Non-syndromic vestibular disorder with otoconial agenesis in tilted/mergulhador mice caused by mutations in otopetrin 1

Belen Hurle1, Elena Ignatova2, Silvia M. Massironi3, Tomoji Mashimo4, Xavier Rios1, Isolde Thalmann2, Ruediger Thalmann2 and David M. Ornitz1,*

1Department of Molecular Biology and Pharmacology, and 2Department of Otolaryngology, Washington University Medical School, 660 South Euclid Ave, St Louis, MO 63110, USA, 3Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Av. Professor Lineu Prestes, 1730, Cidade Universitária, CEP 05508-900, São Paulo, SP, Brazil and 4Unite de Genetique des Mammiferes, Institut Pasteur, 25 Rue du Docteur Roux, F-75724 Paris Cedex 15, France

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Otoconia are biominerals within the utricle and saccule of the inner ear that are critical for the perception of gravity and linear acceleration. The classical mouse mutant tilted (tlt) and a new allele, mergulhador (mlh), are recessive mutations that affect balance by impairing otoconial morphogenesis without causing collateral deafness. The mechanisms governing otoconial biosynthesis are not known. Here we show that tlt and mlh are mutant alleles of a novel gene (Otopetrin 1, Otop1), encoding a multi-transmembrane domain protein that is expressed in the macula of the developing otocyst. Both mutants carry single point mutations leading to non-conservative amino acid substitutions that affect two putative transmembrane (TM) domains (tlt, Ala151→Glu in TM3; mlh, Leu408→Gln in TM8). Otop1 and Otop1-like paralogues, Otop2 and Otop3, define a new gene family with homology to the C. elegans and D. melanogaster DUF270 genes.

INTRODUCTION

The vestibular system within the inner ear is responsible for the perception of gravity and motion, and for the maintenance of balance. The peripheral vestibular organs form a complex three-dimensional structure of interconnected chambers located within the inner ear that include three semicircular canals, the utricle and the saccule. Sensory transduction depends on the inertial mass of thousands of tiny biomineral particles, otoconia, embedded in a gelatinous membrane that overlies the sensory epithelium of the saccule and utricle (1,2). Forces due to gravity and linear acceleration act through otoconia to deflect the stereocilia of the sensory hair cells and create vestibular evoked potentials (VsEPs).

Otoconia are critical for the correct processing of orientation and positional information. In humans, dislodging of otoconia due to ototoxic drugs, infection or trauma causes incapacitating chronic recurrent vertigo (3,4). Age-dependent degeneration of otoconia is a high-risk factor for loss of balance, contributing to hip fracture and accidental death in the elderly (5,6). Similarly, the absence of gravitational stimulation of otoconia under microgravity leads to space adaptation syndrome in astronauts, akin to terrestrial motion sickness (7). In mice, congenital absence of otoconia completely abolishes the VsEPs in response to linear acceleration (8).

Almost nothing is known about the development, maintenance and potential for regeneration of otoconia, in part because animal models with non-syndromic vestibular phenotypes are extremely rare (9,10). The original tilted mutant arose spontaneously in the C57BL/6J background and is one of the three classical mouse mutants presenting with a non-syndromic vestibular disorder [tilted (tlt; Chr5) (11); tilted head (thd; Chr1) (12–14); head tilted (het; Chr17) (15)]. Homozygous tlt mice lack a perception of gravity and spatial orientation, show head tilting behavior and an inability to swim. The non-swimming phenotype correlates with the total absence of otoconia in the saccule and utricule or, infrequently, with the presence of a few giant otoconia in the saccule (16). Unlike most available mutants and knockout mice with abnormal otoconia (www.jax.org/hmr/index.html) (17), tlt is not accompanied by deafness, congenital or progressive degeneration of the cochlea, degeneration of the...
Mlh was mapped using a standard interspecific backcross to mouse Chr 5 (Fig. 1) and was found to co-segregate with markers D5Mit13, D5Mit75, D5Mit127, D5Mit149, D5Mit182, D5Mit351 and D5Mit354. This region overlapped with the tlt locus as determined by Ying et al. (19).

