people older than the age of 75 suffer from presbycusis, a high-tone HL that appears and progresses in advanced age. The presence or absence of presbycusis, as well as the age at onset and rate of progression, is thought to have a genetic etiology (Nadol & Merchant, 2001).

More than 1000 deafness-causing mutations have been identified in 63 NSHL genes, and approximately 100 additional loci have been mapped (Hereditary Hearing Loss Homepage). To differentiate between the deafness loci, autosomal dominant loci are named DFNA, autosomal recessive loci DFNB, X-linked loci DFN and modifier loci DFNM; the number following indicates the chronological order in which they were mapped.

The first locus for NSHL, DFNA1, was mapped in 1992 (Leon et al., 1992) and the first mutation in the first gene was identified in 1997 (Kelsell et al., 1997). This gene, *GJB2* encoding connexin 26, has turned out to be the most prevalent deafness gene worldwide. In subsequent years, there has been remarkable progress in the number of deafness genes identified, and each new gene detected has added another layer to the understanding of the molecular basis of hereditary HL.

The genes involved in human hereditary NSHL encode many proteins, such as gap junctions (GJB2, GJB6), transcription factors (POU4F3, POU3F4, TFCP2L3, PAX3), ion channels (KCNQ1, KCNE1, KCNQ4), molecular motors (MYO6, MYO7A, SLC26A4, Prestin), extracellular proteins (TECTA, OTOA, COLL11A2), and structural proteins (OTOF, DIAPH1). Their expression pattern varies from proteins

that are exclusively expressed in the mammalian inner ear (TECTA, COCH, EYA4) to proteins that are expressed in many tissues (POU4F3, WHRN), but surprisingly have been found to be involved only in HL (Hereditary Hearing Loss Homepage).

The most frequent deafness causative gene *GJB2* is followed by other prevalent genes including *SLC26A4*, *MYO15A*, *OTOF*, *CDH23*, and *TMC1*. At least 20 mutations have been reported to be involved in HL for each of these genes. The number of mutations in the other genes is lower, and most of them have been reported in consanguineous families (Hilgert et al., 2009). These numbers are underestimated as a result of several biases. One bias originates from the gene size, as large genes are rarely completely analyzed. A second bias is caused by the methods used for diagnosis, which frequently do not include sequencing but rather mutation-specific assays, leading to underestimation of the numbers of mutations in frequently mutated genes such as *GJB2* and *SLC26A4*. A third bias is caused because of the rarity of many genes; although they may have been found in a particular population, the cost–benefit to examine them is low, and hence they are not examined further in the population. In addition, although families with HL are found all over the world, the majority of families reported with recessive deafness come from the "'consanguinity belt,"' including all the countries in North Africa, through the Middle East, to India. These consanguineous families were easily mapped by linkage analysis and the powerful technique of homozygosity mapping, allowing for locus identification on the basis of a single family. Dominant HL, in contrast, was identified mainly in families originating in Europe, North America, and Australia (Hilgert et al., 2009).

2 Complexity of the Auditory Apparatus

The myriad of proteins required for proper functioning of the inner ear correlates with the complex structure of its six organs: the cochlea, saccule, utricle, and the three semicircular canals. The ear itself is divided into three compartments: the outer, the middle, and the inner ear (Fig. 2). The inner ear includes both the organ of hearing (cochlea) and the vestibular sense organs that control balance and spatial orientation. The cochlea in the inner ear is a coiled snail-shaped organ in the temporal bone (Fig. 2a). It contains the cochlear duct that runs along the spiral shape from base to apex. This coiled duct is divided by two thin membranes into three different sections filled with fluids: the scala tympani and the scala vestibuli filled with perilymph, and between them, the scala media, filled with endolymph (Fig. 2b). The scala media contains the organ of Corti, which is the sensory epithelium of the auditory system (Raphael & Altschuler, 2003b). The organ of Corti, containing hair cells and supporting cells (Fig. 2c), lies on the basilar membrane that separates the scala media from scala tympani. When sound strikes the tympanic membrane, the movement transferred by the footplate of the stapes presses it into the cochlear duct through the oval window, causing the fluids to

