Animal Knockout

A compendium of mouse knockouts with inner ear defects

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Genetically engineered strains of mice, modified by gene targeting (knockouts), are increasingly being employed as alternative effective research tools in elucidating the genetic basis of human deafness. An impressive array of auditory and vestibular mouse knockouts is already available as a valuable resource for studying the ontogenesis, morphogenesis and function of the mammalian inner ear. This article provides a current catalog of mouse knockouts with inner ear morphogenetic malformations and hearing or balance deficits resulting from ablation of genes that are regionally expressed in the inner ear and/or within surrounding tissues, such as the hindbrain, neural crest and mesenchyme.

In mammals, the inner ear originates from the otic placode, which is derived from a thickened area of the surface ectoderm adjacent to the hindbrain. During embryonic development, the otic placode invaginates to form a cup-like structure, which eventually pinches closed to form a spherical epithelial vesicle, the otocyst. Following a series of morphological changes, the otocyst generates several sensory structures: a cochlea, which senses auditory stimuli; a saccule and utricle (the otolith organs) and associated sensory regions (maculae), which sense gravity and linear acceleration; and three semicircular ducts, oriented perpendicular to one another, and associated sensory regions (cristae), which sense angular acceleration. Saccule, utricle and semicircular canals are collectively known as vestibule, and vestibule and cochlea jointly form the membranous labyrinth (Box 1). The sensory neuroepithelia of these organs consist of mechanoreceptive hair cells, supporting cells and nerve endings. The ontogenesis of inner ear sensory organs has been reviewed elsewhere [1–3]. A series of paint-filled membranous labyrinths highlighting the gross anatomical changes that occur in mouse inner ear development from 10.75 days postcoitum (dpc) to postnatal day 1 (P1) is shown in Fig. 1 [2].

Due to the remarkable similarity between the human and mouse auditory systems, analysis of vestibular and auditory mouse mutants has provided valuable insights in the ontogenesis, morphogenesis and function of the mammalian inner ear, and contributed to the identification of candidate deafness-causing genes in human. The utility of spontaneous deafness and circling mouse mutations, many of which have been attributed to vestibular lesions and are effectively null alleles, has already been validated and reviewed elsewhere [4–9]. In addition, *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis [10] has proven a well-suited phenotype-driven strategy for discovering genes involved in hearing or balance defects [11–13]. A large-scale ENU mutagenesis program at The Medical Research Council (Harwell, UK) is presently focusing on the recovery of novel mutations by employing efficient phenotype screens for deafness and balance defects [11,12]. At the same time, mouse knockout strains, modified by gene targeting, are being generated at an impressive rate and used, among other areas, as premiere research tools in deciphering the basis of human genetic deafness [6,14–20].

The aim of this article is to provide a comprehensive catalog of mouse knockouts with inner ear malformations and hearing deficits that result from the selective ablation of currently known endogenous inner ear patterning genes. As normal development of the inner ear depends on several surrounding tissues, such as the hindbrain, neural crest and mesenchyme, several recently identified gene products that have been implicated in hindbrain segmentation, tissue interactions, extracellular matrices, cell-cycle regulation, developmental apoptosis and metabolic processes, are now known to affect auditory and vestibular function, either directly or indirectly [6,14–20]. Here, I elaborate on phenotypes that emerge following targeted disruption of mouse genes that are regionally expressed in the inner ear and/or within surrounding tissues. Spontaneous mutations and chemically induced null alleles that compromise hearing and vestibular function are not included in this article, with the exception of a few spontaneous mutations cited in relevant cases.

Gene disruptions affecting mouse inner ear development and function

Given the reported overlap in the expression pattern and action of inner ear patterning genes, it is not surprising that single null mutations typically result in subtle phenotypes of reduced penetrance and/or variable expressivity, rather than in a drastic morphological effect. Here, I focus on inner ear defects resulting from single and/or multiple gene disruptions, with little or no reference to abnormalities observed in other parts of a particular knockout animal. Description of the phenotype is primarily based on the affected structural or cellular component and/or physiological process, although some degree of intersection with other affected organs is clearly inevitable. An alphabetical listing of these mouse genes, accompanied by TBASE accession numbers (see below), bibliographical references and related human disorders, is provided in Table 1. Investigators within the hearing-research community are also encouraged to consult and relay recent experimental data to electronic sites and databases that host information on classical and/or targeted mutant mouse models of genetic hearing impairment. These include: the Transgenic and Targeted Mutation Database (TBASE; http://tbase.jax.org/) [21], the Hereditary Hearing Impairment in Mice Site (HHIM; http://www.jax.org/research/hhim/), the Institute of Hearing Research Site (http://www.ihr.mrc.ac.uk/hereditary/MutantsTable.shtml), and The Hereditary Hearing Loss Homepage (http://dnalab-www.uia.ac.be/dnalab/hhh).

Inner ear development: morphogenetic patterning

Hindbrain segmentation and rhombomere identity

Recent loss-of-function studies have revealed new roles for genes that control hindbrain segmentation or rhombomere identity (particularly rhombomeres r5

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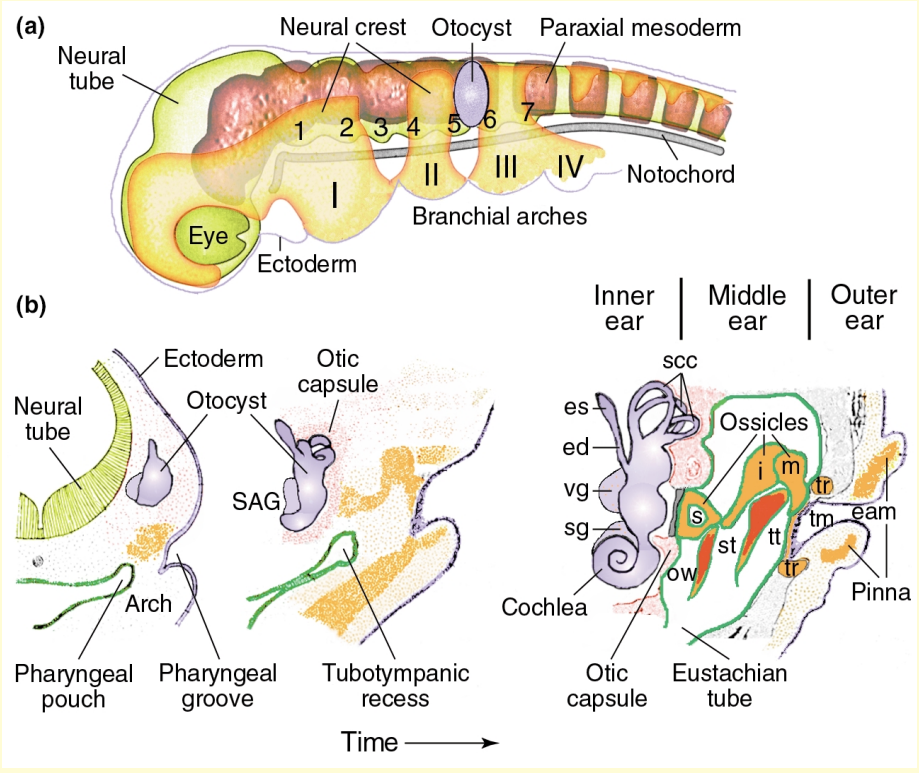
Box 1. Development of the vertebrate ear

The vertebrate ear develops from a complex convergence of tissues from all three germ layers as well as the neural crest. A major contri-bution to the outer ear is made from the first and second branchial arches, which include arch ectoderm and mesoderm. Figure Ia shows a lateral view of a developing vertebrate embryo and Fig. Ib shows sections through the ear and how it develops over time (left to right). The epithelium that overlies the arches is derived from head ectoderm (violet). The mesenchymal tissue of each branchial arch consists of a small core of cells (not shown) derived from paraxial mesoderm (red) surrounded by a larger population of cells derived from the neural crest (orange). The middle ear is formed from cells arising from either the neural crest or paraxial mesoderm that initially migrated to the branchial arches and then coalesced to form the middle-ear elements. It also contains a significant contribution from the endoderm (green), as it is lined by mucosal tissue that expands into the middle ear from the oral cavity (embryologically this is derived from the first pharyngeal pouch) and is innervated by cranial nerve IX in the same way as the oral mucosa. The epithelial tissues and neurons of the inner ear are formed almost exclusively of placodal ectoderm, which invaginates to form the otocyst. A relatively small contribution made to the inner ear by the neural crest gives rise to the melanocytes of the stria vascularis in the cochlea, the Schwann cells of the statoacoustic ganglion and other mesenchymal tissues (not shown). A cartilaginous capsule that eventually ossifies into bone surrounds the epithelial core of the inner ear. Most of the otic capsule is derived from paraxial mesoderm, and forms through epithelial–mesenchymal interactions with the membranous labyrinth that it surrounds and protects. There is also a minor contribution of neural crest to the otic capsule (not shown), at least in birds [a]. Abbreviations: 1–7, rhombomeres 1–7; I–IV, branchial arches I–IV; eam, external auditory meatus; ed, endolymphatic duct; es, endolymphatic sac; i, incus; m, malleus; ow, oval window; s, stapes; SAG, statoacoustic ganglion; scc, semicircular canals; sg, spiral ganglion; st, stapedius muscle; tm, tympanic membrane; tt, tensor tympani muscle; tr, tympanic ring; vg, vestibular ganglion. Reproduced from Ref. [b].