**RESULTS**

Tlt and mlh mutations are allelic

Mlh arose from a medium-scale ENU mutagenesis screen (18). Mlh/mlh mice are viable and fertile. Homozygotes hold their head tilted to one side, shake when suspended by the tail, and exhibit non-swimming behavior indicative of vestibular dysfunction. Mlh and tlt phenotypes are indistinguishable. Mlh was mapped using a standard interspecific backcross to

vestibular organs, abnormal central vestibular function and/or multi-organ involvement (16). Thus, the identification of the gene responsible for the tlt phenotype could provide, for the first time, a tool to investigate the specific mechanisms that regulate otococh development and function.

Through a positional cloning strategy, we have characterized tlt as a mutant allele of a novel gene designated Otopetrin 1 (Otop1). A second mutant allele, Mergulhador (mlh), or diver in Portuguese, arose in a medium-scale ENU (N-ethyl-N-nitrosourea) mutagenesis screen (18), and exhibits non-swimming behavior and lack of otocochia, similar to the tlt mutant mouse. Both tlt and mlh carry single-point mutations leading to non-conservative amino acid substitutions that affect two putative transmembrane domains of Otop1. This is the first molecular characterization of mutations that specifically impair the morphogenesis of otocochia without affecting other organs, other inner ear structures or hearing function.

**Evaluation of candidate genes within the tlt interval**

To pinpoint the position of tlt within the available 900 kb physical map spanning the tlt locus (20), new SSLP markers were developed and mapped throughout the minimum tiling contig. The minimum genetic interval for tlt was narrowed to a 450 kb region delineated by D5Dmo9 and D5Dmo10, with only one crossover between tlt and each marker in 894 meioses (Fig. 2A, haplotype data not shown). Three known genes (Drd5, Wdr1 and Glut9) lie within this segment. BlastN (21) and GENSCAN (22) inspection of the remaining genomic sequence suggested an additional new gene on BAC RPC23-426E16 that partially matched UniGene cluster Mm.204765. Expression of Wdr1 and Mm.204765 (but not Drd5 or Glut9) was observed in E18.5 mouse otocyst and P0 mouse inner ear RNA by RT–PCR (data not shown). Sequencing of the exons and splice junctions of Drd5, Wdr1 and Glut9 in tlt/mlh mice, and the parental C57BL/6J strain, did not detect abnormalities that could be related to
Figure 2. Physical, transcription and mutation map of the tlt locus. (A) The tlt physical map from D5Dmo3 to D5Dmo5 (20) links five overlapping RPCI-23 BAC clones (241E18, 426E16, 268D19, 115A12 and 456A210) that span ~900 kb of genomic DNA. The asterisks indicate the T7 end of each clone, the open circles represent relevant SSLP markers within the contig. D5Dmo9 and D5Mit10 delimit the tlt minimum region with only one crossover between tlt and each marker in the F2 intercross panel (19). Candidate genes within this interval (Drd5, Wdr1 and Glut9 and UniGene Mm.204765) and their transcriptional orientation are shown (bold arrows). (B) Map of UniGene Mm.204765 showing coding exons (black boxes); untranslated regions (white boxes); CpG dinucleotide rich regions (orange boxes). The positions of the tlt and mlh mutations (exons 3 and 6, respectively) are indicated by asterisks. (C) Sequence chromatographs spanning the tlt (top) and mlh (bottom) mutation sites compared with that of wild-type controls. Nucleotide changes and resulting non-conservative amino acid changes are highlighted in red. Amino acids are numbered according to the position of the first methionine in the most abundant transcript (Otop1-a). The tlt and mlh point mutations were reconfirmed by restriction analysis that detects a new TaqI restriction site in the Otop1tlt allele and loss of a MscI restriction site in the Otop1mlh allele. The sizes of the PCR products (PCR) and endonuclease restriction (RE) fragments are indicated.
the tlt phenotype (data not shown). We therefore focused on characterization of the Mm.204765 cluster.