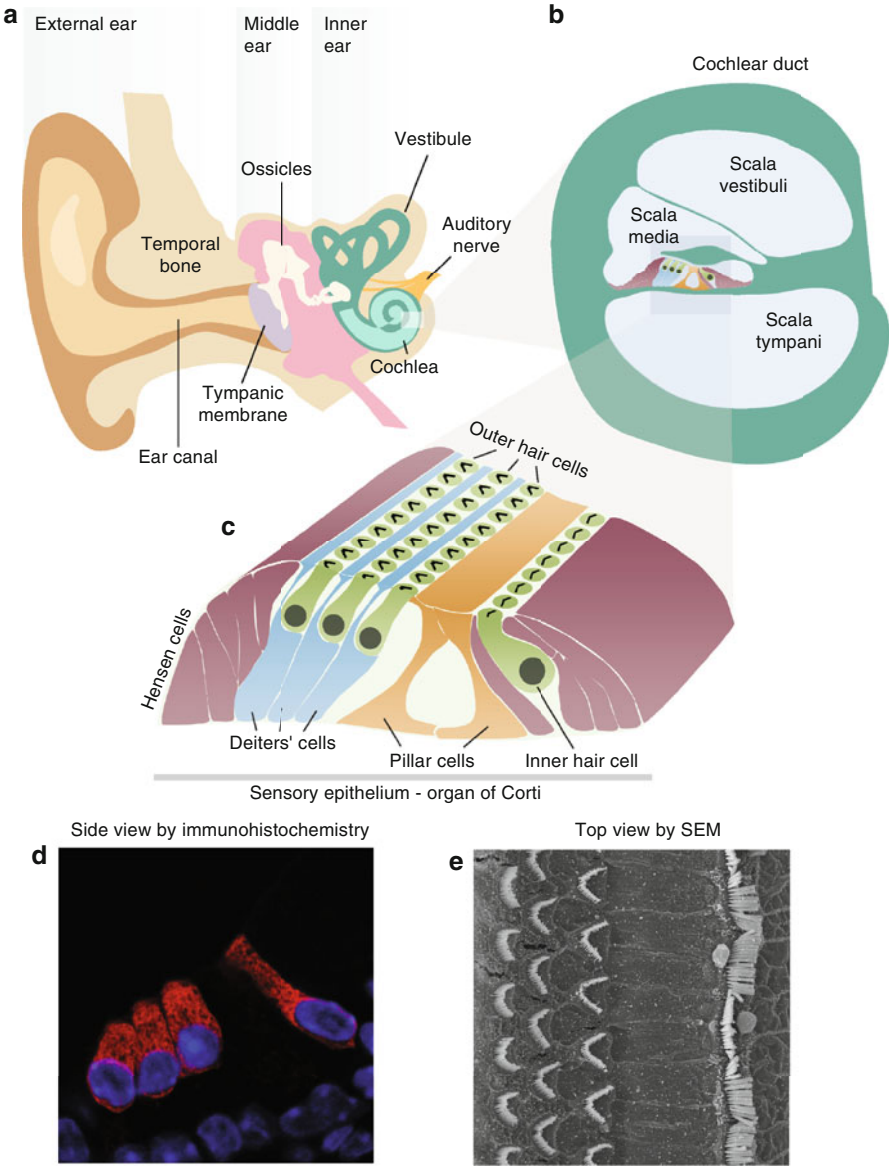

Fig. 2 Illustration of the human ear. (**a**) The ear is divided into the outer, middle, and inner ear. (**b**) A section through the cochlear duct demonstrating the fluid-filled compartments of the inner ear. (**c**) Enlargement of the organ of Corti showing the sensory hair cells surrounded by supporting cells, including Deiters', Hensen, and pillar cells. (**d**) Immunohistochemistry with myosin VI marks the cytoplasm of inner and OHCs, and 4′,6-diamidino-2-phenylindole (DAPI) marks the nuclei. (**e**) Scanning electron microscopy (SEM) image of the top view of the inner ear sensory epithelium, including one row of IHCs and three rows of OHCs separated by pillar cells. (Modified from Dror & Avraham, 2009a, with permission)

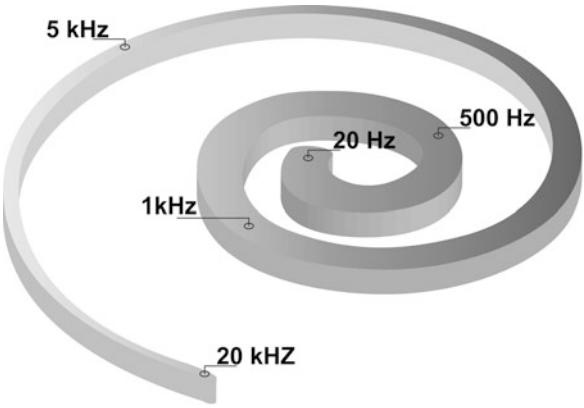

Fig. 3 Frequency distribution along the human cochlea basilar membrane shown by passive tonotopy. Some characteristic frequencies are indicated from base (20 kHz) to apex (20 Hz). Note the progressive enlargement of the basilar membrane. (Modified from http://www.cochlea.org/)

move through the cochlear duct, flowing against the receptor cells (hair cells) of the organ of Corti. The hair cells in the organ of Corti are composed of an inner row and three outer rows of hair cells (Fig. 2c–e). The vibrations caused by sound activate mechanoelectrical transduction, triggering the hair cells via deflection of the hair bundles and enabling potassium influx through the apical transduction channels that depolarize the cells. This sensory transduction is dependent on the ionic composition of the endolymph (reviewed in (Dror & Avraham, 2009a).

Ears are constantly exposed to a variety of sounds from the environment, composed of a wide spectrum of frequencies and different intensities. The tonotopic organization, meaning organization by frequency, in the basilar membrane allows the detection of the components of the incoming sound. The apical end of the basilar membrane vibrates most at low-frequency tones, and the basal end of the membrane vibrates most at high-frequency tones (Fig. 3). Based on these initial processes of acoustic features and temporal and spatial resolution, the brain can begin the complex task of assigning meaning to the sounds heard (reviewed in the chapters by Leake & Stakhovskaya; Kral & Shepherd).

The development, differentiation, and maintenance of this complex machinery explain the involvement of such a large number of genes in HL. HL can in principle result from pathological changes in different parts of the hearing apparatus. For example, morphological changes and degeneration of the stereocilia are associated with several forms of deafness due to impaired cytoskeleton and actin structure, and both tip links and lateral links may be affected, leading to defects in mechanotransduction. In the stria vascularis, pathological changes include defects that may affect secretion of potassium into the endolymph and maintenance of the endocochlear potential. Defects in the auditory ribbon synapse may lead to impairment of synaptic vesicle exocytosis, leading to deafness. In each of these cases, mutations in genes encoding essential proteins in these portions of the inner ear may have a critical impact on hearing.

3 Old and New Technologies to Identify Genes and Mutations

The molecular genetics revolution marked by Sanger sequencing, first described in 1977 (Sanger et al., 1977), and by the polymerase chain reaction (PCR), developed in 1983 (Bartlett & Stirling, 2003), made the detection of genes and mutations feasible. Other crucial landmarks were the Human Genome Project (HGP), completed in 2001, and the most recent development of the Massively Parallel Sequencing (MPS), also called Next-Generation Sequencing (NGS) and deep sequencing, that enhanced the ability to identify mutations in terms of both time and cost dramatically. Before the MPS era, Sanger sequencing yielded a 24-hour output of 120,000 base pairs (bp) for the cost of $4000 per megabase (Mb) sequenced (Metzker, 2010). Thus, Sanger sequencing, using one sequencer, would take 73 years and cost $200,000 to sequence the 3.2 gigabase (Gb) in a single human genome. In contrast, the output of a single MPS machine is larger than 30 Gb in 24 hours and costs less than $2 per Mb, so that a human genome can be sequenced in one day for a far lower cost (Shearer et al., 2011). However, it is important to remember that MPS generates massive quantities of sequencing data, with an increased error rate when compared with Sanger sequencing (Glenn, 2011). This immense amount of data requires intensive bioinformatics analysis, as well as validations of mutations by Sanger sequencing of specific regions, which prolongs the process and has to be taken into account in order to achieve results.