References

a Couly, G.F. *et al.* (1993) The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* 117, 409–429

b Fekete, D.M. (1999) Development of the vertebrate ear: insights from knockouts and mutants. *Trends Neurosci.* 22, 263–269

and r6), as well as genes expressed in the neural crest cells of the branchial arches, in inner ear development. None of these tissues supplies cells to the inner ear directly, with the exception of the neural crest-derived melanocytes and Schwann cells. Nonetheless, it has been postulated that inductive signals emanating from the rhombencephalon are essential for early induction of the otic placode and subsequent differentiation of the otocyst. To illustrate, *Hoxa1* (homeobox A1) is never

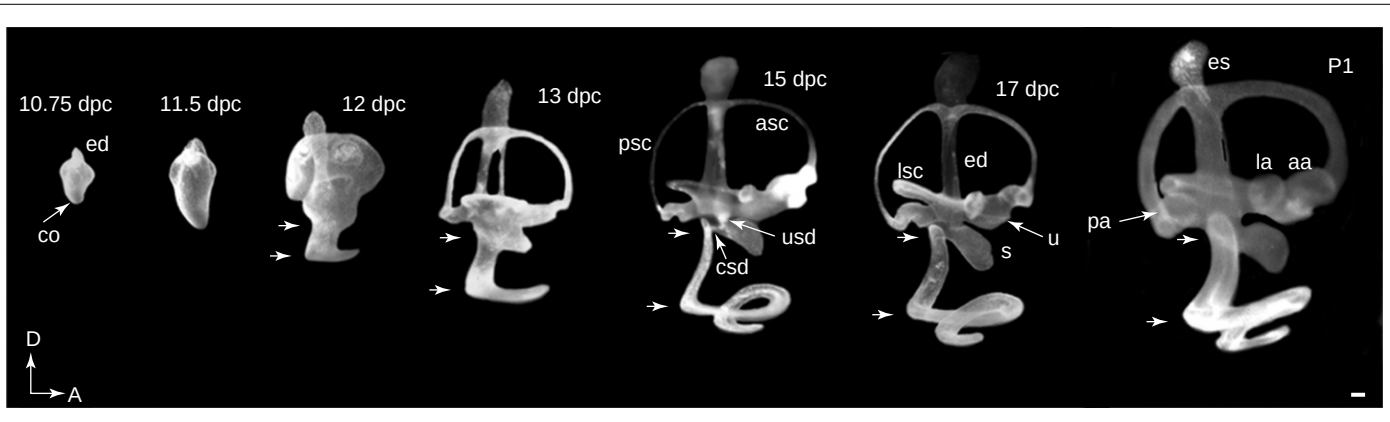


Fig. 1. Lateral views of paint-filled membranous labyrinths. Membranous labyrinths of mouse inner ears from 10.75 days post-coitus (dpc) to postnatal day 1 (P1) were filled with latex paint solution as described in [2]. At 10.75 dpc, the protrusions of the endolymphatic duct in the dorsal and the cochlear anlage in the ventral portion of the otocyst are evident. By 17 dpc, the gross anatomy of the inner ear is mature. Arrowheads identify the proximal region of the cochlea. aa, anterior ampulla; asc, anterior semicircular canal; co, cochlea; csd, cochleosaccular duct; ed, endolymphatic duct; es, endolymphatic sac; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle; usd, utriculosaccular duct. Orientation: D, dorsal; A, anterior. Scale bar: 100 μm. Adapted with permission from Ref. [2] ©1998, the Society for Neuroscience.

Table 1. Gene disruptions affecting mouse inner ear development and function

Gene symbol[a]	Gene name[b]	Chromosomal location (chromosome number; cM position; band)	TBASE Id number[c]	Human disorder (where applicable)[d]	Refs
Abcb1a[e]	ATP-binding cassette, sub-family B (MDR/TAP), member 1A (P-glycoprotein)	5 (1.0)	7052, 7053		[206,207]
Aldh1a2 (*Raldh2*)	Aldehyde dehydrogenase family 1, subfamily A2	9 (42.0)	5218, 5219		[39,40]
Apaf1	Apoptotic protease activating factor 1	10 (48.0; C3-D1)	6720, 6721, 7111, 7112	Noonan Syndrome	[111–113]
Aqp4	Aquaporin 4	18 (6.0)	6590, 6591, 7161		[161,162]
As2	Arylsulfatase A	15 (E)	7062, 7063	Metachromatic leukodystrophy (MLD)	[201–203]
Atf2 (*Creb2*)	Activating transcription factor 2	2 (syntenic)	3149, 3150		[109]
Atoh1 (*Math1*)	Atonal homolog 1 (*Drosophila*)	6 (29.7)	5429, 5430, 6925, 6926, 7046, 7047		[87–90]
Atp2b2 (*Pmca2*)	ATPase, Ca^{2+} transporting, plasma membrane 2	6 (49.5)	6970, 6971		[157]
Bdnf	Brain derived neurotrophic factor	2 (62.0)	1149, 1151, 3199, 3819, 5098, 5630, 5631		[187,189, 193,194]
Bmp4	Bone morphogenetic protein 4	14 (15.0)	4544		[2,208]
Cacna1d	Calcium channel, voltage-dependent, L type, α 1D subunit	14 (8.0)	6191, 6192		[137]
Calb1[e]	Calbindin-28K	4 (10.5)	7000		[135]
Casp3	Caspase 3, apoptosis related cysteine protease	8 (26.0)	4147, 4148, 6994, 6995, 7027	DFNA24	[114–116]
Cdkn1b (*p27, Kip1*)	Cyclin-dependent kinase inhibitor 1B (P27)	6 (62.0)	3666, 3667, 5395, 7064, 7065		[117–119]
Chrna9 (*Acra9*)	Cholinergic receptor, nicotinic, α polypeptide 9	5 (41.0)	7096, 7097		[198]
Col4a3	Procollagen, type IV, α 3	1 (syntenic)	7087, 7088	Alport syndrome	[142]
Col11a2	Procollagen, type XI, α 2	17 (18.5)	6967, 6968	DFNA13 (also called Stickler syndrome, type II, STLII)	[141]
Crabp1[e]	Cellular retinoic acid binding protein I	9 (31.0)	7100		[85,86]
Dlx2	Distal-less homeobox 2	2 (44.0)	7082, 7083, 7084		[81]
Dlx5	Distal-less homeobox 5	6 (2.0)	5739, 5740, 5741, 5742, 5743		[79,80]
Edn3	Endothelin 3	2 (104.0)	–	Waardenburg syndrome (WS4)	[168]
Ednrb	Endothelin receptor type B	14 (51.0)	–	Waardenburg syndrome (WS4)	[167]
Ephb2	Ephrin receptor B2	4 (65.7; D-E)	–		[164]
Eya1	Eyes absent 1 homolog (*Drosophila*)	1 (10.4)	5547, 5548	Branchio-oto-renal (BOR) syndrome	[43–45]
Fgf3	Fibroblast growth factor 3	7 (72.4)	1202, 1204		[49,50]
Fgfr2	Fibroblast growth factor receptor 2	7 (63.0)	7033, 7034, 7035		[57–60]
Fgfr3	Fibroblast growth factor receptor 3	5 (20.0; B)	3467, 3468	Craniosynostosis	[52]
Foxi1 (*Fkh10*)	Forkhead box I1 (forkhead homolog 10, (*Drosophila*))	Unknown	5437, 5438		[42]
Fzd4 (*Fz4*)	Frizzled homolog 4 (*Drosophila*)	7 (44.5)	6988, 6989		[204]
Gata3	GATA-binding protein 3	2 (7.0)	7108, 7109, 7113		[63]
Gjb2[f]	Gap junction membrane channel protein β 2 (connexin 26)	14 (21.0; D1-E1)	6791, 6792	DFNA3, DFNB1	[177]
Gjb3[f]	Gap junction membrane channel protein β 3 (connexin 31)	4 (syntenic)	6436, 6437	DFNA2	[178]
Gpx1	Glutathione peroxidase 1	9 (57.0)	7057		[134]
Hes1 (*Hry*)	Hairy and enhancer of split 1 (*Drosophila*)	16 (27.0)	5652, 7028, 7030		[91–93]
Hes5	Hairy and enhancer of split 5 (*Drosophila*)	4 (81.5)	7167		[93]
Hmx2 (*Nkx5-2*)	H6 homeobox 2	7 (65.0)	7334, 7335		[72]
Hmx3 (*Nkx5-1*)	H6 homeobox 3	7 (65.0)	5386, 5388, 4833, 6121		[70,71]
Hoxa1 (*Hox-1.6*)	Homeobox A1	6 (26.28)	783, 785, 1205, 1207, 1208, 1210		[22–27,29]
Hoxa2 (*Hox-1.11*)	Homeobox A2	6 (26.29)	2669, 2670, 3433, 3434		[30–33]
Hoxb1 (*Hox-2.9*)	Homeobox B1	11 (56.2)	–		[22,28]
Igf1	Insulin-like growth factor 1	10 (48.0)	2613, 2614, 7098		[197]

Table 1 continued. Gene disruptions affecting mouse inner ear development and function

Gene symbol[a]	Gene name[b]	Chromosomal location (chromosome number; cM position; band)	TBASE Id number[c]	Human disorder (where applicable)[d]	Refs
Itga8	Integrin α8	2 (7.0)	5772, 5773		[110]
Jag1[e]	Jagged 1	2 (77.0)	7159, 7160		[98,100]
Jag2	Jagged 2	12 (57.9)	4899, 4900, 5145, 5146, 7164, 7165		[95,99, 102]
Kcne1 (*Isk, MinK*)	Potassium voltage-gated channel, Isk-related Subfamily, member 1	16 (64.4)	6998, 6999, 4966, 4967, 7086	Jervell and Lange-Nielsen syndrome, locus 2 (JLNS2)	[147,150, 151]
Kcnj10 (*Kir4.1*)	Potassium inwardly-rectifying channel, subfamily J, member 10	1 (93.5)	7310, 7311, 7312		[146]
Kcnq1 (*Kvlqt1, Kcna9*)	Potassium voltage-gated channel, subfamily Q, member 1	7 (69.3)	6298, 6299, 6973, 6974	Jervell and Lange-Nielsen syndrome, locus 1 (JLNS1)	[147–149]
Lfng	Lunatic fringe gene homolog (*Drosophila*)	5 (82.0)	5204, 5205, 7163, 7164, 7165		[95,103]
Ndph[e]	Norrie disease homolog	X (5.3)	7104, 7105	Norrie disease	[209]
Neurod1 (*BETA2/ Neurod*)	Neurogenic differentiation 1	2 (46.0)	5616, 5617, 5904, 5966, 5969, 6936, 7016		[181–184]
Neurod3 (*Ngn1*)	Neurogenic differentiation 3 (neurogenin 1)	13 (35.0)	7031		[180]
Notch1	Notch gene homolog 1 (*Drosophila*)	2 (15.0)	3247, 3248, 7162		[95,98, 101]
Nr4a3 (*Nor1*)	Nuclear receptor subfamily 4, group A, member 3 (neural orphan receptor 1)	Unknown	7307, 7308		[84]
Ntf3	Neurotrophin 3	6 (61.0)	1183, 1185, 7106		[186–189]
Ntn1	Netrin 1	Unknown	7008, 7009		[64]
Ntrk2 (*TrkB*)	Neurotrophic tyrosine kinase, receptor, type 2	13 (36.0)	1023, 1025, 7140, 7141		[185,189–192]
Ntrk3 (*TrkC*)	Neurotrophic tyrosine kinase, receptor, type 3	7 (39.0)	1155, 1157, 6214, 7140, 7141		[185,189–192]
Otog	Otogelin	7 (B4-C)	5669, 5671		[140]
Otx1	Orthodenticle homolog 1	11 (12.0)	4075, 4088, 7066, 7067, 7068, 7069, 7072, 7074, 7075		[17,73–77]
Otx2	Orthodenticle homolog 2	14 (19.0)	3184, 3187, 7067, 7069, 7072		[17,73,77, 78]
Pax2	Paired box gene 2	19 (43.0)	4092, 7061	Renal-coloboma syndrome	[41]
Pnoc (*Npnc1*)	Prepronociceptin	Unknown	4378, 4379		[210]
Pou3f4 (*Brn4.0, Slf*)	POU domain, class 3, transcription factor 4	X (48.4)	7005, 7006, 7014, 7015	DFN3	[65–67]
Pou4f1 (*Brn3.0, Brn3a*)	POU domain, class 4, transcription factor 1	14 (E1-E3)	4008, 4009		[195,196]
Pou4f3 (*Brn3.1, Brn3c*)	POU domain, class 4, transcription factor 3	18 (24.0; B3-E1)	4010, 4011, 5500, 5501, 6996, 6997	DFNA15	[104–107]
Prrx1 (*Prx1*)	Paired related homeobox 1	1 (85.4)	1972, 1973		[68,69]
Prrx2 (*Prx2*)	Paired related homeobox 2	2 (19.0)	7022, 7023		[68]
Prrx1/Prrx2 double knockout	Prrx1: paired related homeobox 1 Prrx2: paired related homeobox 2	2 (19.0)	7024, 7025, 7026		[68]
Rara/Rarb double knockout	Rara: retinoic acid receptor, α Rarb: retinoic acid receptor, β	14 (1.5; A1-A3)	7139		[34–37]
Rara/Rarg double knockout	Rara: retinoic acid receptor, α Rarg: retinoic acid receptor, γ	15 (57.4; E-F3)	7138		[34]
Scnn1a (*ENaC-α*)[e]	Sodium channel, nonvoltage-gated, type I, α polypeptide	6 (60.6)	7085		[136]
Slc1a3 (*GLAST*)	Solute carrier family 1, member 3	15 (6.7; A2)	6991, 6992		[205]
Slc12a2 (*Nkcc1*)	Solute carrier family 12, member 2	18 (32.0)	5316, 5318, 5723, 5724, 5725, 5726, 7018, 7019		[152–155]
Slc12a7 (*Kcc4*)	Solute carrier family 12, member 7	Unknown	7337, 7338		[174]
Slc26a4 (*Pds*)	Solute carrier family 26, member 4 (pendrin)	Unknown	6340, 6342	DFNB4 (also called Pendred syndrome)	[156]
Sod1 (*Cu/Zn SOD*)	Superoxide dismutase 1, soluble (copper/zinc superoxide dismutase)	16 (61.0; B5-C3)	3725, 3726, 7054		[130–133]
Tcfap2a (*Ap2-α*)	Transcription factor AP-2, α	13 (25.0; A5-B1)	4023, 4024, 4025, 4026, 4978		[46,47]
Tecta (*α-tectorin, Tctna*)	Tectorin α	9 (25.0)	7011, 7012	DFNA8/DFNA12/ DFNB21	[138]

