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Characterization of the transmembrane channel-like (*TMC*) gene family: functional clues from hearing loss and epidermodysplasia verruciformis☆

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Abstract

Mutations of *TMC1* cause deafness in humans and mice. *TMC1* and a related gene, *TMC2*, are the founding members of a novel gene family. Here we describe six additional *TMC* paralogs (*TMC3* to *TMC8*) in humans and mice, as well as homologs in other species. cDNAs spanning the full length of the predicted open reading frames of the mammalian genes were cloned and sequenced. All are strongly predicted to encode proteins with 6 to 10 transmembrane domains and a novel conserved 120-amino-acid sequence that we termed the TMC domain. *TMC1*, *TMC2*, and *TMC3* comprise a distinct subfamily expressed at low levels, whereas *TMC4* to *TMC8* are expressed at higher levels in multiple tissues. *TMC6* and *TMC8* are identical to the *EVER1* and *EVER2* genes implicated in epidermodysplasia verruciformis, a recessive disorder comprising susceptibility to cutaneous human papilloma virus infections and associated nonmelanoma skin cancers, providing additional genetic and tissue systems in which to study the *TMC* gene family.

Keywords: Deafness; Epidermodysplasia verruciformis; EVER1; EVER2; Hearing; Ion channel; Papillomavirus; Receptor; TMC domain; Transmembrane

The *TMC* gene family was recently discovered through positional cloning of the gene underlying both dominant and recessive nonsyndromic sensorineural hearing loss at the DFNA36 and DFNB7/B11 loci, respectively, on chromosome 9q13–q21 [1]. These were shown to be allelic disorders caused by mutations of *TMC1*, a novel gene of unknown function. The mouse ortholog, *Tmc1*, also has dominant and recessive mutant alleles that cause hearing loss in the Beethoven (*Bth*) and deafness (*dn*) mutant mouse strains, respectively [1,2]. The dominant mutations are missense substitutions that likely act via dominant negative or gain-of-function mechanisms, since recessive mutations appear to be functional null alleles of *TMC1* or *Tmc1*, and heterozygous carriers of recessive mutations are unaffected [1].

TMC1 was initially identified among unassembled genomic sequence fragments with similarity to a second predicted gene of unknown function, *TMC2*, on chromosome 20 [1]. Neither *TMC1* nor *TMC2*, nor their mouse orthologs *Tmc1* and *Tmc2*, has nucleotide or amino acid sequence similarity to any known genes or domains. All four genes are strongly predicted to encode at least six conserved transmembrane domains, raising the possibility that TMC proteins may function as ion channels, pumps, or transporters [1]. The cochlear electrophysiologic phenotype of homozygous deafness mice, which segregate a partial genomic deletion of *Tmc1* [1], is consistent with an ion channel defect [3].

☆ Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession Nos. AY236490 (*TMC3*), AY236491 (*Tmc3*), AY236492 (*TMC4*), AY236493 (*Tmc4*), AY236494 (*TMC5*), AY236495 (*Tmc5*), AY236496 (*TMC6*), AY236497 (*Tmc6*), AY236498 (*TMC7*), AY236499 (*Tmc7*), AY236500 (*TMC8*), and AY236501 (*Tmc8*).