To identify the Mm.204765 full-length transcript, overlapping cDNA products were obtained from E18.5 mouse otocyst RNA by RT–PCR using primers positioned in exons 2–7 and a start of the gene was characterized by 5’ RACE. A total of seven exons were identified and assembled into two ORFs, differing in their 5’-ends (a and b, Figs 2B and 3A). The most abundant transcript in mouse otocyst mRNA, (a), joined exons 2–7 and contained a 1802 bp ORF and a 1313 bp 3’ UTR with three consensus polyadenylation sites (positions 2416, 3113 and 3129) all within exon 7. The putative translation initiation codon in Otop1-a is an in-frame methionine in exon 2 at position 25 bp in the cDNA. A rare alternative splice form, (b), in which exon 1 is joined to an internal splice site at position +130 within exon 2 to produce an alternative ORF of 1846 bp with a different 5’ end was also detected. The first in-frame methionine for this transcript is located in exon 1, within a favorable context for initiation with a purine at position −3 and a guanine at +4 (23). The existence of exon 1 is consistent with the Mm.204765 gene structure predicted by the Celera algorithm (www.celera.com).

Mutation screening of the Mm.204765 gene revealed that the tlt allele contained a C476→A change in exon 3, leading to an Ala151→Glu substitution. The mlh allele carried a T1247→A change in exon 6 leading to a Leu408→Gln substitution (Fig. 2C). To genotype these alleles, two independent PCR/restriction endonuclease assays were developed that specifically target the mutation sites in tlt and mlh. These assays were conducted on genomic DNA amplified from strains SJL/J, C3H/H, BALB/CJ, 101/H and C57BL/6J, and mutations were only found in DNA from heterozygous or homozygous tlt and mlh mice (Fig. 2C). To investigate further whether the tlt and mlh substitutions could be naturally occurring polymorphisms, 43 high-throughput genomic reads from the Celera mouse trace database that partially span the Mm.204765 gene in three additional backgrounds (DBA/2J, A/J and 129/SVJ) were retrieved and analyzed. Overall, nine strain-specific polymorphisms (seven silent and two non-silent) were found but in no case were the tlt or mlh sequence-variants detected. We therefore concluded that the observed non-conservative substitutions in Mm.204765 (subsequently named Otopetrin 1, Otop1) are not neutral polymorphisms, and account for the observed phenotype of tlt and mlh mice.

**Otop1 belongs to a novel gene family**

tBLastN searches, using murine Otop1 amino-acid sequence as a query, revealed multiple Otop1 orthologous and Otop1-like paralogous sequences in the EST and genomic databases. Comparison of Otop1 homologous sequences was carried out through EST clustering and the alignment of EST assemblies to corresponding genomic contigs, combined with GENSCAN predictions and RT–PCR amplification, to resolve ambiguities in gene structures.

Orthologous sequences were identified for mouse Otop1 in rat, human, *F. rubripes* (fugu) and *D. rerio* (zebrafish), showing 95, 77, 44 and 41% amino acid identity, respectively (Table 1). Complementary mapping data from the Ensembl projects (wwwensembl.org/), the WUGZGR integrated zebrafish map (http://zfish.wustl.edu/) and the Ratmap database (http://ratmap.gen.gu.se/) confirmed that these genes map to syntenic regions in mouse Chr5, human Chr4p16.2, rat Chr14p21-14q21 and zebrafish LG14. No orthologous mapping information was available for Fugu scaffold_3725, where Otop1 was electronically identified. Additionally, tandem assemblies representing two Otop1-like genes (hereafter named Otop2 and Otop3), were found on mouse Chr11q21 and its syntenic location on human Chr17q25.3. Mouse Otop2 and Otop3 showed 34 and 30% amino acid identity with Otop1, respectively.

Comparison of the cDNA assemblies and genomic sequence indicated that all Otop family members share a common gene structure, with conserved splice-site positions. Some variability was observed in the structure of the 5’ UTR and N-terminal coding exons, and in the length of exon 6 (Fig. 3A). Among the Otop1 and Otop1-like proteins that have been identified, only mouse Otop1-b showed a recognizable N-terminal signal peptide. Pattern recognition algorithms TMHMM2.0 (24) and TMPred (25), and the protein localization program PSORT II (26), predicted a variable number of transmembrane (TM) domains. TM domain annotations were accepted according to the number of algorithms predicting them, and their occurrence in an orthologous gene. A conserved pattern of seven TM domains (namely TM1, TM2, TM3, TM6, TM7, TM9 and TM10) was strongly predicted in all family members by all algorithms used. Three additional TMs, in conserved positions, were predicted by either TMHMM2.0 (TM5) or TMPred (TM8) or both (TM4). Based on these annotations we suggest that the Otop family members are 10-span transmembrane domain proteins, with membrane topology type 3a, and a cytoplasmic orientation of the N- and C-termini. The tlt and mlh mutations occur in TM3 and TM8 of mouse Otop1, respectively, and could therefore affect the transmembrane domain structure, and/or tertiary structure of the protein (Fig. 3B).