Table 1 continued. Gene disruptions affecting mouse inner ear development and function

Gene symbol[a]	Gene name[b]	Chromosomal location (chromosome number; cM position; band)	TBASE Id number[c]	Human disorder (where applicable)[d]	Refs
Tgfb2	Transforming growth factor, β2	1 (101.5)	6525, 6526		[48]
Thra[e]	Thyroid hormone receptor, α1 isoform	11 (57.0; D-E)	4814, 4815, 7055		[121–123]
Thrb	Thyroid hormone receptor β	Unknown	3869, 3870, 7056	Thyroid hormone resistance	[122–126]
Thrb[e]	Thyroid hormone receptor, β2 isoform	Unknown	7049, 7050		[124,126]
Tnc	Tenascin C	4 (32.2)	7020		[199,200]
Tulp1[e]	Tubby like protein 1	17 (syntenic)	6773, 6774, 6776, 6777		[211,212]

[a]Gene symbol refers to the official symbol of the targeted gene (with abbreviated and/or alternative gene symbols in parentheses), as indicated in the Mouse Genome Database (MGD; http://www.informatics.jax.org/).
[b]Gene name refers to the name of the protein product of the targeted gene (with abbreviated and/or alternative names in parentheses), as indicated in MGD.
[c]TBASE id number refers to the accession number of each relevant record listed in TBASE (http://tbase.jax.org/). Users may search the database by TBASE id number to view the full phenotypic profile of a given mouse knockout.
[d]Human disorder refers to the general human condition, syndrome or form of hearing loss modeled by a given gene knockout.
[e]The gene has been knocked out but no specific inner ear defect or hearing impairment has been reported.
[f]The knockout phenotype does not faithfully recapitulate the human disorder.

expressed in the otocyst, but rather in the rhombencephalon adjacent to the otocyst. Yet in *Hoxa1*-null mice the entire endolymphatic labyrinth fails to undergo normal morphogenesis, resulting in complete disruption of the vestibular and cochlear components. These malformations are variable [22], and can be rescued by a single maternal administration of a subteratogenic dose of the vitamin A metabolite retinoic acid (RA) [23]. The ensuing hindbrain reorganization is characterized by the complete [24,25] or quasi-complete absence of r5 [26,27] and a severe reduction in r4, suggesting a role for *Hoxa1* in the maintenance and/or generation of hindbrain segments rather than fate specification. By contrast, *Hoxb1* (homeobox B1) appears to be crucial in conferring specific identity to r4 cells [28]. When combined with a loss-of-function mutation in *Hoxb1,* the *Hoxa1*-null/*Hoxb1*$^{(3RARE)}$ mutant phenotype is fully penetrant, displaying exacerbated molecular changes in the r4–r6 region and in the second pharyngeal arch neural crest cells, absence of the cochlea, aplasia of the vestibular apparatus, and total lack of fenestration in the mutant ear (Fig. 2), indicating that the products of *Hoxa1* and *Hoxb1* function synergistically on inner ear development [22,29]. Lastly, inactivation of *Hoxa2* (homeobox A2) leads to disruption of the dorsal r2–r3 patterning with concomitant loss of cochlear nuclei and enlargement of the lateral part of the cerebellum [30–33] (Fig. 2).

Although RA receptors are synthesized extensively in both the inner ear and the brain, inner ear malformations are not evident unless two or more receptors are ablated, as shown by compound null mutants [34]. Examination of *Rara*/*Rarb* double knockouts, lacking RA receptors α and β, at early embryonic stages (E8.5–E10.5) reveals defects of post-otic pharyngeal arches, and a fully penetrant disorganization of the post-otic cranial nerves that is not evident in the corresponding single knockouts [34–37]. No significant alterations in the number and migratory paths of neural crest cells are observed in the pharyngeal arches. Notably, the initial formation of the third and fourth branchial pouches and of the third, fourth and sixth arch arteries (i.e. processes known to be independent of the neural crest) are perturbed. Moreover, r5 is enlarged, probably accounting for the observed induction of supernumerary

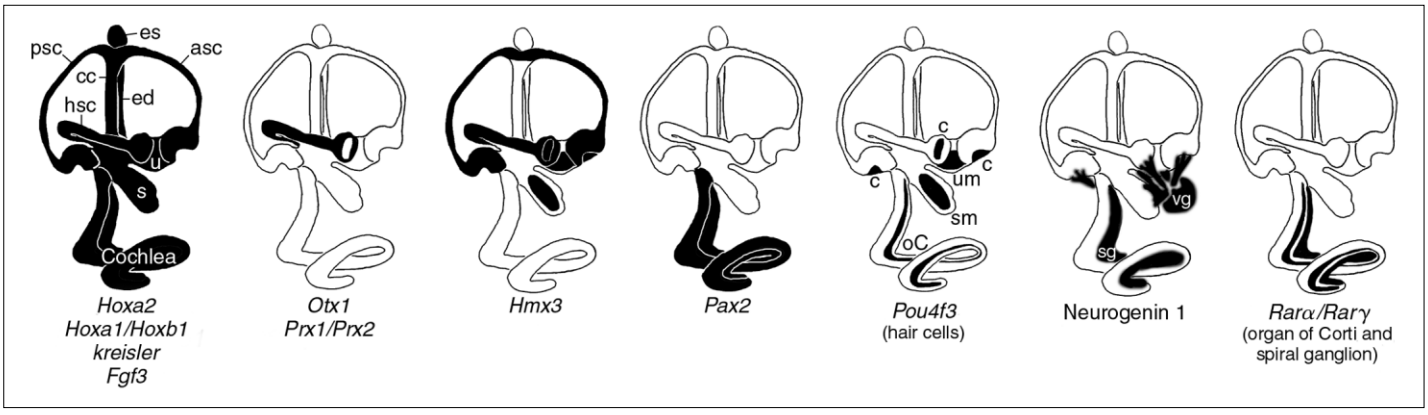


Fig. 2. Mutations that alter morphology of the inner ear, its sensory organs or the statoacoustic ganglion neurons. All genes listed represent homozygous null mutants unless otherwise indicated. Structures reported to be altered (including hypomorphisms and missing elements) are indicated in black. Full penetrance of the phenotypes can vary between individual knockout mice, between the strains of mice and for different mutant alleles. The most severe phenotypes reported are indicated here. Note the structures that are not shown in black are not necessarily entirely normal, as they might not have been studied in sufficient detail to rule out subtle defects. Abbreviations: asc, anterior semicircular canal; c, crista; cc, common crus; ed, endolymphatic duct; es, endolymphatic sac; hsc, horizontal semicircular canal; oC, organ of Corti; psc, posterior semicircular canal; s, stapes; sg, spiral ganglion; sm, saccular macula; u, utricle; um, utricular macula; vg, vestibular ganglion. Reproduced from Ref. [16].

otocysts, whereas the r5–r6 boundary is lost. Thus, RA signaling has a significant role in patterning of the post-otic hindbrain and caudal pharyngeal arches.

Supernumerary otocysts are also detected in embryos lacking both *Rxra* and *Rxrb* (retinoid X receptors α and β), establishing a key role for RA-activated RXR/RAR heterodimers in the specification of the otic placode from the ectoderm [38]. Surprisingly, the putative otogenic signals emanating from the neural tube following otocyst formation are apparently unaffected, as E14.5 and E18.5 *Rara*/*Rarb*-null mice display normal inner ears [37]. By contrast, *Rara*/*Rarg* double knockouts, lacking RA receptors α and γ, display a consistently hypoplastic otocyst at E10.5, and a complete absence of the spiral organ of Corti and the spiral ganglion at E18.5 [34] (Fig. 2). Finally, *Aldh1a2* (aldehyde dehydrogenase family 1, subfamily A2; also called *Raldh2*), whose product is involved in the enzymatic generation of RA, displays a restricted expression pattern during mouse inner ear ontogenesis [39]. Its absence results in midgestational lethality, abnormal r3 (and, to a lesser extent, r4) identity of the caudal hindbrain cells, and otocysts that are abnormally distant from the hindbrain neuroepithelium and remain hypoplastic even following the administration of maternal RA [40].

Morphogenesis of the cochlear and vestibular apparati

Genesis of the cochlear and vestibular apparati requires the undisturbed spatiotemporal expression of several patterning genes in the otic epithelium. A classic patterning gene is *Pax2* (paired box gene 2), the expression of which is restricted to the ventral half of the developing otocyst from which the cochlear and saccular regions derive. *Pax2* targeted inactivation leads to agenesis of the cochlea and the spiral ganglion, while the vestibular and saccular structures develop properly [41]. As a result, none of the organ of Corti cell types can be identified and cochlear innervation is curtailed (Fig. 2).