More than 100 loci have been mapped and more than 60 genes have been identified that are involved in HL since Sanger sequencing was first developed, prior to the MPS era. This was done mainly by genome-wide linkage analysis using genetic markers such as microsatellites or SNPs. Microsatellites are DNA regions with a variable number of short tandem repeats flanked by a unique sequence (Weber & May, 1989). The tandem repeats are usually simple dinucleotides (CA_n) repeated several times, and the number of repeats varies from one person to another. This variation makes the microsatellites highly informative for mapping. Because the microsatellites are locus specific, highly polymorphic, and randomly distributed throughout the genome, a set of approximately 400 polymorphic DNA microsatellite markers, spaced across the genome at 10 centimorgan (cM; approximately 10 Mb, measured as genetic distance) intervals, have been used for linkage analysis. These markers are commercially available and amenable to automation. In many cases, this distance of 10 cM is too large and fails to map the disease locus. Therefore, to reduce the critical region, additional microsatellites need to be genotyped, adding time and expense. To overcome this problem, several single nucleotide polymorphism (SNP) genotyping arrays have been developed. A SNP is a nonpathogenic change of a single nucleotide in the DNA sequence (Sachidanandam et al., 2001). SNPs have a prevalence of more than 1% in the human population. The human genome contains about 10–30 million SNPs, with a SNP present on average every 100–300 bases. The disadvantage is that SNPs are biallelic and therefore less

informative as compared to microsatellites. But the very high density of SNPs in the human genome, and the SNP technologies that offer highly automated and rapid methods of genotyping, as compared with genotyping microsatellites by PCR, overcome the limitations due to the low heterozygosity of SNPs (Vignal et al., 2002). While microsatellites are considerably more informative, SNPs are far more numerous across the human genome and with the advent of array-based typing technologies, are more economical to use (Polasek et al., 2010).

For disease locus identification, genetic linkage data can be analyzed by various methods, such as parametric multipoint linkage analysis and, when relevant, homozygosity mapping. Homozygosity mapping is a powerful tool to detect disease loci for autosomal recessive disorders, particularly in consanguineous pedigrees (Lander & Botstein, 1987). Homozygosity mapping, performed on families with related parents, or at least with both parents of the same descent, is based on the assumption that homozygosity of genes inherited by the offspring is due to a common ancestor or a founder effect. A significant limitation to this approach for identifying mutations is that it is suitable only for families with recessive diseases. Another disadvantage is the need for at least two affected offspring, preferably with related parents. Nevertheless, the fact that consanguinity increases the likelihood of the presence of mutations in a homozygous state made homozygosity mapping an effective gene discovery approach for recessive diseases and a powerful tool in clinical genetics. This approach led to the identification of many deafness genes (Borck et al., 2011; Shahin et al., 2010), particularly in populations with social preference for endogamous or consanguineous marriage and large family size (Christianson & Modell, 2004).

Despite the impressive contribution of linkage analysis approaches for deafness gene discovery, many cases remained unsolved and the list of unresolved human loci linked with HL remain longer than the list of cloned genes (Hereditary Hearing Loss Homepage). This can be partly explained by the limitations of the linkage methods that require large families for analysis and the lengthy time and cost required for gene identification. As a result, usually only one gene has been identified at a time and in many of these cases, mutations have been found in only one family, while in many other cases the causative gene has remained unknown. To overcome this obstacle, efforts for large-scale screening of deafness genes have emerged, for example, by genotyping 198 mutations with a primer extension array (Rodriguez-Paris et al., 2010). This Hereditary Hearing Loss Arrayed Primer Extension (APEX, Asper Biotech) microarray included 198 mutations across eight genes (*GJB2*, *GJB6*, *GJB3*, *GJA1*, *SLC26A4*, *SLC26A5*, *MTRNR1*, and *MTTS1*) in a single test. This microarray no doubt added diagnostic value beyond the customary testing of the single common *GJB2* gene, but still was not comprehensive enough, as it had only a limited number of mutations included for each gene and a limited number of genes. Further, even if such microarrays were to be expanded to include a larger number of mutations and genes, it would be limited to detection of known mutations only. Moreover, complex mutations such as duplications or deletions of entire genes cannot be

assessed by this method. These chromosomal imbalances can be identified by array comparative genomic hybridization (array CGH), a clinical diagnostic tool used to detect aneuploidies, microdeletion/microduplication syndromes, unbalanced chromosomal rearrangements, and copy number variants (CNVs) (Shinawi & Cheung, 2008). For example, an inverted genomic duplication of the *TJP2* gene was identified as the cause of progressive NSHL in DFNA51 individuals by means of this method (Walsh et al., 2010a). Array CGH was used after failure to detect mutations by other methods. However, a systematic study of unsolved deafness cases has not been undertaken using array CGH, so it is not known what proportion of deafness is due to large duplications or deletions. Clearly, there is a need to develop a technique for large-scale screening of a larger number of genes in a reasonable amount of time and more cost-effective manner, which can detect all types of mutations.