The extent of the sequence conservation in the Otop family was examined by CLUSTALW alignment (27) of the amino acid sequences followed by manual adjustment (Fig. 3C). Amino acid sequence divergence within the family occurred primarily in the N-terminus and in the loop between TM8 and TM9. Two recognizable regions showed higher levels of amino acid sequence conservation. These regions spanned TM3–TM5 in the middle of the protein, and TM9 and TM10 in the C-terminus (Fig. 3B). Searches of the NCBI Conserved Domain Database (28) with these two regions identified significant homology with the Pfam03189 motif. Pfam03189 is a 404 amino acid consensus sequence domain of unknown function that defines the DUF270 family, with members in *C. elegans* and *D. melanogaster* (Table 1). The two regions of maximum homology with the Otop family (hereafter designated DUF270-1 and DUF270-2), match positions 1–184 and 351–404 of the Pfam03189 consensus sequence, respectively (Figs 3B and 4). PSORT II also identified a leucine zipper pattern (PS00029) in several Otop sequences, although its functionality is unclear because of its partial overlap with either TM4 or TM9 (data not shown). No other identifiable functional motifs were detected.

**Otop1 and Oe90/95 exhibit complementary mRNA expression patterns**

The inorganic phase of otoconia consists of CaCO₃ crystals arranged in a calcite lattice (29). Accordingly, Ca²⁺ and CO₃²⁻
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NA, non available.

*EST not grouped into a UNIGENE cluster.

aElectronic annotation.

bDouble UNIGENE entries represent 5’ and 3’ end sequences of the same gene.
ions constitute the most important building material. Because of the low concentration of these ions in endolymph, nucleation and growth of CaCO₃ crystals can only proceed with the help of organic facilitators, mainly in the form of acidic glycoproteins (1). The principal otoconial matrix protein in mammals, otoconin-90/95 (Oc90/95), is a heavily glycosylated 90 kDa acidic protein that is closely related to phospholipase A₂ (PLA₂) (30,31).

In the developing otocyst, Oc90/95 is expressed exclusively in the nonsensory epithelia, adjacent and opposite to the macular sensory region (Fig. 5A) (30,31). In contrast to Oc90/95, expression of Otop1 is detectable in macular epithelia (most likely in supporting cells), and is absent from nonsensory regions (Fig. 5B). OC90/95 protein, like its corresponding mRNA, is synthesized in the nonsensory epithelia (Fig. 5C). Beginning on embryonic day 15.5, OC90/95 is secreted into the endolymphatic lumen, where it aggregates into the supramacular region, and becomes incorporated into the matrix of developing otoconia. In contrast, Otop1 protein was not detected at its site of synthesis in the macular epithelia. However, strong expression was visible in the overlying gelatinous membrane (Fig. 5D). Because Otop1 appears to be a multi-transmembrane domain protein, its localization in the gelatinous membrane suggests that it may be integral to the membrane vesicles that are released.
into the gelatinous membrane. Although Otop1 is expressed in tlt/tlt mice, the protein no longer appears localized to the gelatinous membrane (data not shown).

In addition to expression in the inner ear, Otop1 appears to be expressed in a number of other tissues. An RT–PCR expression screen of a panel of mouse tissues identified expression of Otop1 in thymus, heart, kidney, skin, stomach, adrenal gland and lactating mammary gland (data not shown). Furthermore, Otop1 and related sequences were represented in a wide range of EST libraries (Table 1).

**DISCUSSION**

The embryonic development, anatomy and physiology of the auditory and vestibular organs are intricately related, making it difficult to study vestibular function apart from hearing. Many genes affecting auditory function have been identified due to the abundance of well characterized human and mouse mutations (17). In contrast, the study of genes affecting vestibular function has lagged behind, in part because of the paucity of animal models with non-syndromic vestibular dysfunction. Nevertheless, otocional pathology is a significant medical problem that can lead to mild to severe vestibular impairment, benign paroxysmal positional vertigo, dizziness, ataxia and, in the elderly, loss of balance and resulting injury or accidental death (1). Chronic recurrent vertigo, unassociated with auditory or neurological symptoms, is one of the most common reasons for referral to otolaryngology services (4).