Severe cochlear and vestibular malformations are noted in *Foxi1* (also called *Fkhl0*) knockouts, lacking forkhead box I1, a member of the forkhead family of winged helix transcriptional regulators which is exclusively expressed in the otocyst at E9.5. These structures are replaced by a single continuous cavity in which neither proper semicircular ducts nor cochlea can be recognized. Such mutants are deaf and show circling behavior, poor swimming ability and abnormal reaching response, consistent with vestibular dysfunction [42]. These observations implicate *Foxi1* as an early regulator required for both cochlear and vestibular development, and identify its human homologue, FOXI1, as a previously unknown candidate deafness-causing gene at 5q34. Recent findings presented at The Molecular Biology of Hearing and Deafness Meeting (October 4–7, 2001, Bethesda, Maryland; Abstract 208) support the notion that *Foxi1* might also play a role in fluid homeostasis of the inner ear by regulating the *Slc26a4*-encoded product, pendrin (see section on endolymph homeostasis and ion balance). Thus, certain phenotypic aspects of the *Foxi1*-null mice are now postulated to result from an ensuing lack of pendrin (Sven Enerbäck, pers. commun.).

Eya1-deficient homozygotes, lacking the *Drosophila* 'eyes absent 1' homolog, display severe malformations in the outer, middle and inner ear as well as bilateral renal agenesis caused by defective inductive tissue interactions and apoptotic regression of the organ primordia [43–45]. Inner ear development arrests at the otocyst stage and all components of the inner ear and specific cranial sensory ganglia fail to form [45]. The facio-acoustic (VII/VIII) ganglion is absent, and the endolymphatic duct is missing or misshapen. Importantly, *Eya1* heterozygotes show renal abnormalities and a conductive hearing loss similar to the human branchio-oto-renal (BOR) syndrome (Online Mendelian Inheritance in Man™ database [http://www.ncbi.nlm.nih.gov/Omim/] OMIM: 113650), typified by craniofacial anomalies and hearing loss [45]. Similar to the *Eya1* knockout anotia, functional loss of *Tcfap2a* (transcription factor AP-2, α) leads to anencephaly, absence of ventral craniofacial structures, including a recognizable ear, and a variable morphological disruption of the VII/VIII ganglion complex [46,47].

Targeted deletions of growth factor/receptor genes are also beginning to disclose information on the morphological patterning of the inner ear. For instance, transforming growth factor, β2 (TGFB2) mRNA has been localized in the developing cochlear epithelium as well as in the underlying mesenchyme. At E18.5, all *Tgfb2*-null mice fail to display mesenchymal condensation in the spiral limbus and differentiation of the overlying interdental cells. In conjunction with an incomplete canalization of the scala vestibuli, these findings suggest that *Tgfb2* has an instrumental paracrine role in guiding epithelial and mesenchymal differentiation and patterning in the inner ear [48]. Moreover, functional disruption of *Fgf3* (fibroblast growth factor 3), which is normally expressed in the hindbrain adjacent to the prospective ear in r5 and r6, alters the coiling of the cochlea (Fig. 2) and reduces spiral ganglion cell numbers [49,50]. Malformations of the inner ear in *Fgf3* knockouts show reduced penetrance and variable expressivity and are generally similar to those encountered in *kreisler* mice [51], involving failure of the development of the endolymphatic appendage (Fig. 2).

It appears that a prerequisite for defining FGF3 function in the inner ear is to characterize its receptor(s) and the putative FGF compensatory signals that could account for the partially penetrant phenotype. Expression of *Fgfr3* (fibroblast growth factor receptor 3) has been detected in differentiating hair cells and their underlying supporting cells. *Fgfr3*-null mice are deaf and display developmental defects in the organ of Corti, including failure of pillar cell differentiation and the complete absence of the triangular, fluid-filled space normally found between the inner and outer rows of supporting pillar cells, known as the tunnel of Corti [52]. Exogenous FGF2 stimulates neuronal migration and differentiation in explants of the early chick inner ear [53]. In mice, the mesenchyme-derived FGF10 appears to be the key factor that initiates and/or maintains outgrowth of FGFR2-expressing epithelial appendages [54]. Persuasive evidence for this assumption has emerged from *Fgf10*-null mutations [55,56] and from different approaches to ablate the *Fgfr2* gene [57–59]. A null mutation and a hypomorphic mutation of the entire *Fgfr2* gene (encompassing both the IIIb and IIIc isoforms) result in either early or midgestational lethality, respectively [57,60]. The latter mutation causes demise at the early otocyst stage, at the time when its morphogenesis is initiated. Mice lacking *Fgfr2* (IIIb)–the only FGFR2 isoform known to bind FGF10 and FGF3–develop otocysts from ectodermal placodes [58], indicating that

FGFR2 (IIIb) signaling is not essential for the earliest stages of inner ear formation. However, *Fgfr2* (IIIb)-specific targeted deletion leads to severe dysgenesis of the cochleovestibular membranous labyrinth, attributed to a morphogenetic arrest at the otocyst stage [59]. Notably, sensory patches in these mutants are rudimentary, suggesting that nonsensory tissues might also feedback on sensory tissues for their subsequent development.

In conjunction with the observed knockout data, detailed localization of *Fgfr2, Fgf10* and *Fgf3* mRNAs in the developing mouse cochlea provide supporting evidence that FGF3 and FGF10 function as paracrine regulators of gross morphological patterning of the inner ear by activating the IIIb isoform of *Fgfr2* [59]. Recently, *Fgf8* transcripts have been detected in two distinct short waves, first at the otic placode stage and later at the otocyst stage, and ectopic application of FGF2 and FGF8 has been shown to result in enlargement of the vestibulo-cochlear ganglion [61]. As more and more of these signaling molecules are identified, it should be feasible to establish a timely orchestration of molecular events starting from otic induction to a mature inner ear.

The zinc finger *Gata3* gene, encoding GATA-binding protein 3, is expressed both in the inner ear and in afferent and efferent auditory neurons. Specifically, *Gata3* is expressed in developing and adult olivocochlear and vestibular efferent neurons but not in the adjacent facial branchial motoneurons. *Gata3* is also detected inside the otocyst and the surrounding periotic mesenchyme [62]. Not surprisingly, *Gata3*-null ears remain cystic, with a single extension of the endolymphatic duct, and show absence of semicircular canals as well as loss of saccular and utricular processes [63].

Fusion plate formation and the subsequent removal of the fused cells are crucial events in semicircular canal morphogenesis. *Ntn1* (netrin 1) is highly expressed in the otic epithelium, in cells destined to form a fusion plate. A severely hypomorphic loss-of-function allele of *Ntn1* results in a significantly reduced anterior semicircular canal and the total absence of the posterior and lateral canals [64].

Pou3f4 (POU domain, class 3, transcription factor 4; also called *Brn4.0* or *slf*) is initially expressed in the otic capsule at 10.5 dpc, when the capsule initiates the mesenchymal condensation that gives rise to the temporal bone. As the otic mesenchyme condenses around the otocyst, *Pou3f4* is expressed throughout the otic capsule. Targeted inactivation of *Pou3f4* results in multiple functional deficits in both the auditory and vestibular systems, including gait abnormalities, head bobbing and hearing loss. Cochlear malformations encompass hypoplasia of the spiral limbus, the scala tympani and strial fibrocytes, as well as a marked reduction in cochlear coiling, which suggests a disruption of cell signaling between the mesenchyme and epithelium. In addition, a constriction or dysplastic phenotype is prominent in the *Pou3f4*-null anterior semicircular canal and, occasionally, in the lateral semicircular canal, whereas the posterior canal is invariably spared [65,66]. Recent mapping data has demonstrated that the 'sex-linked fidget' (*slf*) mouse mutation, showing an identical vertical head-shaking phenotype, is an X chromosomal inversion with one breakpoint close to *Pou3f4,* which selectively abolishes *Pou3f4* expression in the developing inner ear. Collectively, these findings indicate that the *slf* mutant is a plausible mouse model for the most prevalent form of X-linked, nonsyndromic congenital deafness in human (DFN3; OMIM: 304400), which is associated with mutations in the human ortholog, POU3F4 [67].

Prrx2 (paired related homeobox 2; also called *Prx2*) knockouts have a normal inner ear structure, despite the observed expression of *Prrx2* in the lateral wall of the otocyst at E9.5 and E10.5 [68]. *Prrx1* (paired related homeobox 1; also called *Prx1*) is not expressed in the otocyst from E9.5 to E12.5; however, *Prrx1* and *Prrx2* are both highly expressed in the mesenchyme surrounding the lateral aspect of the otocyst and the diverticula of the developing ducts. Inactivation of both genes leads to a size reduction of the entire otic capsule and loss of the lateral semicircular canal. In *Prrx1*/*Prrx2* double knockouts [68], the formation of the diverticula of the prospective semicircular ducts is retarded and incomplete, whereas delamination and fusion of the walls of the diverticula occurs relatively uneventfully (Fig. 2). By contrast, only a slight size reduction is noted in the otic capsule of *Prrx1* single knockouts [68,69]. These findings suggest that *Prrx* genes are involved in epitheliomesenchymal interactions that induce inner ear morphogenesis.

Complete loss of the lateral semicircular canal and lack (or large reduction) of the posterior and anterior canals is observed in an *Hmx3* (H6 homeobox 3) knockout strain which features hyperactivity and circling movements reminiscent of the shaker/waltzer phenotype [70]. *Hmx3* is initially expressed in the rostral part of the otic placode at E8.5, and relocates during otocyst formation from a medial domain to the dorsolateral wall, the region that later gives rise to the vestibular apparatus. The severity of dysgenesis is variable for the posterior and anterior canals among individual mutants, and between both ears within individuals (Fig. 2). The maculae of the utriculus and sacculus appear unaffected, and normal hearing is preserved in the absence of detectable alterations in the cochlea and the endolymphatic duct. Interestingly, Wang *et al.* [71] attribute similar vestibular defects observed in a second *Hmx3* knockout strain to the severe depletion of sensory cells in the saccule and utricle, as well as the complete loss of the lateral semicircular canal crista and fusion of the utricle and saccule endolymphatic spaces into a common utriculosaccular cavity. In this strain, all of the semicircular ducts are present and appear normal, with the exception of the lateral crista and the associated lateral ampullary chamber [71].