The latest technology, targeted genomic capture and MPS, was used recently for identifying deafness genes (Shearer et al., 2010) and appears to be the ideal tool to address these challenges: it enables the detection of all types of mutations underlying a heterogeneous disease such as HL; it allows for screening of large genes that have heretofore been largely untested; it can include all known deafness genes in a single test; and it can be used in cases of isolated deafness. The *DFNB79* gene, encoding taperin, was identified using a combination of targeted capture and MPS technology (Rehman et al., 2010). Multiple mutations responsible for HL were identified using targeted genomic capture and MPS of 246 genes responsible for either human or mouse deafness (Brownstein et al., 2011). In this study, screening multiple families for alleles first identified by MPS in five probands led to the identification of causative alleles for deafness in a total of 25 families. This approach exploits the high-throughput nature of targeted MPS to make a single fully comprehensive test for all known deafness genes.

On a larger scale, whole exome sequencing (WES) is even more promising, as it screens the exons of all genes in the human genome, allowing for the discovery of completely novel genes. It is estimated that approximately 60% of genes for Mendelian disease may be discovered using this technology(Gilissen et al., 2012). However, the data analysis is quite tedious, and strategies are being devised to ease this analysis. For example, homozygosity mapping has been used in parallel to exome sequencing. Although sequencing is done on the entire exome, only the linked region found by mapping needs to be analyzed for the mutation, making the bioinformatics analysis much easier. This strategy led to the identification of a *GPSM2* mutation as the cause of DFNB82 (Walsh et al., 2010b). Overall, for clinical and genetic diagnosis of HL, the deep sequencing strategy will undoubtedly enable further prediction of phenotypes and enhance rehabilitation by leading to the discovery of new deafness genes and mutations. Characterization of the proteins encoded by these genes will shed light on the biological mechanisms involved in the pathophysiology of hearing loss, which is the basis for genetic-based therapeutics.

4 Genes, Mutations, and Consequences on the Inner Ear

The discovery of genes involved in hearing and the detection of deafness-causing mutations have paved the way to deciphering the molecular mechanisms underlying the development and function of the auditory system. Distinctive studies based on both experimental research and bioinformatics tools have integrated groups of proteins encoded by these genes into networks and pathways in the ear, explaining similar phenotypes of different genes of the same network. One such example is the Usher network of proteins, with mutations in nine different genes underlying this most common syndrome of deafness and blindness, including *MYO7A*, *USH1C* (harmonin), *CDH23*, *PCDH15*, *USH1G* (sans), found in Usher syndrome type 1 (USH1); and *USH2A*, *GPR98* (VLGR1), *DFNB31* (WHRN), and *CLRN1* genes involved in USH2-3 (Mahboubi et al., 2012; Saihan et al., 2009). Many more genes are predicted to be involved using the HEarSpike bioinformatics tool, based on interactions between genes in other systems (Paz et al., 2011).

Usher syndrome is an autosomal-recessive disorder involving HL and blindness due to retinitis pigmentosa (RP). The most severe form is USH1 with severe to profound congenital hearing impairment, onset of RP in first decade of life, and vestibular symptoms. In spite of this, mutations in four out of the five USH1 genes may cause only NSHL. The USH1 proteins are considered key components of the mechanoelectrical transduction machinery. The phenotype of USH1 patients caused by mutations in different USH1 genes is similar, which suggests that the proteins encoded by these genes may all be involved in the same cellular functions (Petit, 2001). Mutations in several of these genes lead to abnormal phenotypes in the mouse cochlea, which consist of fragmented and misoriented hair bundles (Fig. 4a) (El-Amraoui & Petit, 2010). The USH1 proteins are expressed in the hair bundle from early development, and by postnatal day 1 (P1) in the mouse, they colocalize to the tip of the hair bundle. Moreover, direct interactions between the USH1 proteins have been seen in vitro; myosin VIIa and sans are required for the targeting of harmonin-b onto the stereocilia, where it binds to F-actin and anchors the links made of cadherin-23 and protocadherin-15 (Fig. 4b) to the actin core of the stereocilium (reviewed in (Richardson et al., 2011). The colocalization in the hair bundle and direct in vitro interactions of these proteins underlies the conclusion that a similar mechanism causes deafness in all forms of USH1 cases. As all five USH1 proteins interact together to achieve the same function of mechanoelectrical transduction (Fig. 4c), it explains why a mutation in any of these proteins results in the same phenotype (El-Amraoui & Petit, 2010; Lefevre et al., 2008).

The fact that mutations in many of the USH genes underlies NSHL as well as Usher syndrome indicates that variants of the same gene may result in clinical heterogeneity. There are many more examples for this phenomenon; one of them is the *SLC26A4* gene, the second most frequent cause of NSHL worldwide (Hilgert et al., 2009). Mutations in the *SLC26A4* gene are linked with either NSHL, DFNB4, with or without enlarged vestibular aqueduct (EVA) (Fig. 5a); Mondini; or a syndromic form known as Pendred's syndrome (PS) with enlargement of the