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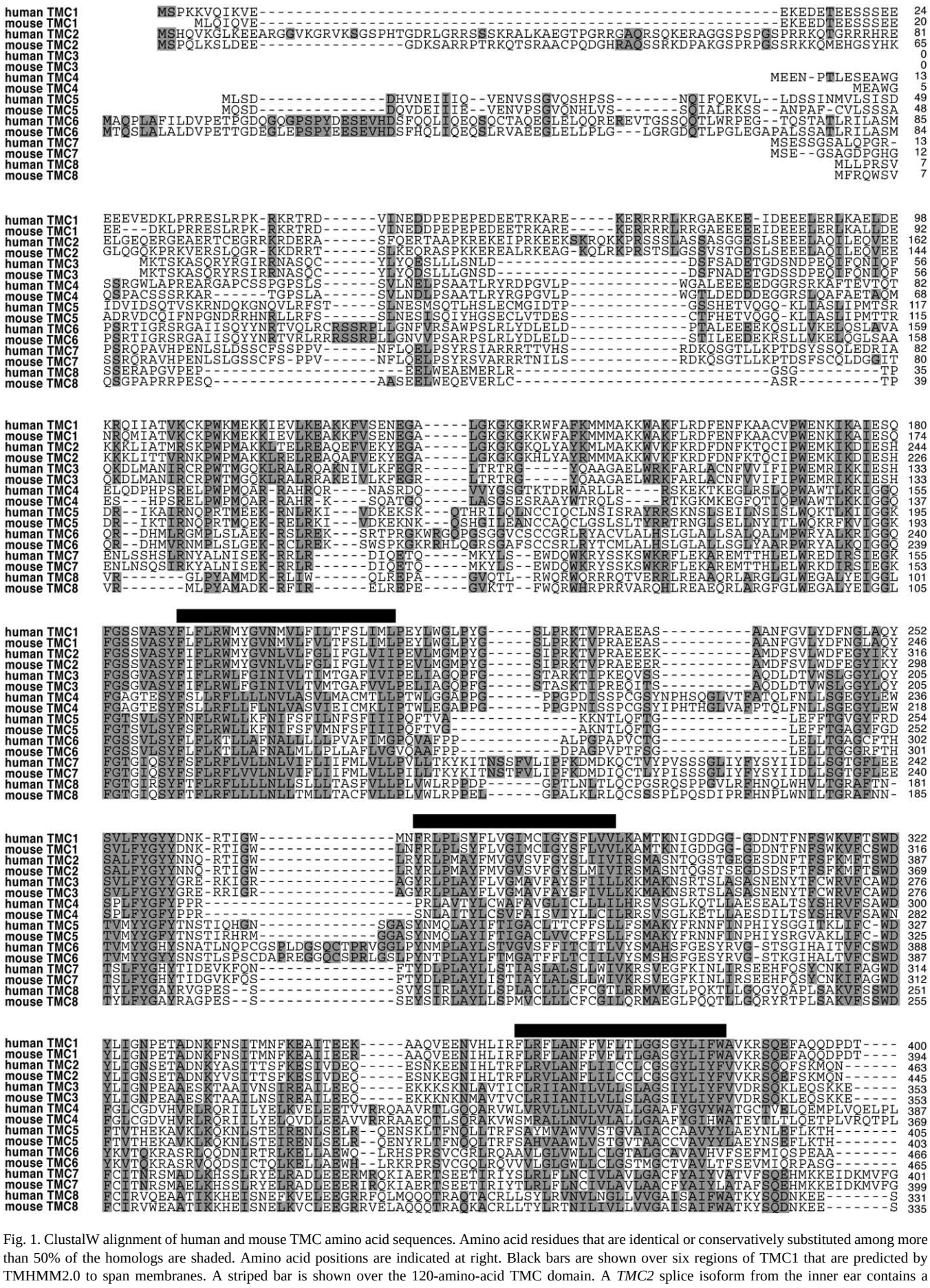

Fig. 1. ClustalW alignment of human and mouse TMC amino acid sequences. Amino acid residues that are identical or conservatively substituted among more than 50% of the homologs are shaded. Amino acid positions are indicated at right. Black bars are shown over six regions of TMC1 that are predicted by TMHMM2.0 to span membranes. A striped bar is shown over the 120-amino-acid TMC domain. A *TMC2* splice isoform from the inner ear contains a previously unreported exon encoding 16 in-frame amino acids near the N-terminus; the corresponding isoform and exon could not be detected in the mouse.