Another member of the *Hmx* homeobox gene family, *Hmx2* (H6 homeobox 2) is coexpressed with *Hmx3* in the developing central nervous as well as in the dorsolateral otic epithelium. Specifically, *Hmx2* is strongly expressed in the anterior aspect of the otocyst at E9.5, and in the anterodorsal portion of the otocyst at E10.5. By E12.5, the entire dorsal portion (pars superior) of the otocyst shows robust expression of *Hmx2*. In the dorsal endolymphatic duct, *Hmx2* expression is pronounced at E12.5, after the overall structure has already been established [72]. Approximately 65% of *Hmx2* knockouts exhibit hyperactivity, circling and head tilting, in the absence of observable abnormalities in the central nervous system. *Hmx2* disruption leads to failure of semicircular duct formation, persistence of the primordial vestibular diverticula, formation of a common macula in a fused utriculosaccular chamber, as well as marked loss of epithelial cells (both sensory and nonsensory) in the developing vestibule. The initial stages of fusion plate

formation (thinning of the otic epithelial layer, loss of epithelial morphology, and detachment from the underlying basement membrane) are delayed, while the eventual fusion of the apposing walls of the otocyst never occurs. The reduced rate of cell proliferation observed at E11.5 in both the otic epithelium and the adjacent periotic mesenchyme, together with the altered expression profiles of specific developmental regulators, including *Bmp4, Dlx5* and *Pax2,* indicate that *Hmx2* controls the specification and commitment of epithelial cells in the dorsal portion of the otocyst to undergo the proliferative growth and fusion processes required to generate a functional vestibular apparatus [72]. Analysis of a combined mutation in *Hmx2* and *Hmx3* will undoubtedly elucidate the unique and overlapping functions of these genes in inner ear and central nervous system development.

Both *Otx1* and *Otx2* (orthodenticle homologs 1 and 2, respectively) are activated during the otocyst stage. At E10.25, *Otx1* is detected in the ventrolateral wall of the otocyst while *Otx2* is detected in the ventral tip of the otocyst within a portion of the *Otx1*-expressing domain. As development proceeds, the most dorsal boundary of the *Otx1* domain corresponds to the presumptive lateral canal level, and the dorsal boundary of *Otx2* expression domain corresponds to the middle of the utricular anlage [73]. *Otx1* knockouts display an absence of the lateral semicircular canal, lateral ampulla, utriculosaccular duct and cochleosaccular duct, and a malformed cochlear hook [17,73–77] (Fig. 2). Notably, saccular and cochlear dysgenesis exhibits variable expressivity in *Otx1*-null mice [73]. *Otx2* knockouts die at E10, before any appreciable inner ear patterning [77,78]. Analysis of *Otx1* (–/–)/*Otx2* (+/–) compound mutants reveals an aggravated phenotype, particularly in ventral structures, including the saccule and cochlea which normally express *Otx2* [73]. The differences observed between the *Otx1*-null and the *Otx1* (–/–)/*Otx2* (+/–) compound null phenotype as well as the inability of human OTX2 cDNA to rescue the *Otx1*-null lateral canal and ampulla phenotypes, suggest that these genes have both overlapping and specific functions in the patterning of the inner ear. Thus, *Otx1* is critical in the formation of the lateral canal and ampulla, whereas *Otx2* is required in the patterning of cochlea and saccule.

Dlx5 (distal-less homeobox 5) transcripts are detected during otic placodogenesis and otic pit invagination. Starting at E8.0, *Dlx5* is expressed in the dorsoposterior region of the otocyst and subsequently in the semicircular canals and in the endolymphatic duct and vesicle of the vestibular organ. Agenesis of the anterior and posterior semicircular canals and reduction of the lateral semicircular canal is independently demonstrated in two distinct strains of *Dlx5* knockouts, generated by Acampora *et al.* [79] and Depew *et al.* [80], respectively. Notably, these two strains display differences in terms of endolymphatic duct development and cochlear morphology. Both these structures appear essentially unaffected in the first strain; the lesion appears to be more severe in the latter strain, as shown by altered cochlear coiling and failure to from the dorsally located endolymphatic duct. It is interesting to note that although *Dlx2*, *-3*, *-5* and *-6* are all expressed in the mouse inner ear [81–83], so far only the *Dlx5* knockout has been reported to display inner ear defects.

Beginning at E11.5, expression of *Nr4a3* (nuclear receptor subfamily 4, group A, member 3; previously known as *Nor1,* for neural orphan receptor 1) is localized in the prospective fusion plate-forming cells of the otocyst. Following formation of the membranous labyrinth at E13.5, and continuing through P1, *Nr4a3* expression is restricted to nonsensory epithelial cells localized at the inner edge of the semicircular canals, as well as nonsensory epithelial cells that form the roof of the ampulla and the utricle. Targeted ablation of *Nr4a3* results in normal vestibular sensory areas and normal fusion of the vestibular walls. However, *Nr4a3*-null mice display diminished canal size and flattened ampullae in addition to partially penetrant hyperactive and bidirectional circling behaviors. The reduction in canal size is associated with a defect in proliferative continual growth of the semicircular canals, once they are formed, and an ensuing reduction in the endolymphatic fluid space within the canals and their corresponding ampullae [84].

The early embryonic expression of retinoid binding proteins in cochlear structures suggests important roles for these proteins during ontogenesis and morphogenesis of the inner ear. Surprisingly, analyses of *Crabp1*-null and *Crabp2*-null mice, lacking cellular RA binding protein I and II, respectively, as well as *Rbp1*-null mice, lacking cellular retinol binding protein 1, do not reveal any modifications in cochlear morphology or auditory thresholds [85,86]. It appears that these retinoid-binding proteins are not directly involved in cochlear development and hair cell differentiation.

Generation and survival of neuroepithelial components

Failure to generate cochlear and vestibular hair cells is detected in mice lacking the mouse homolog of the *Drosophila* proneural gene *atonal* (*Atoh1*; also called *Math1*), which is specifically expressed in the sensory epithelia. *Atoh1* is required for the development of certain components of the proprioceptive pathway, including cerebellar granule neurons and the pontine nuclei [87–89]. The sensory epithelia of *Atoh1* knockouts lack hair cells entirely, but display functional supporting cells of normal morphology, in the absence of apoptotic cell death. *Atoh1* is the first gene shown to be essential for the specification of hair cells. Furthermore, *Atoh1*-null embryos lack the D1 interneurons, which give rise to a subset of proprioceptor interneurons and the spinocerebellar and cuneocerebellar tracts [90].

Interestingly, targeted inactivation of *Hes1* (the mouse homolog of the *Drosophila* 'hairy and enhancer of split 1'), which is a negative regulator of neurogenesis, leads to generation of supernumerary hair cells in the cochlea and utricle [91,92]. *Hes1* expression is detected during hair cell differentiation, becomes elevated perinatally, and persists into adulthood. Within the vestibular sensory epithelium, *Hes1* is expressed in supporting cells, but not in hair cells. In the cochlea, *Hes1* is selectively expressed in the greater epithelial ridge and the lesser epithelial ridge. Similar to *Hes1*, *Hes5* (the mouse homolog of the *Drosophila* 'hairy and enhancer of split 5') is not expressed in hair cells, but is predominantly found in the lesser epithelial ridge, in supporting cells, and in a narrow band of cells within the greater epithelial ridge. Independent experiments by Zine *et al.* [93] demonstrate that cochleae from their *Hes1*-null mice exhibit a significant increase in the number of inner hair cells (IHCs), which derive from progenitor cells located in the most distal

domain of the greater epithelial ridge, whereas cochleae from *Hes5*-null mice display a significant increase in the number of outer hair cells (OHCs), which derive from progenitor cells located in the proximal domain of the lesser epithelial ridge. In the vestibular organs, inactivation of *Hes1* and to a lesser extent *Hes5* results in the generation of supernumerary hair cells in the saccule and utricle. Moreover, the supernumerary hair cells in *Hes1* and *Hes5* knockouts display an upregulation of *Atoh1*, suggesting that *Hes1* and *Hes5* activities are pivotal in repressing the commitment of progenitor cells to IHCs and OHCs fates, respectively, possibly by antagonizing *Atoh1* [93].

Lineage tracing using retroviral transfection suggests that the hair and supporting cell lineages arise from a common progenitor and do not segregate until late in inner ear development [94]. Although the molecular mechanism underlying the developmental choice between hair versus supporting cells remains elusive, recent data implicates the *Notch–Delta* signaling pathway in the determination of cell fates in the cochlear mosaic [95]. *Notch* proteins are ligand-activated transmembrane receptors participating in cell fate selection throughout development of both *Drosophila* and vertebrates [96]. *Notch* activation results in the subsequent activation of *Hes1* and *Hes5* genes [97]. Moreover, the *Notch* signaling components show both distinct and overlapping expression patterns of the *Notch1* receptor and its ligands *Jagged 1* (*Jag1*) and *Jagged 2* (*Jag2*) in the developing auditory epithelium of the rat [98].

In mice, targeted mutations in different components of this pathway have been shown to differentially affect the IHC and OHC populations [95]. *Notch1* expression is detected throughout the sensory cochlear epithelium before hair cell differentiation, and is downregulated in differentiating hair cells [99], whereas *Jag1* expression is restricted to non-sensory supporting cells surrounding IHCs and OHCs by E18 [100]. *Notch1* and *Jag1* homozygous null embryos die well before cochlear formation, thus precluding assessment of hair cell differentiation [100,101]. *Notch1* heterozygous neonates display supernumerary hair cells; however, these are specifically confined to the OHC rows, which retain an evenly patterned arrangement [95]. Although inner ear development has not been investigated in *Jag1* heterozygotes, it is worth noting that these mice are not hyperactive and do not circle or head-bob [100]. Examination of *Jag2*-null neonates reveals that supernumerary hair cells differentiate in both the inner and outer hair cell rows of the cochlea presumably as a result of a decrease in *Notch* activation [99,102]. Specifically, *Jag2*-null cochleae show a significant increase in the numbers of IHCs, and a smaller increase in the numbers of OHCs, and the patterning in OHC rows is highly irregular compared with the patterning in wild-type littermates [95].

Simlar to *Jag1*, the *Lfng* (*lunatic fringe*) gene, which encodes an extracellular modulator of the Notch signaling pathway, is expressed in non-sensory supporting cells in the cochlea at E18. *Lfng* homozygous null mice do not exhibit significant differences in hair cell numbers or patterning compared to littermate controls [95,103]. However, in double knockouts lacking both *Jag2* and *Lfng*, the generation of supernumerary hair cells in the IHC row noted in *Jag2* single knockouts is suppressed, whereas supernumerary hair cells in the OHC rows remain unaffected, indicating that the *Lfng* mutation partially suppresses the consequences of *Jag2* inactivation on IHC development [95].

Pou4f3 (POU domain, class 4, transcription factor 3; also, *Brn3.1* or *Brn3c*) is specifically expressed in postmitotic hair cells. *Pou4f3* expression is initially detected at E14, shortly after the hair cells differentiate from the epithelial cells of the organ of Corti, and persists throughout adulthood. Young *Pou4f3* homozygous null mice exhibit a circling behavior, hyperactivity and deafness. The initial stages of cochlear morphogenesis occur uneventfully, and the hair cells initiate the expression of hair cell-specific markers, such as myosin VI, myosin VIIa, calretinin and parvalbumin [104]. However, the *Pou4f3*-null hair cells die shortly thereafter, before overt morphological differentiation occurs and, eventually, many of the spiral ganglion neurons undergo degeneration [105,106] (Fig. 2). *Pou4f3* haploinsufficiency has no effect on cochlear morphology, as 18- and 24-month-old heterozygotes do not display greater hearing loss or greater cochlear degeneration than their age-matched wild-type littermates [107]. In human, there is a corresponding gene for deafness, POU4F3, at the DFNA15 locus (OMIM: 602459 and [108]). In contrast to the mouse homozygous knockout, the mutant phenotype is dominant in human, and heterozygotes have a relatively late onset of hearing loss (age 18–30 years). It is rather unlikely that the human mutant allele can obstruct hair cell differentiation, but it could very well affect long-term survival of hair cells.