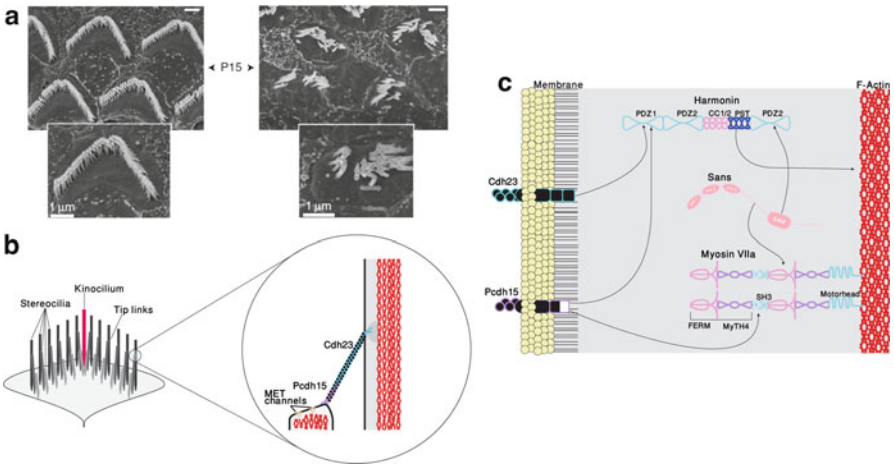

Fig. 4 The USH1 molecular network. (**a**) An example of one mutant mouse in the USH1 network, deficient in protocadherin 15. SEM of OHC bundles in wild-type and mutant mice at postnatal day 15 (P15). The hair bundles of the mutant mice are disorganized. (From El-Amraoui & Petit, 2010. Used with permission of Michel Leibovici [El-Amraoui & Petit, 2010].) (**b**) Hair cell bundle showing the staircase structure of the sterocilia and position of the kinocilium. The tip link structure, composed of cadherin 23 and protocadherin 15, is enlarged. Protocadherin 15 in the lower stereocilia is presumed to be associated with the MET (mechanoelectrical transduction channel) (El-Amraoui & Petit, 2010). (Source: S. Shivatzki, Tel Aviv University, Israel.) (**c**) A schematic summary of the interactions between USH1 proteins (El-Amraoui & Petit, 2010). (Source: S. Shivatzki, Tel Aviv University, Israel)

thyroid gland (Pera et al., 2008). This clinical heterogeneity is usually explained by the severity and type of mutations, whereas nonsense and frameshift mutations, particularly in the beginning of the gene, tend to be involved in more severe forms of the disease, in this case, in SHL, while missense and nonsense or frameshift mutations toward the end of the gene usually cause less damage and are involved in NSHL (McHugh & Friedman, 2006). However, this is not always the case, as the same combination of mutations in the *SLC26A4* gene have been described that result in variable phenotypic expression. These phenotypes range from isolated NSHL to non-syndromic EVA to Mondini dysplasia to PS, suggesting that the same etiology underlies all conditions (Suzuki et al., 2007; Tsukamoto et al., 2003). Further, phenotypes are variable, even with the same mutations. Several mutations, including D28R, L236P, T416P, L445W, L676Q, and more are involved in either PS or NSHL (Dossena et al., 2011) (Fig. 5b). Moreover, even intrafamilial phenotypic variability was observed, for example, the L445W mutation was identified in all affected individuals of a large family, either with PS or with NSHL(Masmoudi et al., 2000). The lack of genotype–phenotype correlation suggests that NSHL/EVA/PS is a disease involving other genetic factors including digenic inheritance, modifier genes, or epigenetic changes. This assumption led to the detection of digenic heterozygosity of *SLC26A4*/*FOXI1* and *SLC26A4*/*KCNJ10* mutations (Yang et al., 2009; Yang et al., 2007). FOXI1, a transcriptional regulating

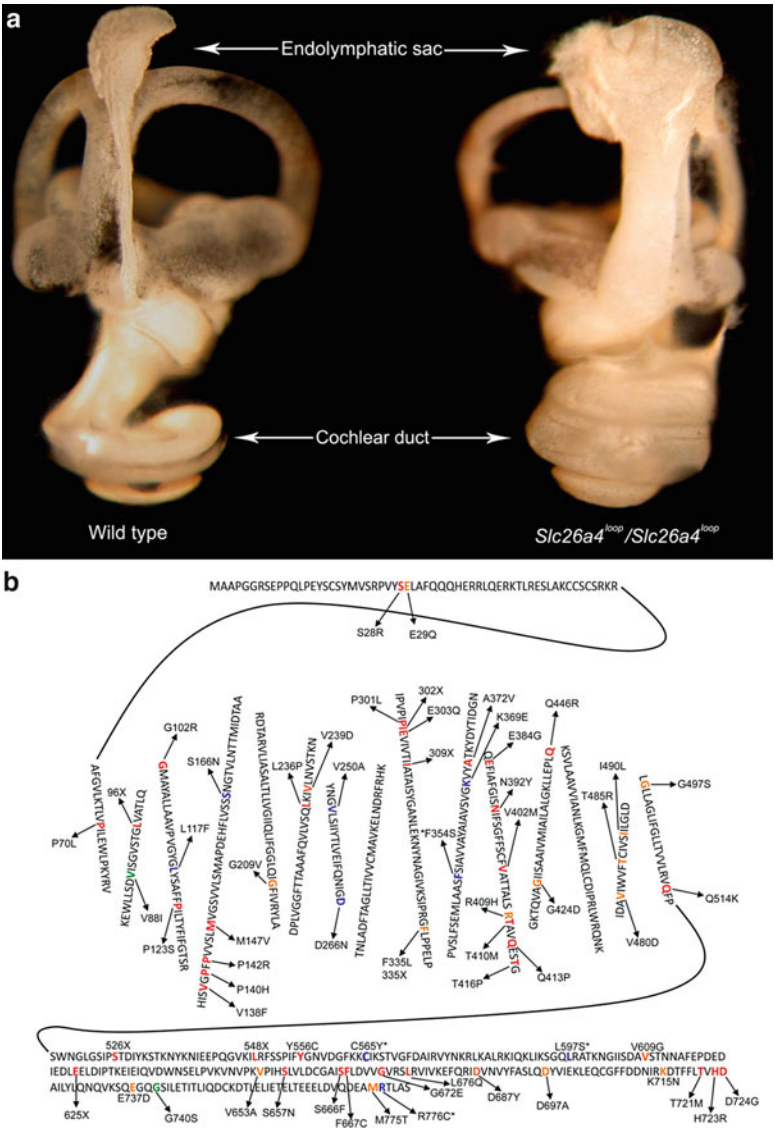

Fig. 5 *SLC26A4* mutations, usually causing EVA, with NSHL or as part of PS. (**a**) Paint-fill technique demonstrating the enlarged vestibular aqueduct (EVA) and the enlargement of the endolymphatic sac and duct in a $Slc26a4^{loop}/Slc26a4^{loop}$ mouse (right), compared to a wild-type mouse. (Source: A. Dror, Tel Aviv University, Israel.) (**b**) *SLC26A4* mutations. Red = loss of function mutations; orange = reduction of function; blue = polymorphisms; green = gain of function. Asterisks indicate variants with ambiguous function. Circled mutations are involved in both NS EVA and PS. Note that the variable-phenotypic mutations are located throughout the gene. (From Dossena et al., 2009. © Society for Endocrinology. Reproduced with permission)