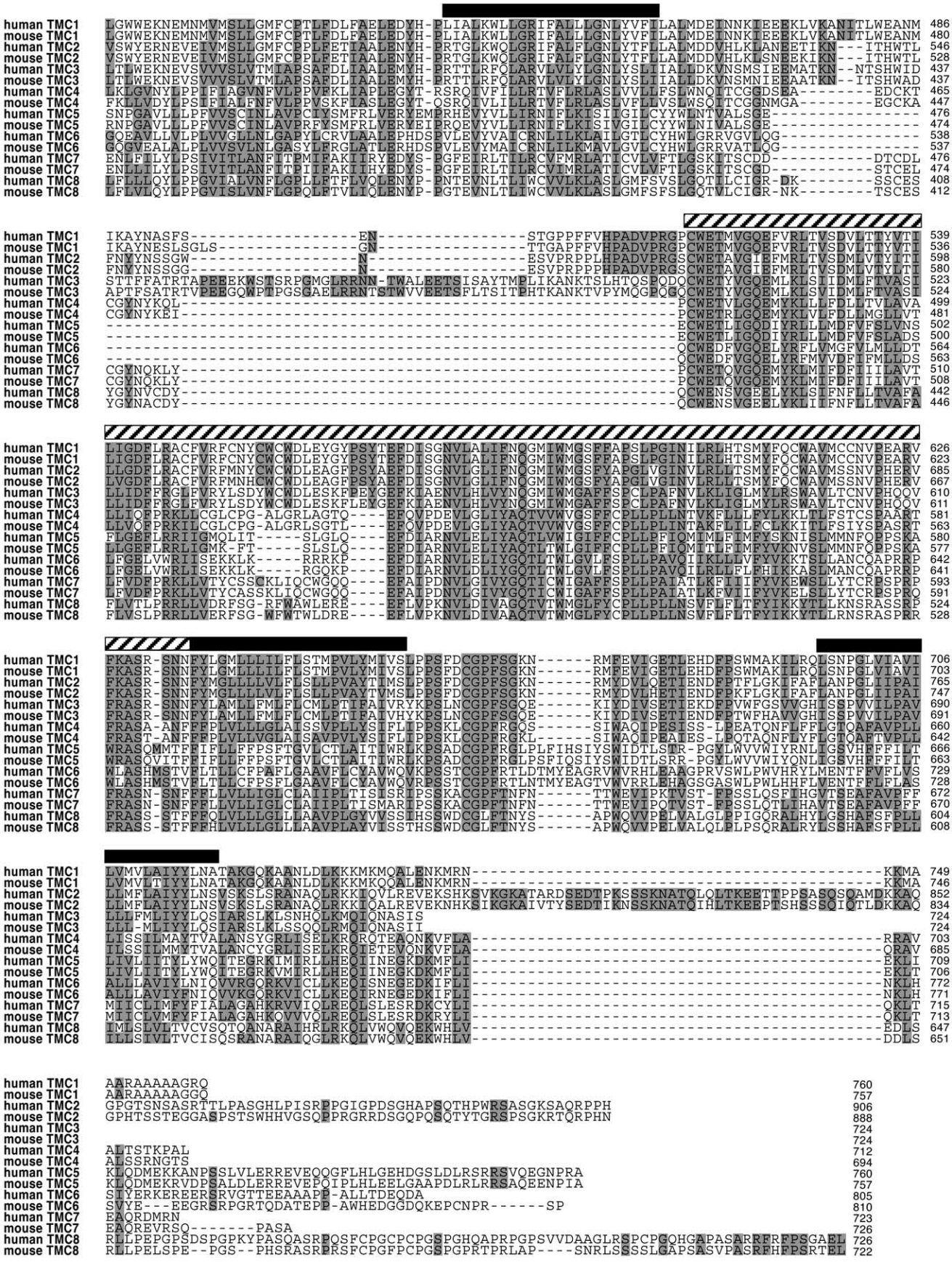

Fig. 1 (*continued*)

Table 1
Pair-wise amino acid sequence conservation among human TMC paralogs

	TMC1	TMC2	TMC3	TMC4	TMC5	TMC6	TMC7	TMC8	
TMC1		57	41	25	23	24	26	25	% amino acid sequence identity
TMC2	73		45	25	22	22	23	25	
TMC3	61	65		25	24	23	27	24	
TMC4	43	43	42		26	26	37	35	
TMC5	42	40	38	37		36	27	25	
TMC6	40	38	37	36	49		25	28	
TMC7	45	42	43	56	47	43		34	
TMC8	42	43	41	50	45	43	54		
	% amino acid sequence similarity[a]								

[a] Summed percentage of identical and conservatively substituted residues.

Tmc1 mRNA is specifically expressed in the neurosensory hair cells of the mouse cochlea [1,2], and therefore this gene and its ortholog were named transmembrane cochlear-expressed gene 1. The development and subsequent rapid degeneration of cochlear hair cells in affected Beethoven and deafness mice indicate a direct and essential role for *Tmc1* in normal hair cell physiology [2,4], but the low levels and restricted tissue distribution of its expression are significant obstacles for in situ studies of its function. We thus sought to identify and clone additional *TMC* genes to identify additional genetic models, tissues, and molecular reagents with which to explore the molecular and physiologic function(s) associated with this gene family. Our results show that mammalian *TMC* genes are expressed with a broad tissue distribution and some underlie mutant phenotypes that do not include hearing impairment, so they have been renamed transmembrane channel-like genes.