Atf2 (activating transcription factor 2; also called *Creb2*) knockouts exhibit decreased hearing, hyperactivity and vertical head tossing. Their saccular and utricular maculae are atrophic and significant reductions are observed in the numbers of sensory cells, stereocilia and otoconia, whereas nearby vestibular ganglia have decreased neuron cell bodies [109]. Balance impairment is also reported for *Itga8* (integrin α8) deficiency. Utricular hair cells of surviving *Itga8* knockouts lack stereocilia or contain malformed stereocilia, resulting in defective mechanosensation [110].

At E12–E13, *Apaf1* (apoptotic protease activating factor 1) is strongly expressed in the inner ear epithelium, where apoptosis occurs upon regulation by *Apaf1* and the anti-apoptotic molecule Bcl-X_L (M. Salminen and F. Cecconi, unpublished). *Apaf1* and the apoptosome are thus implicated in a Bcl-X_L-dependent pathway of programmed cell death, which contributes to the morphogenetic remodeling of the developing inner ear. Disruption of *Apaf1* results in embryonic demise with a phenotype affecting several aspects of developmental apoptosis, which is particularly prominent in the brain, inner ear and retinal tissues [111,112]. Preliminary characterization of *Apaf1* mouse knockouts shows that, at E9.5, the neuroepithelium lining the otocysts is thickened and disorganized. Moreover, the few surviving adult mutants display marked hyperactivity, compatible with a pronounced inner ear defect [112,113].

Deficiency of caspase 3 (a cysteine protease also implicated in apoptosis) leads to severe, age-dependent hearing loss, hyperplasia of supporting cells and degeneration of sensory hair cells and stereocilia [114–116]. Furthermore, *Casp3*-null mice exhibit progressive degeneration of the spiral ganglion neuron in the absence of apoptotic manifestations [115]. The greater epithelial ridge, a

remnant of the primordial organ of Corti that normally disappears within ten days after birth, is shown to persist throughout all of the turns of the cochlea in two-week-old knockouts, denoting immature cochlear morphology. The mapping of both the human CASP3 gene and the locus responsible for an autosomal dominant, nonsyndromic form of hearing loss (DFNA24; OMIM: 606282) to chromosome 4q35 suggests that *Casp3* knockout mice could be a model for this human condition. Although no hearing impairment is noted in *Casp3* heterozygotes, it is possible that individuals with DFNA24 might harbor a dominant–negative mutation in CASP3.

Similar to *Casp3*-deficient mice, *Cdkn1b*-deficient mice, lacking cyclin-dependent kinase inhibitor 1B (also called *p27* or *Kip1*), show postnatal hyperplasia of both supporting cells and hair cells. In adulthood, *Cdkn1b* knockouts display aberrant stereocilia, absence of hair cells and a severe hearing impairment [117–119]. *Cdkn1b* expression is normally induced in the primordial organ of Corti, correlating with the cessation of cell division of the progenitors of the hair cells and supporting cells at E12–E14. *Cdkn1b* expression is rapidly downregulated in differentiating hair cells, although it persists in postmitotic supporting cells of the mature organ of Corti. In *Cdkn1b*-targeted mice, the cells of the developing neuroepithelium undergo a period of prolonged cell division leading to the formation of supernumerary hair cells and supporting cells. The presence of proliferating cells in the postnatal organ of Corti, as well as the persistence of *Cdkn1b* expression in mature supporting cells, could contribute to the maintenance of quiescence in this tissue, and perhaps, to its ability to regenerate [118].

The development of normal auditory function requires thyroid hormone and thyroid hormone receptors (TRs). Congenital thyroid disorders are known to impair hearing, and severe deafness is often associated with iodine deficiency [120]. During embryonic and postnatal cochlear development, *Thra* (encoding TRα) and *Thrb* (encoding TRβ) are expressed in an overlapping fashion in the organ of Corti. Selective deletion of either the TRα1 isoform [121–123] or the TRα2 isoform [124], results in normal cochlear morphology, physiology and hearing. Interestingly, knockouts lacking both isoforms of *Thrb* (TRβ1 and TRβ2), display thyroid hyperactivity and profound hearing loss in the absence of cochlear malformations [125], whereas knockouts lacking specifically the TRβ2 isoform show no evidence of hearing impairment [126]. Recently, deletion of TRα2 has been shown to result in overexpression of TRα1. Moreover, when the TRα2 mutant allele is introduced into the *Thrb*-null background, the auditory and thyroid phenotypes caused by loss of TRβ are suppressed, suggesting that overexpression of TRα1 could substitute for the absence of TRβ [124]. The physiological differentiation of IHCs appears to be critically dependent on a *Thrb*-mediating pathway, as *Thrb* knockouts, which have normal hair cell transducer conductances, OHC electromotility and endocochlear potentials, show retarded expression of a potassium current, $I_{K,f}$, in cochlear IHCs [122]. Importantly, deletion of all known TRs generates new and aggravated phenotypes that include retarded differentiation of the sensory epithelium, aberrant formation of the tectorial membrane, defective electromechanical transduction in OHCs, and a low endocochlear potential [127]. It appears that distinct pathways mediated by TRβ alone or by TRβ and TRα1 together are involved in cochlear maturation.

Superoxide dismutases (SODs) are a class of antioxidant enzymes that convert the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide. Generation of $O_2^{\bullet-}$ during ischemia and noise exposure has been linked to cochlear damage [128,129]. Inactivation of *Sod1* (superoxide dismutase 1), which is abundantly expressed in the cochlea, results in increased susceptibility to noise-induced hearing loss and to central nervous system damage caused by axonal injury, ischemia and exposure to glutamate analogs [130–132]. In the absence of exposure to noise or ototoxic agents, both wild-type and *Sod1* knockouts exhibit similar patterns of sensory hair cell loss with advancing age; that is, a baso-apical progression of hair cell loss, with greater loss of OHCs than IHCs. However, the magnitude of degeneration is considerably greater in *Sod1*-null mice, indicating that *Sod1* exerts a modulatory effect on the rate and/or degree of cochlear hair cell loss, presumably through metabolic pathways that engage $O_2^{\bullet-}$ [133]. Increased noise-induced hearing loss is also reported for *Gpx1*-null mice, lacking glutathione peroxidase 1 (another major antioxidant enzyme), thus linking genetic impairment of antioxidant defenses, vulnerability of cochlear injury and cochlear degeneration, and recapitulating some features of age-related hearing loss in human [134].

Calbindin-D28k is a cytosolic calcium-binding protein located in the IHCs and OHCs, as well as in the vestibular hair cells of the apex of the cristae and in the striolae of the maculae. It is also abundant in all cochlear and some vestibular ganglion neurons and in distinct neurons in brainstem auditory nuclei. Despite its abundance in hair cells, calbindin-D28k appears to be dispensable in the basic hearing function of the inner ear, as shown by normal auditory brainstem evoked responses and distortion product otoacoustic emissions in *Calb1*-null mice. Moreover, its calcium buffering capacity does not seem to provide any physiological protection against a moderate acoustic inner ear trauma [135].

Functional loss of *Scnn1a* (sodium channel, nonvoltage-gated, type I, α polypeptide) appears to be of little consequence for IHC transduction in *Scnn1a*-null newborns, which display mechanically sensitive hair cells and produce normal transducer currents compared with controls [136]. By contrast, *Cacna1d* (calcium channel, voltage-dependent, L type, α1D subunit) inactivation leads to congenital deafness due to the complete lack of L-type currents in cochlear IHCs and degeneration of OHCs and IHCs [137].

Inner ear function: mechanotransduction

Architectural organization of the tectorial membrane

The tectorial membrane is an extracellular matrix of the inner ear that contacts the stereocilia bundles of specialized sensory hair cells. Sound induces movement of these hair cells relative to the tectorial membrane, deflects the stereocilia, and leads to fluctuations in hair cell membrane potential, transducing sound into electrical signals. α-tectorin, encoded by *Tecta*, is one of the major non-collagenous components of the tectorial membrane. Adult *Tecta*-null mice do not circle or head-bob, but possess tectorial membranes that are abnormally detached from the spiral limbus and lack all non-collagenous matrix. Normally, basilar membrane responses display a second resonance, indicating that the tectorial membrane provides a structure

against which OHC hair bundles can react. In *Tecta*-null mice, basilar membrane responses are tuned but less sensitive, and cochlear microphonics show clear differences in both phase and symmetry relative to those of controls, indicating that the tectorial membrane is essential for the appropriate gain and timing of cochlear feedback [138]. Currently, two distinct autosomal–dominant forms, DFNA8 (OMIM: 601543) and DFNA12 (OMIM: 601842), and one autosomal–recessive form, DFNB21 (OMIM: 603629), of sensorineural prelingual nonsyndromic deafness are known to result from mutations in the human TECTA gene [138,139].

Otogelin is an inner ear-specific glycoprotein expressed in all the acellular structures involved in the mechanotransduction process. *Otog*-null mice are deaf and exhibit remarkable imbalance. In the vestibule, the otoconial membranes and cupulae are detached from the neuroepithelia indicating that otogelin is essential for the anchoring of these membranes to the underlying neuroepithelia. Conversely, otogelin appears to be dispensable for the anchoring of the tectorial membrane to the spiral limbus and for the incorporation of major components of the tectorial membrane. Finally, ultrastructural analysis of type-A and type-B fibers in *Otog*-null cochlea implicates otogelin in the organization of its fibrillar network [140].

Mutations in the human COL11A2 (procollagen, type XI, α2) result in a congenital, non-progressive, non-syndromic sensorineural hearing loss (DFNA13; OMIM: 601868) which is mostly noted in the mid-frequencies [141]. Similar to the human condition, *Col11a2*-null mice show a moderate-to-severe hearing deficit that is related to architectural alterations in the tectorial membrane. Collagen fibrils course through the mutant tectorial membrane in an atypical and disorderly manner in the absence of other observable changes in the IHCs, OHCs, non-sensory epithelial cells, organ of Corti, neural structures or stria vascularis [141]. By contrast, the predominant otopathology observed in *Col4a3*-null mice, lacking procollagen, type IV, α3, is progressive tissue damage in the basement membranes surrounding the vessels of the stria vascularis, accompanied, in extreme cases, by the loss of basolateral infoldings in the marginal cells [142]. In humans, Alport syndrome is characterized by progressive glomerulonephritis culminating in renal failure and death, as well as progressive, high-frequency sensorineural hearing loss, and anterior lenticonus (OMIM: 301050). In *Col4a3* knockouts, the alterations noted in the strial vasculature are consistent with a frequency-independent hearing loss that contrasts with the high-frequency hearing loss reported in Alport patients. The correlation between ultrastructural abnormalities within the cochlea and hearing loss of individual knockouts warrants further investigation to elucidate the cause of the physiological effects and to resolve whether the *Col4a3* knockout represents a plausible model of the ear pathology noted in Alport syndrome.