factor of SLC26A4 and KCNJ10, have also been implicated in the development of inner ear pathology. Interestingly, Kcnj10 expression is down-regulated in *Slc26a4*-depleted mice, proving they both share the same pathway (Wangemann et al., 2004). Mutations in both *FOXI1* and *KCNJ10* were observed in PS and nonsyndromic EVA patients, as well as in non-syndromic EVA patients in a double heterozygous state with *SLC26A4*. Thus, *FOXI1* and *KCNJ10* are two genes that may contribute to the understanding of the phenotypic heterogeneity. This is one example showing that many genes and different factors are most likely to be identified, including modifier genes, which may determine the phenotype and the differences between and within SHL and NSHL.

Another, more frequent digenic (double) heterozygosity condition is known in several populations between the most prevalent *GJB2* gene and the *GJB6* gene (del Castillo et al., 2005). Both *GJB2* and *GJB6* genes map to the same chromosomal region, 13q11–q12, contain only one coding exon, and share 76% identity (Grifa et al., 1999). *GJB2* and *GJB6* encode the gap junction proteins, connexin 26 (Cx26) and connexin 30 (Cx30), respectively. Both belong to a family of more than 20 members that share a common structure of four transmembrane segments. Most cell types express more than one connexin species, which may form homomeric or heteromeric connexons. In the auditory system, intercellular channels are formed predominantly by Cx26 but also by Cx30, Cx31, and Cx43. Cx30 colocalizes with Cx26 in the same inner ear structures: in the supporting cells of the organ of Corti, in the stria vascularis, and in the spiral ligament (Forge et al., 2003). Connexons composed of Cx26 can bind connexons composed of Cx30 to form heterotypic gap junction channels (Dahl et al., 1996). Cx26 is involved in maintaining a high-extracellular K concentration in the endolymph by facilitating the circulation of K^+ ions (Beltramello et al., 2005).

More than 200 mutations in *GJB2* are responsible for up to 50% of severe to profound prelingual recessive deafness in several worldwide populations (Denoyelle et al., 1999). These mutations manifest clinical heterogeneity as they include mostly recessive mutations for congenital severe to profound NSHL, but some cause mild to-moderate or progressive NSHL (Chan et al., 2010). Moreover, dominant mutations for NSHL and for SHL, including skin disease and deafness, are encountered as well (Connexin-Deafness Homepage). Three deletions were reported in the *GJB6* gene (Mahdieh et al., 2010). The most common deletion of 342 kb, del(*GJB6*-D13S1830), was found to accompany a *GJB2* mutant allele in trans in up to 50% of heterozygote deaf *GJB2* cases in different world populations. del(*GJB6*-D13S1830 has been found less frequently in homozygosity (del Castillo et al., 2003). Double heterozygotes for *GJB2* and *GJB6* mutations manifest the same phenotypes of congenital profound NSHL as homozygotes for *GJB2* or *GJB6*. As the deletion does not directly affect the coding region of *GJB2*, but truncates the adjacent *GJB6* gene, it is not clear if the HL is a result of a digenic mode of inheritance or abolishes control elements that are important for the expression of *GJB2*. Findings of a study on skin disease support the latter hypothesis, suggesting that this deletion eradicates *GJB2* expression, probably by deleting a regulatory element (Common et al., 2005).

Another gene, *OTOF*, is involved in HL and auditory neuropathy (AN). AN is also named AN spectrum disorder (ANSD), as it affects the temporal coding of acoustic signals in the auditory nerve, causing poor auditory perception. In patients with AN, otoacoustic emissions (OAEs) are normal or partly normal, reflecting the preserved function of the outer hair cells (OHCs), but the auditory brainstem responses (ABRs) are abnormal or absent, indicating that the disorder originates from lesions in the inner hair cells (IHCs), in the intervening synapse (presynaptic AN), or in the auditory nerve (postsynaptic AN) (Starr et al., 2000), all resulting in disruption of auditory nerve activity. With the progression of HL, OHC function is lost as well, as is the OAE response. AN patients show impairment of speech perception beyond what is expected from the severity of HL. AN can be an isolated disorder or part of a syndrome including peripheral and optic neuropathies. Mutations in several nuclear and mitochondrial genes underlie AN. A partial list includes *DIAPH3*, *OTOF*, and *PJVK* for nonsyndromic AN and *OPA1* for syndromic AN (Santarelli, 2010). Otoferlin, encoded by *OTOF*, is crucial for vesicle release at the synapse between IHCs and auditory nerve fibers by interacting with syntaxin1 and SNAP25 (Roux et al., 2006), and for replenishing synaptic vesicles (Pangrsic et al., 2010). *OTOF* mutations are the major cause of AN, leading to prelingual, profound NSHL, accompanied by AN in about half of cases with biallelic *OTOF* mutations (Rodriguez-Ballesteros et al., 2008). The other AN genes lead to the same phenotype, although the outcomes of cochlear implants in these patients point to differences in the location of the lesion between the genes. The cochlear implant outcome in AN children is not as good as in children with SNHL, but it still might be the best option, as they benefit even less from hearing aids. For these AN patients, the results of cochlear implant probably depend on the location of the damage in the auditory pathway. Cochlear implants aim to improve the synchronicity of the neural activity by providing supraphysiologic electrical stimulation to the auditory nerve. Therefore, presynaptic AN patients may benefit from cochlear implants, whereas patients with postsynaptic AN may not (Gibson & Graham, 2008). Thus, patients with *OTOF* mutations show good cochlear implant outcome, in contrast to patients with mutations in other AN genes that benefit very little from cochlear implants (Rouillon et al., 2006; Wang et al., 2011), suggesting that different genes work in functionally separate cells. This has a crucial impact for rehabilitation, as molecular screening and the identification of specific gene mutations may help to localize the lesion and predict cochlear implant results. For many AN patients, cochlear implantation, bypassing the site of the lesion, may be the only way for restoration of speech perception. Cochlear implantation is predicted to be successful in patients with mutations underlying presynaptic (*OTOF*) and postsynaptic (*OPA1* and *DIAPH3*) AN, with less benefit for AN patients that involves the entire auditory nerve (Santarelli, 2010) (presynaptic and postsynaptic mechanisms are reviewed in the chapter by Sanes).