Despite the prevalence of vestibular dysfunction in humans, of the 150 murine loci linked to deafness and/or vestibular dysfunction, only three (tlt, thd and het) specifically affect otocional development without causing collateral deafness, disrupted inner ear morphogenesis or stereocilia degeneration (17). The identification of Otop1 as the gene underlying the tlt and mlh mutations provides, for the first time, a tool to investigate the specific mechanisms that regulate otocional development and function.

Morphogenesis of otocional depends on a complex interaction between organic and inorganic processes that are not well understood (1,30). Sensory epithelia within the inner ear organs are covered by an extracellular matrix which transmits forces to the underlying hair cells. Three types of extracellular structures have been described, the cupula (covering the crista within the
ampullae of the semicircular canals), the otolithic membrane
(coversing the macula of the saccule and utricle), and the
tectorial membrane (covering the organ of Corti). These
acellular structures are tailored to the specific function
of each sensory epithelium. Non-collagenous matrix protein
components of the various membranes include
\( \alpha \)- and \( \beta \)-tectorin, otogelin, otoancorin and OC90/95.
OC90/95, the principal organic constituent of
otoconia, is synthesized by the nonsensory epithelia throughout
all the divisions of the inner ear, including the saccule,
semicircular canal system and cochlea, and is secreted into the
endolymphatic lumen. Because otoconia are only
formed over the macula of the utricle and saccule, it is
necessary to postulate that otoconial nucleation and maturation
requires an interaction between OC90/95 and other factors
unique to the gravity receptor organs. The most likely
site of such an interaction is the otolithic membrane.

Otop1 mRNA was expressed in the macular sensory
epithelium, and Otop1 protein was detected in the overlying
gelatinous membrane, but not in the sensory epithelium. The localization of Otop1 in the gelatinous
membrane was detected by efficient transport of Otop1 into microvesicles
that are abundantly secreted from the supporting cells, and the accumulation of the protein at its postulated site of interaction
with OC90/95 or other otocanial matrix proteins. Importantly,
the spatial and temporal expression pattern of Ocot1 appears
to be unchanged in \( tlt/tlt \) mutants. Deficiencies of OC90/95 are therefore unlikely to be
responsible for the otoconial agenesis in \( tlt/tlt \) mice.

Secondary structure predictions for Otop1 suggest a topology
that is compatible with a transporter, ion channel or receptor. An
attractive hypothesis is that Otop1 regulates the local ionic
environment in the vestibular organs, critical for the formation of
otoconia. Two examples of integral transmembrane
domain proteins that affect inner ear fluid homeostasis and
otoconia development are the plasma membrane Ca\(^{2+}\)ATPase (Pmca2) and pendrin (Pds). Pmca2 regulates the
intracellular free Ca\(^{2+}\) concentration of the hair bundle by
extruding Ca\(^{2+}\) from the cell into the endolymph. Pmca2 has
been proposed as a source of the calcium used in the formation
and maintenance of otoconia. Similar to \( tlt/tlt \) and \( mlh/mlh \) mice,
Pmca2\(^{-/-}\) mice show an intact macular epithelium and overlying
gelatinous membrane but lack otoconia in both the saccule and
utricle. Unlike \( tlt/tlt \) and \( mlh/mlh \) mice, Pmca2 null mice have
deficits in cochlear function. Pds is an anion transporter thought
to be important for endolymphatic fluid resorption in the inner
ear. Like with \( tlt/tlt \) mice, Pds\(^{-/-}\) mice also lack otoconia or
develop giant otoconia. However, unlike the \( tlt/tlt \) mutants, Pds\(^{-/-}\) mice develop stereocilia degeneration, and both
Pds\(^{-/-}\) and Pmca2\(^{-/-}\) mice show a range of abnormalities in the
anatomy of the organ of Corti, and are profoundly deaf. Tlt and
mlh mice differ from Pds\(^{-/-}\) and Pmca2\(^{-/-}\) mice and the vast