Endolymph homeostasis and ion balance

Recycling of K^+ ions from the hair cells back into the endolymph maintains the appropriate high K^+ concentration ($[K^+]$) in the endolymphatic fluid and is therefore critical for normal hair cell transduction. K^+ ions are then presumably secreted into the endolymph by distinct regions of the labyrinth epithelium, namely the stria vascularis of the cochlea and the dark cells of the vestibular system. The stria vascularis normally generates an endocochlear potential of about +80 mV, which in conjunction with the high $[K^+]$ of the endolymph (~150 mM) drives the sensory transduction that potentiates hearing. The stria vacularis is composed of two epithelial barriers, the marginal cells, which secrete K^+ into the endolymph by an active mechanism, and the basal cells, which are coupled by gap junctions to intermediate cells. Of note, *Kcnj10* (potassium inwardly rectifying channel, subfamily J, member 10) transcripts have been localized in the membrane of strial intermediate cells [143], and not in the basolateral membrane of marginal cells, as originally reported [144]. Furthermore, a comparison of a drug sensitivity profile between the endocochlear potential and the membrane voltage of isolated intermediate cells has implicated KCNJ10 in the generation of the endocochlear potential [145]. Consistent with the reported absence of the KCNJ10 K^+ channel in the vestibular labyrinth [144], *Kcnj10*-null mice show a normal vestibular endolymphatic $[K^+]$ and volume. However, they exhibit profound hearing loss, fail to generate an endocochlear potential, and have a reduced cochlear endolymphatic $[K^+]$ and volume [146]. These findings establish that the KCNJ10 K^+ channel supplies the electromotive force for the generation of the endocochlear potential in concert with other transport pathways that establish the $[K^+]$ difference across the channel, and is an important mechanism for the delivery of K^+ to the strial marginal cells.

A similar collapse of the endolymphatic space has been noted in mice lacking either subunit of the apical KCNQ1-KCNE1 K^+ channel. KCNQ1 (potassium voltage-gated channel, subfamily Q, member 1) associates with the regulatory subunit, KCNE1 (potassium voltage-gated channel, Isk-related subfamily, member 1) to generate the cardiac repolarizing current, I_{Ks}. I_{Ks} is a candidate for endolymph secretion, as *Kcnq1* and *Kcne1* are both expressed in the marginal cells of the stria vascularis, and in the vestibular dark cells which have been shown to produce an I_{Ks}-like current at their apical membranes [147]. A prerequisite for I_{Ks} is further corroborated by inner ear defects associated with the Jervell and Lange-Nielsen syndrome (JLNS; OMIM: 220400), a human disorder characterized by profound bilateral deafness and a cardiac phenotype that has been linked to loss-of-function mutations in KCNQ1. In addition to cardiac dysfunction, *Kcnq1* knockouts exhibit a shaker/waltzer phenotype and are deaf [147–149]. By P3, the Reissner's membrane, which normally separates the scala media from the scala vestibuli, has collapsed onto the spiral limbus and the tectorial membrane of the organ of Corti. The ensuing reduction in endolymph volume continues postnatally, when extensive degeneration of the IHCs and OHCs in all turns of the cochlea is observed. The deafness and collapse of the endolymphatic spaces noted in *Kcnq1*-null mice resembles that observed in *Kcne1*-null mice [147,150,151] and *Slc12a2* (solute carrier family 12, member 2)-null mice [152–154]. *Slc12a2* is highly expressed on the basolateral membrane of marginal cells of the stria vascularis and encodes a Na^+-K^+-Cl^- co-transporter thought to be required in the penultimate step of recycling of K^+ back into the endolymph from the hair cells. Ultrastructural analysis of *Slc12a2* knockouts indicates defects including collapse of the Reissner's membrane, absence of hair cells and supporting cells in the tunnel of Corti, and mislocation of the tectorial membrane [155].

Signs of vestibular dysfunction (circling behavior, head-tilting and head-bobbing) and early-onset profound deafness are noted in *Slc26a4*-null mice lacking solute carrier family 26, member 4, also called pendrin [156]. Consistent with pendrin's anion transport role, *Slc26a4* (also called *Pds*) is expressed in discreet areas of the inner ear that are putatively critical in the regulation of endolymphatic fluid composition. Abnormal fluid homeostasis is already evident by E15.5, as shown by the dilation of the endolymphatic duct and sac, of the cochlea and saccule, and in some cases of the semicircular canals. In the organ of Corti, both the OHCs and IHCs exhibit severe, albeit varying extents of degeneration, the latter often associated with enlarged stereocilia. Degeneration of sensory hair cells exacerbates with age. Normal otoconia are mostly absent while giant otoconia are occasionally observed. *Slc26a4*-null mice show no evidence of thyroid disease and thus resemble patients with non-syndromic deafness associated with SLC26A4 (PDS) mutations identified in the deafness/goiter Pendred syndrome (OMIM: 274600).

A striking absence of otoconia is observed in the vestibular system of *Atp2b2* (also called *Pmca2*)-targeted mice, lacking ATPase, Ca^{2+} transporting, plasma membrane 2, known to be highly expressed in cochlear OHCs and spiral ganglian cells. The loss of otoconia implies that these calcium carbonate crystals might be formed and/or maintained, in part, by Ca^{2+} extruded into the endolymph by *Atp2b2*. Homozygous null mutants are deaf, and display a range of abnormalities in the organ of Corti as well as severe ataxia, balance impairment and cerebellar alterations. A range of histopathology is also detected in *Atp2b2* heterozygous null cochlear ducts, coincident with significant hearing loss [157]. In accord with these findings, spontaneous, deafness-causing mutations affecting *Atp2b2* have been reported for deafwaddler (*dfw*) mutant mice, which waddle and head-bob [158–160]. Heterozygous deafwaddlers exhibit elevated distortion product otoacoustic emission (DPOAE) thresholds and reduced amplitudes at high frequencies, compared with control mice that have been treated with furosemide, a drug known to cause a transient reduction of DPOAEs [159]. Further experimentation is required to define the molecular mechanisms underlying the aberrant histopathology and determine whether *Atp2b2* plays a direct role in mechanoelectrical transduction by hair cells.

Targeted deletion of *Aqp4* (aquaporin 4) leads to elevated auditory brainstem response (ABR) thresholds and a frequency-independent hearing deficit in the absence of abnormal ABR wave patterns or cochlear morphological changes, disclosing that an aquaporin water channel is involved in hearing [161,162]. Given the selective expression of *Aqp4* in epithelial cells (Hensen's cells, Claudius cells and inner sulcus cells) in the organ of Corti, these findings implicate AQP4 in the maintenance of osmotic balance during K^+ recycling. The hearing impairment in *Aqp4* knockouts is likely to result from modifications in the basal ionic composition of endolymph and/or OHC volume during mechano-electric signal transduction. Notably, loss of other water channel proteins expressed in the inner ear (including AQP1, AQP3 and AQP5) leaves ABR thresholds relatively unaffected [162].

Ephrins (Ephs) and their receptors have been implicated in guidance and fasciculation of axons, boundary formation in the brain, and neural crest cell migration [163]. Specifically, the cytoplasmic domain of *Ephb2* (Eph receptor B2 tyrosine kinase) is crucial for axon guidance and endolymph production [164]. *Ephb2*-null mice show aberrations in the early midline navigation of contralateral inner ear efferent growth cones, as well as a strain-specific hyperactive circling behavior and vestibular malfunction that is associated with ultrastructural abnormalities in the endolymph-producing vestibular dark cells, which normally express *Ephb2*. In adulthood, the ducts of *Ephb2*-null semicircular canals are collapsed, apparently because of reduced endolymph fluid production. In accord with a cell-autonomous role in fluid regulation, PDZ domain-containing proteins that bind the C-terminus of *Ephb2* appear to recognize the C-termini of certain anion exchangers and aquaporins (such as AQP1). These observations unveil a potentially novel mechanism by which Ephs and their receptors are linked to the ionic homeostasis and endolymph fluid production in the inner ear [164].

Cochlear melanocytes exist as neural crest-derived intermediate cells of the stria vascularis and are required for normal development of the cochlea, as shown by studies of mutant mice with congenital melanocyte anomalies [165]. Furthermore, Na^+K^+-ATPase and potassium channels of intermediate cells are required for production of endocochlear potential and for preparation of ionic milieu in the stria [166]. Consistent with this notion, the melanocyte deficiency due to some gene disruptions results in hearing impairment in mice and humans. Null mutations in the mouse gene encoding endothelin receptor type B (*Ednrb*) [167] or its ligand endothelin-3 (*Edn3*) [168] have identified a key role for this pathway in the development of two neural crest-derived cell lineages, myenteric ganglion neurons and epidermal melanocytes. *Ednrb* and *Edn3* knockouts display aganglionic megacolon associated with white-spotted coat color [167,168], closely resembling the phenotype manifested by the natural mouse mutants *piebald-lethal* and *lethal spotting*, respectively [169]. In fact, crossbreeding analyses have confirmed that *Ednrb* is allelic to the *piebald* locus [167], and *Edn3* is allelic to the *lethal spotting* locus [168]. Earlier observations suggested that *piebald-lethal* mutants exhibit an inner ear abnormality, possibly as a result of defects in the part of the acoustic ganglion derived from the neural crest, or as a secondary consequence of the absence of neural crest-derived melanocytes of the inner ear [170]. In human, the Waardenburg–Shah syndrome (also called WS4; OMIM: 277580), is an auditory-pigmentary syndrome which comprises Waardenburg syndrome and Hirschsprung disease, and is associated with hearing impairment and pigmentation anomalies of the skin and the iris. To date, three genes responsible for WS4 have been cloned: *SOX10* (SRY-box containing gene 10), *EDN3* and *EDNRB*.

It has been postulated that K^+ ions are recycled from the endolymph through the sensory epithelium and back to the stria vascularis by moving from cell to cell through gap junctions [171]. K^+ ions exit from OHCs presumably through KCNQ4 (potassium voltage-gated channel, subfamily Q, member 4), a new K^+ channel which is expressed in OHCs and mutated in a form of non-syndromic dominant deafness (DFNA2; OMIM: 600101) in humans [172,173]. Following their exit from OHCs, K^+ must be removed, partially by uptake into the supporting Dieters' cells. In the current K^+ recycling model,

K^+ then diffuses through a gap junction system connecting Deiters' cells and fibrocytes back to the stria vascularis [171]. New evidence suggests that *Slc12a7* (solute carrier family 12, member 7, also called *Kcc4*) is instrumental in siphoning K^+ ions after they exit from OHCs into Deiters' cells, where K^+ enters the gap junction pathway [174]. At P8, before the onset of hearing, the mouse *Slc12a7* gene is expressed in the stria vascularis and in almost all cells of the organ of Corti. At P14, when the organ has matured to allow hearing, *Slc12a7* expression is uniquely restricted to the Deiters' cells, which support OHCs at their basal pole, and the phalangeal cells enveloping IHCs. During the third postnatal week, *Slc12a7*-null mice exhibit progressive hearing loss, and display a striking degeneration of OHCs in the basal turns of the cochlea as well as neuronal degeneration in the spiral ganglion, in the presence of an intact Reissner's membrane. Limited survival of OHCs in the apical turns of the cochlea is likely to account for the residual hearing ability observed in adult knockouts [174]. Notably, deafness is associated with renal tubular acidosis, indicating a role for *Slc12a7* in Cl^- extrusion across the basolateral membrane of acid-secreting α-intercalated cells in the nephron. Given that the intracellular anions chloride and bicarbonate act as extrinsic voltage sensors of the OHC motor protein prestin [175], it is plausible that alterations in $[Cl^-]$ within the organ of Corti might further compromise OHC function in *Slc12a7*-null mice.