The genes described in the preceding text are only a minor part of the complete list of genes identified until now, but they represent the crucial significance that each and every one of them has in the complex auditory system and the consequences of mutations that interrupt their normal function. The genes and

proteins they encode are extremely diverse in terms of size, structure, expression, and function. Nevertheless, mutations in different genes may lead to the same phenotype, which may be explained by a shared pathway or network. In contrast, mutations in the same gene, or even identical mutations, may cause different phenotypes, a phenomenon suggested to be influenced by the involvement of other factors such as modifier genes or epigenetics. These factors highlight the importance of the identification of genes, early detection by molecular screening and protein characterization for clinical rehabilitation and comprehensive understanding of the auditory system.

5 Mouse Models for Human Deafness

The use of the advanced MPS technique in research and in the clinic is expected to identify most of the genes in the approximately 100 unresolved deafness loci and to add many more genes to the list of human genes for HL in a short period. However, understanding the mechanisms leading to deafness will still remain an open question, and one best answered using animal models. Zebrafish, the chick, and the mouse have provided a significant understanding of the functions of the ear. The mouse has turned out to be optimal to study the genetics of deafness for several reasons. The mouse genome sequence, completed in 2002, was found to have 80% homology with the human genome and 99% of mouse genes have orthologues in humans with large syntenic regions (Waterston et al., 2002). Not surprisingly, the remarkable similarity between the mouse and human genomes is reflected in the similar structure and function in many systems of the two species, including the ear. Further, the similar functions of the orthologous genes in human and mice result in a similar anatomy, physiology, and metabolism, as well as in many similar genetic disease pathologies. Other advantages of the mouse as a model include the circling or head bobbing phenotype that is usually characteristic of deaf mice and enables easy detection of deaf mice; the gestation time, allowing for breeding of large colonies in a short time; and the size of mice, making them easy to handle and cost effective (Vrijens et al., 2008). Another great advantage of mouse models is the availability of molecular techniques that have been adapted for mice research and for the study of the ear in particular. Several techniques have been developed and optimized to construct transgenic and knock-out/knock-in mice (Capecchi, 2005), allowing one to mimic human mutations in the mouse genome. This includes the ability to delete or duplicate genomic regions, to knock-out or knock-in single genes, and to make single nucleotide substitutions (targeted mutagenesis), providing optimal models for HL research. To date, the homologous recombination technology for creating knock-out/knock-in mice is applied on a routine basis in the mouse only (Vrijens et al., 2008).

In addition, extensive studies were performed beginning with the mouse, and then moving toward human deafness. Spontaneous mutations, as well as chemically induced mutants generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, have

facilitated the discovery of new deafness genes in mice, followed by the discovery of their human orthologues (Brown et al., 2009). For example, identification of a recessive mutation in the *Loxhd1* gene in the samba deaf ENU mice led to the discovery of its human orthologue *LOXHD1* within the previously mapped DFNB77 locus responsible for autosomal recessive NSHL (Grillet et al., 2009). This phenotype-driven approach has not only enriched the list of known deafness genes, but has also enabled scientists to study further the pathophysiology underlying different mutations. Once a phenotype is created, imaging techniques such as scanning electron microscopy (SEM) and computed tomography (CT) scanning, as well as immunohistochemical methods, are used to study the mouse inner ear and characterize the specific gene/protein. For all these reasons, the main advancement in deciphering the role of the genes in the hearing system was achieved by using mouse models.

Gene discovery in humans led to the construction of a long list of mouse models for HL, covered in several reviews (Friedman et al., 2007; Leibovici et al., 2008; Vrijens et al., 2008). For example, included in the list are two mouse models with targeted mutations in *Tecta* (α-tectorin). One mutation, a targeted deletion, $Tecta^{\Delta ENT}$, leads to a defective tectorial membrane completely detached from the organ of Corti and spiral limbus, resulting in HL (Legan et al., 2000). The second mouse model had a missense mutation in *Tecta* (Legan et al., 2005), identical to the Y1870C mutation involved in human HL (Verhoeven et al., 1998). Homozygous $Tecta^{Y1870C/Y1870C}$ mice presented the same phenotype as the $Tecta^{\Delta ENT/\Delta ENT}$ mice. The power of this phenotype-driven approach is demonstrated by the conclusions that could be drawn from the analysis of these mouse models. These models helped define the role of the tectorial membrane in hearing, including the enabling of the OHCs to act as amplifiers, synchronized with the basilar membrane, and guaranteeing that the IHCs are maximally responding at their characteristic frequency by the basilar-membrane vibrations, and thus allowing the frequency tuning and temporal resolution of the neural output of the cochlea (reviewed in the chapter by Kral and Shepherd and in (Petit, 2006) Another example is the *Gjb2*/*Cx26* mouse model. The first two different approaches used to knock out the *Gjb2* gene in mice, targeted mutagenesis (Gabriel et al., 1998) and ENU-induced mutagenesis (Coghill et al., 2002), failed to produce deaf embryos because homozygous embryos died in utero due to placental defects. Two other strategies succeeded to generate viable, hearing impaired, mutant *Gjb2* mice. By one technique, the conditional *cre-loxP* system (Fig. 6), *Gjb2* was locally knocked out in the cochlear epithelium (supporting and flanking epithelial cells), generating mice homozygous for *Gjb2-loxP* that carry *Cre* following an *Otog* promoter, which is expressed only in cochlear epithelial cells (Cohen-Salmon et al., 2002). In a second approach, targeted point mutagenesis was used to mimic the Cx26 R75W mutation (Kudo et al., 2003) involved in autosomal dominant SHL (HL and skin disease) in humans. The heterozygous R75W dominant-negative mutation inhibits the function of the wild type (WT) protein encoded by the *WT* allele (Richard et al., 1998). Both *Gjb2* knockout homozygotes and Cx26R75W heterozygotes exhibited similar HL in adults and similar histological phenotypes. In both models, inner ear development