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**Figure 4.** Alignment of the DUF270 homology regions. The DUF270-I and DUF270-II homology domains from the three mouse ototetins are aligned with
five \( C. \) elegans DUF270 sequences. The shared amino acid residues and the position of the TM domains are as indicated Figure 3C. The corresponding Pfam03189
consensus sequence, as deduced from the alignment of 17 invertebrate sequences (\( C. \) elegans, nine proteins; \( D. \) melanogaster two proteins; \( A. \) gambiae five proteins) is indicated in red. DUF270-I spans positions 1–184 and DUF270-II spans positions 351–404 of the Pfam03189 sequence.
majority of the other inner ear mouse mutants in that their phenotype only affects otoconia morphogenesis. This may be due to the very restricted expression of Otop1 in the macular sensory epithelium, to expression of Otop1 in a restricted developmental stage, or to the possible hypomorphic nature of the missense mutations in Otop1.

The vestibular system is one of the phylogenetically oldest sensory systems in vertebrates (39,40). In zebrafish, the ability to initiate otolith formation is limited to a critical period from 18.5 to 24 h post fertilization (41,42). In contrast to the scarcity of mouse otoconia mutants, mutations that cause loss or abnormal development of otoliths, without affecting other ear structures, are well represented in zebrafish (43). A variety of phenotypes have been identified, including complete bilateral loss of otoliths, only one otolith and supernumerary or misplaced otoliths (44,45). Bilateral loss of utricular otoliths severely impairs both balance and motor coordination, and is invariably lethal. The presence of one utricular otolith in at least one inner ear is necessary and sufficient for vestibular function and survival (46). Overall, 12 different complementation groups that only have defective otolith development have been described, although the genes underlying these mutations remain to be identified (44,45). We have cloned the Otop1 cDNA in zebrafish, reported its linkage to LG14, and partially characterized its genomic structure. However, Otop1 ESTs were not identified in the zebrafish embryonic inner ear EST database using accession number queries (www.genoscope.cns.fr/zie) (47). Unfortunately, this database cannot currently be queried by a direct BlastN search. Further studies are in progress to determine the expression pattern of Otop1 in zebrafish. A direct-candidate gene screen would clarify the potential involvement of zebrafish Otop1 in any of the available otolith mutants. Alternatively, morpholino antisense strategies, already proven successful in dissecting early otic placode induction pathways (48,49), will be tested.

In humans, three disease loci causing hearing impairment and vestibular dysfunction have been assigned to Chr17q25 (Fig. 6). The recessive disease, USHG1, is characterized by profound congenital hearing loss, vestibular dysfunction and prepubertal onset of retinitis pigmentosa (50). The USH1G genomic locus overlaps with the candidate region for DFNA26 (www.uia.ac.be/dnalab/hhh/), and on its distal side with the DFNA20 locus (51), two forms of dominant non-syndromic late-onset bilateral and progressive sensorineural hearing loss (52). It is not yet clear whether these disorders are attributable to mutations in the same gene. Two Otop1-like genes, OTOP2 and OTOP3, map within the DFNA26 and USH1G candidate regions, and lie in close proximity to the DFNA20 candidate region (Fig. 6). Interestingly, OTOP2 and OTOP3 representation was found in human retinoblastoma and retina EST databases (ESTs BM466860 and BQ188901, respectively). Based on available information, we suggest OTOP2 and OTOP3 should be screened as positional candidates for these disease loci. Inner ear mutants mapped to the orthologous region of mouse Chr11, include shaker-2 (sh2) and jackson-shaker (js). Sh2 is caused by a deletion in the

Figure 5. Expression of Otop1 and Otoconin-90/95 in the utricle of E16.5 mouse embryos. (A) Expression of Otoconin-90/95 (Oc90/95) in the nonsensory epithelium (arrowheads) (non-radioactive in situ). (B) Expression of Otop1 in the sensory epithelium (se) (radioactive in situ). (C) Immunolocalization of OC90/95 in the gelatinous membrane/otoconia (arrow) and nonsensory epithelium (arrowhead). (D) Immunolocalization of Otop1 in the gelatinous membrane/otoconia (arrow). Similar results were obtained in the saccule (data not shown).
myosin XV gene (53). Jb is characterized by deafness, head-bobbing and circling behavior, indicative of vestibular dysfunction (54), and has been proposed as a model for DFNA26/20 and USH1G (50). Although mutation screening of exon and splice junction sequences of Otop2 and Otop3 in Jb/Jb mice did not identify abnormalities that could account for the observed phenotype (data not shown), it is possible that other ‘regulatory’ mutations could affect the expression of these genes.