Mutations in the human GJB2 gene, encoding gap junction membrane channel protein β2 (also called connexin 26), account for ~50% of autosomal-recessive non-syndromic deafness (DFNA3, DFNB1) in humans [176]. GJB2 has been implicated in the recycling of K^+ ions back to the endolymph of the cochlear duct following stimulation of the sensory hair cells (see above). *Gjb2*-null embryos die at E11 because of impaired transplacental uptake of glucose [177]. Thus, a knockout directed specifically to the inner ear might address human DFNB1/GJB2 pathology more appropriately. Similarly, mutations in the human GJB3 gene (gap junction membrane channel protein β3; also called connexin 31), have recently been reported to cause deafness. In mice, *Gjb3* inactivation leads to embryonic demise and transient placental dysmorphogenesis; however, no morphological or functional defects of the inner ear are noted in surviving knockouts [178].

Axon pathfinding and innervation of inner ear sensory neurons

Recent evidence suggests that a cascade of transcription factors is required for neuronal survival and differentiation of inner ear sensory neurons [179]. To illustrate, *Neurod3* (neurogenic differentiation 3; also called neurogenin 1), is specifically expressed in the VIIIth cranial ganglion/neurogenic region at the otocyst stage. *Neurod3* deletion results in the development of smaller sensory epithelia that are devoid of innervation, with fewer, morphologically unaffected hair cells, suggesting that normal innervation is not required for hair cell differentiation, at least until birth [180]. The VIIIth ganglion fails to form, and the maculae of both the utricle and saccule are reduced in size. The modiolus (the bony tube that forms the central axis of the cochlea) is missing, and the saccule forms only a tiny extension on the utricle leading to the ductus reuniens connecting to the cochlea. The *Neudo3*-null cochlea shows only 1.25 turns instead of the 1.75 turns found in wild-type ears (Fig. 2). Importantly, knockouts preserve only the distal, epibranchial, placode-derived visceral afferents in the facial and stato-acoustic nerve that project to the solitary tract [180].

Neurod1 (neurogenic differentiation 1), a downstream target of *Neurod3*, is also critical for the survival of inner ear sensory neurons during the early phases of differentiation. In addition to motor dysfunction resulting from cerebellar defects, *Neurod1* knockouts show severe ataxia and deafness [181–184]. In contrast to *Neurod3* knockouts, where a complete loss of both afferent and efferent fiber projections is observed [180], some sensory neurons still remain in *Neurod1*-null mice. The pattern of remaining sensory neurons and afferent innervation shows an early onset of developmental loss that stabilizes by E14.5. Strikingly, the migration of remaining vestibular sensory neurons is misdirected, and the projection of fibers to all the vestibular epithelia is highly irregular [184]. The failure of *Neurod1*-null sensory neurons to express *Ntrk2* (neurotrophic tyrosine kinase, receptor, type 2; also called *TrkB*) and *Ntrk3* (neurotrophic tyrosine kinase, receptor, type 3; also called *TrkC*) implicates Trk signaling in the survival of young differentiating neurons [184]. The *Neurod1* knockout phenotype is similar to that observed in compound 'Trk' mutations [185]. In the cochlea, the loss of basal turn innervation and spiraling of afferent and efferent fibers along the IHCs toward the basal tip of the cochlea resembles the defect in *Ntf3* (neurotrophin 3)-null [186–189] and *Ntrk3*-null mutants [185,189–192]. However, the pattern of neuronal loss in *Neurod1* knockouts is most similar to the one observed when the *Ntrk3*-null mutation is combined with *Ntrk2* heterozygosity: these compound mutants show a high degree of inter-individual variation in the density of remaining afferent and efferent fibers [185]. Notably, functional deletion of either *Bdnf* (brain derived neurotrophic factor) [187,189,193,194] or its associated receptor, *Ntrk2* [185,189–192], results in a severe reduction in the number of vestibular neurons and a loss of all innervation to the semicircular canals [189]. Furthermore, mice lacking both *Bdnf* and *Ntf3*, or *Ntrk2* and *Ntrk3* lose all innervation to the inner ear, thereby establishing that these neurotrophins and their associated receptors are required for normal afferent innervation of the inner ear [189].

Pou4f1 (POU domain, class 4, transcription factor 1; also called *Brn3.0* and *Brn3a*) is expressed in the facial-stato-acoustic ganglion before sensory neuron differentiation and innervation of the otocyst [195]. Targeted loss of *Pou4f1* leads to diminished neuronal size and downregulation of gene expression, including that of *Ntrk3*, *Pva* (parvalbumin) and *Pou4f2* (POU domain, class 4, transcription factor 2; also called *Brn-3.2* and *Brn3b*) in the spiral ganglion, indicating that these are downstream targets of *Pou4f1* [195,196]. Afferent innervation is ultimately established, despite an observed delay in the development of axon projections to the cochlea and the posterior vertical canal at E13.5. The selective loss of *Ntrk3* neurons in the spiral ganglion of *Pou4f1*-null cochlea leads to an innervation abnormality similar to that of *Ntrk3*-null mice (see above). Also, efferent axons that use the afferent fibers as a scaffold during pathfinding display irregular routing to the inner ear. Collectively, these findings unveil new roles for *Pou4f1* in the control of survival, differentiation, migration and axon pathfinding of inner ear sensory

neurons by regulation of downstream genes [195].

Postnatally, *Igf1* (insulin-like growth factor-1) is strongly expressed in the cochlea and in a subpopulation of neurons of the cochlear ganglion, as well as in the stria vascularis, spiral limbus and supporting cells of the organ of Corti. Analysis of young *Igf1*-null mice reveals a shortened cochlea and cochlear ganglion, an immature tectorial membrane and a significant loss of auditory neurons. Loss of IGF-1 severely affects postnatal survival, differentiation and maturation of the cochlear ganglion cells, and causes abnormal innervation of the sensory cells in the organ of Corti [197].

Chrna9 (cholinergic receptor, nicotinic, α polypeptide 9; also called *Acra9*) appears to be the only nicotinic receptor subunit expressed by cochlear OHCs, which are contacted by descending, primarily cholinergic, efferent fibers originating in the central nervous system. *Chrna9*-deficient mice are externally normal and show a Preyer's reflex with no apparent deficits in balance or movement. However, most mutant OHCs are innervated by a large single terminal instead of multiple smaller terminals, suggesting a role for *Chrna9* in normal synaptic connections between efferent fibers and hair cells. *Chrna9*-null mice are functionally de-efferented and fail to exhibit suppression of cochlear responses during efferent fiber activation [198].

The presence of tenascin-C along the growth routes of nerve fibers in the spiral lamina and in the organ of Corti suggests that this glycoprotein could participate in afferent synaptogenesis, and serve as a substrate for neural growth in the cochlea, particularly for those fibers growing towards the OHC region. However, tenascin-C knockouts are not hearing-impaired and have normal cochlear anatomy [199], although some behavioral deficits have been noted [200]. Perturbation studies *in vitro* and analyses of additional *Tnc*-knockout strains should help clarify the role of tenascin-C in cochlear development and regeneration.

Deafness and neuromotor deficits are observed in a knockout model of metachromatic leukodystrophy, a lysosomal sphingolipid storage disorder caused by the deficiency of *As2* (arylsulfatase A) and typified by progressive demyelination and multiple neurological symptoms (OMIM: 250100). *As2*-null inner ears show a nearly complete demyelination and neurodegeneration of the acoustic ganglion, which is accompanied by the loss of brainstem auditory-evoked potentials at 12 months. The molecular basis of the specific susceptibility of the mouse acoustic ganglion to demyelination and neurodegeneration remains unclear, as these knockouts exhibit an unexpectedly mild phenotype in their central and peripheral nervous system [201–203].

Wnt signaling has been implicated in the control of proliferation and in synapse formation during neural development, and these actions are presumed to be mediated by the Frizzled family of cell surface receptors. The expression of *Fzd4* (frizzled homolog 4, *Drosophila*) in auditory and vestibular hair cells and late-onset hearing loss exhibited by *Fzd4* knockouts in the absence of auditory or vestibular hair cell death, suggest that *Fzd4* is important in maintaining hair cell function but not critical for the initial development and functioning of the inner ear. Auditory brainstem responses in adult mutants localize the hearing deficit to the peripheral auditory system, although the possibility of a coexisting central auditory defect has not been excluded [204].

Finally, exposure of *Slc1a3*-null mice, lacking solute carrier family 1, member 3, to an acoustically traumatizing stimulus results in increased accumulation of glutamate in the perilymph, and aggravates hearing loss due to afferent dendrite swelling below the IHCs. This study confirms that *Slc1a3*, a Na^+-dependent glutamate receptor which is specifically expressed in the region of IHCs as well as in the fibrocytes in the limbus and the spiral ligament, acts as a neuroprotector against glutamate excitotoxicity during acoustic overstimulation. Furthermore, it clearly indicates that glutamate is a neurotransmitter of synapses between IHCs and the auditory nerve [205].

Conclusion

A notable array of auditory and vestibular mouse knockouts is already available as a valuable resource for studying specific auditory gene function and morphological characterization of the mammalian inner ear. The observed phenotypic consequences provide supporting evidence that the otocyst resembles a mosaic in which different structural components are under independent genetic control for their specification and/or morphogenesis. The generation of conditional knockouts, designed to restrict the effects of a given mutation to a specific developmental stage and/or cell type, and development of knock-in models, whereby normal genes are replaced by mutant alleles that faithfully mimic human deafness alleles, will undoubtedly enrich our understanding of the molecular mechanisms involved in the inner ear developmental cascade. Such genetic approaches will in turn help us decipher the causes of congenital forms of human deafness and evaluate the design and efficacy of treatments for hearing loss.

Acknowledgements

My sincere thanks to Kenneth R. Johnson (The Jackson Laboratory, Bar Harbor, Maine, USA) for his valuable suggestions and critical review of the manuscript, and Doris K. Wu (The Laboratory of Molecular Biology, NIDCD, Rockville, Maryland, USA) for her kind contribution of paint-injected series of illustrations on mouse inner ear development.

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