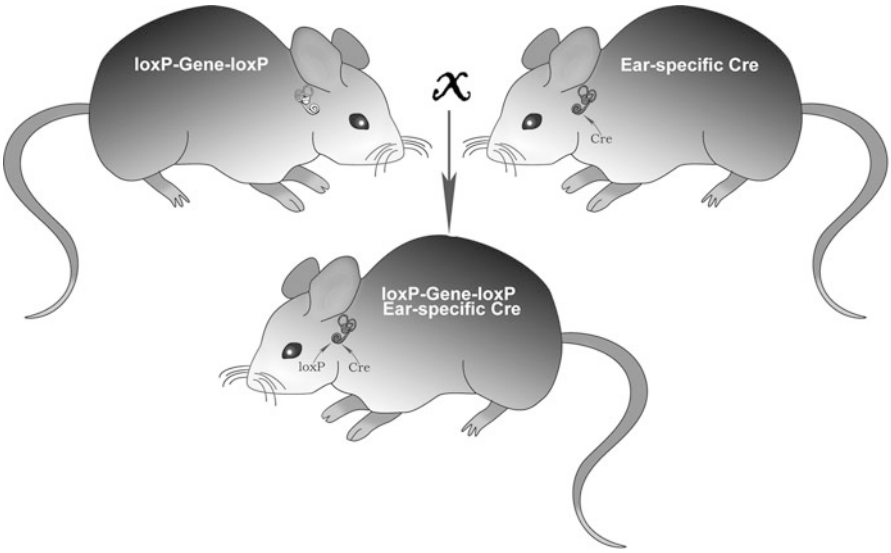

Fig. 6 The conditional *cre-loxP* technique is used to create mouse models for deafness, a homologous recombination technology optimized for creating mice with loss of tissue-specific expression. This has been the method of choice for the connexin 26/*Gjb2* mouse model. (Source: S. Shivatzki, Tel Aviv University, Israel)

was normal until postnatal day 14 (P14) but after the onset of hearing, at P15–P16, epithelial cells began to die due to apoptosis. Surprisingly, the Cx26R75W mice, even though heterozygous, manifested a more severe phenotype, beginning earlier with a relapse of the whole organ of Corti at P14, leading to a complete degeneration of both hair cells and supporting cells by 7 weeks of age. In *Gjb2* knockout mice, IHCs displayed immature synapses but usually survived, dying only in the more profoundly hearing impaired mice. These findings suggest that Cx26 has a role in survival and function, but not in development, of the organ of Corti. The two models showed differences in the maintenance of electric potential difference between the endolymphatic and perilymphatic ducts in the cochlea, measured by the endocochlear potential (EP). As expected, in *Gjb2* knockout homozygous mice, endolymphatic K^+ concentration and EP were much lower than in the Cx26 R75W heterozygotes, supporting the hypothesis that Cx26-based gap junctions are required for K^+ recycling in the cochlea. Moreover, EPs of Cx26R75W heterozygotes were normal, suggesting that impaired K^+ transport by supporting cells leads to apoptosis of organ of Corti cells rather than affecting endolymph homeostasis, as originally thought.

Given the apparent differences between human and mice, one might expect that distinct mutations would cause HL in human but not in mice, and vice versa, or that identical or similar mutations would lead to different degrees of HL in humans and in mice, resulting from the differences in development and physiological

characteristics between the two species. Language skills, for example, must have driven the evolution of the cochlea and the central auditory pathway to a specific direction, with many genes responsible for this difference between humans and mice (Petit, 2006). For this reason, it is also expected that a larger number of modifier genes and more complex gene regulation, perhaps at the level of epigenetics and microRNAs, are involved in humans as compared to mice, as mice do not manifest the diversity of humans.

6 Summary

A combination of advanced molecular biology techniques, including high-throughput sequencing that allows for rapid identification of many genes involved in HL, and the ability to explore the role of these genes in mouse models using homologous recombination technology for creating knock-out/knock-in mice, has led to remarkable progress in the understanding of the auditory machinery. In recent years, the function and expression patterns of many genes and proteins have been elucidated, increasing our understanding of the normal function of the auditory system, as well as its impaired state. Mutations in even more genes, close to 200 to date, are known to lead to HL in mice. Thus, there are many mouse models for HL with mutated genes that have not yet been correlated with human deafness. Furthermore, there are human deafness genes for which no mouse model is yet available, making it harder or almost impossible to complete the protein characterization, as no other tool can compete with mouse models in investigating the molecular and physiological processes taking place in the ear, in health and disease.

In spite of the rapid progress in recent years, deafness-causing genes have been identified for less than half of all mapped loci. Further, the genetics of complex forms of HL has yet to be deciphered. Continued efforts to detect and characterize all deafness genes are crucial to understand the physiology and pathophysiology of hearing, with direct implications for genetic diagnostics and rehabilitation. The ability to predict genotype–phenotype correlations might be crucial for integration of the deaf in a hearing society, as it enables professionals to provide the hearing impaired with the best treatment for a specific lesion. Such is the case for AN, for example, and to do so within the critical period of language development (reviewed in the chapter by Bavelier). Finally, high-throughput diagnostic screening and the elucidation of mechanisms in the auditory pathways are helping pave the way for future development of therapeutic approaches for HL.

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