MATERIALS AND METHODS

Primers

SSLP markers delineating the minimal non-recombinant interval for the llt mutation are as follows: D5Dmo9 [5’-GTT GAC CTC TGA CCT ACA TGC (forward), 5’-GAT AGA GGT CAA TAG TGT GC (reverse)] and D5Dmo10 [5’-TGT ACT TAA TTT ACT ATT TGC (forward), 5’-CTC TAG TGT CTG CCC AAG TGT CTA G (reverse)]. Primers to amplify across the Otop1thC-176 > A mutation are 5’-CAC TGT TGT GTG TGG GTA CC (forward) and 5’-CAG CTC ATT ATT CCT GAC AAG (reverse). Primers to amplify across the Otop1mth T1247 > A mutation are 5’-TGA GAA GTC TCT GTC TGT GGT GTC (forward) and 5’-GAA TAA CCA CAG CTT GTT GGA G (reverse).

Generation and haplotype analysis of the mth mice

The mth mutation was induced using ENU as described previously (18). Briefly, adult BALB/cJ males were injected with a single dose of ENU (250 mg/kg) and mated to wt DBA/2 adult females after 13 weeks. Fertile F1 males were mated with...
wt BALB/cJ females and F2 females were back-crossed to the F1 parents. Homozygous mlh mice were identified in the F2 generation by their inability to swim and head tilting and have been subsequently maintained in a BALB/cJ background for more than 20 generations.

For the genetic mapping of the mlh mutation, a (BALB/cJ × C57BL/6J)F1 × BALB/cJ/mlh interspecific back-cross was established. Twenty-seven back-cross mice were typed with 54 SSLP markers evenly distributed over the whole genome (data not shown). Genotype data were analyzed with MapManager QTLb28 software. When evidence of linkage with marker D5Mit254 became obvious (LodScore 6.0), a high-resolution genetic scan of Chr5 was carried out with 18 additional D5Mit markers (Fig. 1).

**Mutation screening**

The genomic structures of mouse Wdr1 (NM_011715), Drd5 (S77992), Glat9 (NM_145559) and Otop1 (AF548337) were determined by alignment of their full-length cDNAs and available ESTs with the genomic sequence of RPC23 clones 426E16 (AC084071), 268D19, (AC084070) and 376E18 (AC084322). A total of 37 exons (Otop1, 7 exons; Wdr1, 15 exons; Drd5, intronless; Glat9, 14 exons) were identified and subsequently amplified from mt genomic DNA and a control sample of parental strain C57BL/6J using intronic primers flanking the splice junction sequences. In the mlh mutant and its parental strain BALB/cJ, only Otop1 was screened. The 5′ and 3′ point mutations were reconfirmed by a restriction endonuclease assay which detected an introduced TaqI restriction site in the Otop1alle allele and the loss of a MscI restriction site in the Otop1mlh allele (Fig. 2). The restriction endonuclease assay was conducted on the SJ/LJ, C3H/H, 101/H and DBA/2J inbred strains with parental-like results in all cases.

**Characterization of the mouse Otop1 cDNA and gene structure**

Mouse Otop1 oligonucleotide primers spanning multiple exons of the ORF were designed from UNIGENE cluster Mm.204765 (19 ESTs from aorta-vein, retina and mammary gland libraries). RT–PCR and RACE fragments were prepared from total RNA obtained from E18.5 mouse otocyst. PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced using ABI Big Dye terminator chemistry (PE Applied Biosystems, Foster City, CA, USA). The Otop1 full-length cDNA was obtained by assembling overlapping 5′ and 3′ RACE fragments generated with the SMART™ RACE cDNA amplification kit (Clonetech Inc.) following the manufacturer’s instructions. The gene structure of Otop1 was initially predicted by running GENSCAN on the sequence of BAC RP23CI-426E16 and later curated by alignment of the full-length cDNA with the genomic sequence.

**Characterization of Otop family members**

The clustering of ESTs was seeded by an initial tBlastN search using the mouse Otop1 amino-acid sequence masked for low complexity regions as a query against the mouse, human and zebrafish subsets of the EMBL EST databases. All the EST matches with a tBlastN bit-score of 45 or greater were searched against the EST database of the appropriate species using BlastN to identify all the overlapping EST sequences. Overlapping ESTs were assembled into contigs, masked for interspersed repeat elements using RepeatMasker and searched against the EST databases until no new ESTs were obtained.

This analysis identified three human and three mouse EST assemblies matching different UniGene clusters that correspond to the Otop1, Otop2 and Otop3 genes (Table 1). These sequences were electronically aligned to the Ensembl (www.ensembl.org) and Golden Path (http://genome.ucsc.edu/) genome drafts to determine their respective chromosomal locations and the complete gene structures. Conserved splice junctions positions across the gene family and GENSCAN predictions were used to resolve point ambiguities.

In zebrafish, 5 ESTs (B1888217, B1890046, A1544512, A1545839 and B985802) were retrieved and joined with overlapping RT–PCR sequences amplified from RNA from 26 somite embryos into a transcript of 2150 bp encoding the ORF of Otop1 (1760 bp). The genomic structure was partially deduced from the alignment of the cDNA with contigs z06s08939 and z06s018800 from zebrafish assembly06. The mapping information was obtained through EST AI544512 that maps to LG14, centiRay Position 57 on the radiation hybrid panel LN54 or centiRay Position 13 in the GF radiation hybrid panel [the WUZGR integrated zebrafish map (http://zfish.wustl.edu/)]. The zebrafish Otop1 amino acid sequence was used in a tBlastN query against the F. rubripes genomic assembly. A 1781 bp ORF spliced from six exons in fugu scaffold_3725 that encodes a protein 60% identical to zebrafish Otop1 was electronically annotated. Otop1 related sequences were also observed in fugu Scaffold_299 and Scaffold_3932 (data not shown).

Other partial Otop1 orthologs found in the databases included one rat EST (BM387360, UniGene Rn.81980), two genomic draft sequences spanning five exons of the rat Otop1 gene (AC099417 and AC127797) and three Xenopus ESTs (BJ055602, BJ077411, BJ065669, UniGene Xl.14432).

**In situ hybridization**

Sagittal hemi-heads from E16.5 mice where fixed in PBS/4% paraformaldehyde, overnight, paraffin-embedded and sectioned (10 μm). Sections were deparaffinized in xylene and rehydrated in a series of graded ethanols prior to use. A 1800 bp fragment spanning the 3′ UTR of mouse Otop1 was used as a probe for in situ hybridization. [35S]Labeled Otop1 probes (both sense and antisense) were hybridized following the procedure described by Naski et al. (55) with minor modification. Sections were autoradiographed for up to 4 weeks prior to development and counter staining with eosin–hematoxylin. The sense and antisense Oc90/95 probes spanning the full-length cDNA were labeled with DIG, hybridized, and developed according to the manufacturer’s instructions (Roche Diagnostics Corp., Indianapolis, IN, USA).

**Antibody generation**

A 14 amino acid peptide (LDESKNPARKLDVD) from the mouse Otop1 protein sequence was synthesized and conjugated to keyhole limpet hemocyanin (Sigma-Genosys, Woodlands,
Two rabbits were immunized with the peptide-conjugated carrier according to an established schedule (Sigma-Genosys, Woodlands, TX, USA). The antiserum was tested for the presence of the desired antibodies and the titer established by ELISA.

**Immunohistochemistry**

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. Residual endogenous peroxidase activity was blocked by incubating the slides in 7% H<sub>2</sub>O<sub>2</sub>/H<sub>2</sub>O for 15 min. The non-specific binding of the primary antibody was blocked by 5% normal goat serum for 30 min. The primary antibody was diluted to 14 μg/ml in the blocking buffer and incubated overnight. The biotinylated secondary antibody and the color development were performed according to the instructions of the ABC Vectastain kit (Vector laboratories, Burlingame, CA, USA). Control experiments were carried out with antiserum that had been preabsorbed overnight with the antigenic peptide.

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