Results

Mammalian paralogs and orthologs

A tBLASTn search of genomic and expressed sequence tag (EST) databases with human and mouse *TMC1* and *TMC2* sequences identified six additional human *TMC* paralogs and six corresponding mouse orthologs (Fig. 1). Their corresponding cDNA nucleotide sequences each contained large open reading frames predicted to encode polypeptides with 36–73% pair-wise amino acid sequence similarity and 22–57% pair-wise amino acid sequence identity among TMC family members within a species (human comparisons shown in Table 1; mouse comparisons not shown). The human and mouse ortholog pairs had higher (75–95%) pair-wise amino acid sequence identity (Table 2), which, in combination with the syntenic relationships among their human and mouse genomic locations (Table 3), confirms our assignment of orthologous relationships. Each gene comprises 14 to 24 exons whose organization is nearly identical within each pair of human and mouse orthologs (not shown).

None of the mammalian *TMC* genes have significant sequence similarity to any other genes or motifs, although they all encode a conserved 120-amino-acid domain that we have named the TMC domain (Fig. 2). This 120-amino-acid sequence was used in a PSI-BLAST query to identify additional, more distantly related, genes from the NCBI nonredundant sequence database. Despite a low *E*-value threshold of 10, the only additional genes that were identified were *Chlamydomonas reinhardtii* adenosine triphosphatase and Crimean–Congo hemorrhagic fever virus envelope glycoprotein precursor. Direct inspection revealed that the similarity to *TMC* genes was not significant (not shown).

One large predicted exon on human chromosome 1 (GenBank Accession No. AL512353) contained sequence homologous to the entire *TMC6* open reading frame but interrupted by numerous frameshifts and termination codons. There are no corresponding ESTs and an orthologous locus is not present in the mouse genome. This processed pseudogene, *TMC9P*, probably arose through a retrotransposition event after the evolutionary divergence of mice from humans.

Table 2
Pair-wise amino acid sequence conservation between human and mouse TMC orthologs

	TMC1	TMC2	TMC3	TMC4	TMC5	TMC6	TMC7	TMC8
% identity	95	83	90	75	80	75	91	78
% similarity[a]	96	88	93	85	86	83	95	83

[a] Summed percentage of identical and conservatively substituted residues.

Table 3
Human and mouse *TMC* chromosomal locations, mutant phenotypes, and positional candidate phenotypes

Gene(s)	Location	Mutant phenotypes (MIM No.)	Positional candidate phenotypes (MIM No.)
TMC1	9q13	Dominant deafness DFNA36 (606705) Recessive deafness DFNB7/B11 (600974)	
Tmc1	19 (15 cM)	Dominant deafness: Beethoven Recessive deafness: deafness	
TMC2	20p13		Harboyan syndrome (217400) Corneal endothelial dystrophy 2 (217700)
Tmc2	2 (78 cM)		Dominant deafness: Tailchaser Susceptibility to Sindbis virus replication
TMC3	15q25.3		Nocturnal frontal lobe epilepsy 2 (603204)
Tmc3	7 (41 cM)		
TMC4	19q13.4		Primary ciliary dyskinesia 2 (606763) Spinocerebellar ataxia 14 (605361) Hydatidiform mole (231090)
Tmc4	7 (4 cM)		
TMC5, TMC7	16p12.3		Medullary cystic kidney disease 2 (603860) Dominant deafness DFNA40
Tmc5, Tmc7	7 (53 cM)		
TMC6, TMC8	17q25.3	Epidermodysplasia verruciformis (226400)	
Tmc6, Tmc8	11 (75 cM)		

Protein sequence motifs

TMHMM2.0 strongly predicts [5] the presence of 6 to 10 membrane-spanning domains in each of the TMC proteins (Fig. 3). No N-terminal signal peptide sequences or other trafficking signals were identified by PSORT II, and all of the proteins were predicted to reside in the plasma membrane. The C-termini of all TMC proteins, with the exception of human TMC6, were predicted to be cytoplasmic by TMHMM2.0. The predicted topologic orientation of N-termini was not uniform among the different genes, even using other algorithms (TMpred and PSORT).

PROSITE identified numerous potential sites in each human and mouse TMC amino acid sequence for amidation, glycosylation and myristoylation, as well as phosphorylation by protein kinase C, casein kinase II, and cAMP- and cGMP-dependent protein kinases (not shown). Some TMC sequences were also predicted to have potential tyrosine kinase phosphorylation sites and leucine zipper structures (not shown). No potential binding sites for ATP or GTP were identified. The C-termini contained potential class I PDZ ligand sequences in mouse TMC3 (SII) and mouse TMC8 (TEL), and potential class II PDZ ligand sequences in mouse TMC7 (ASA) and human TMC8 (AEL). The N- and C-terminal regions of all mammalian TMC proteins were enriched in charged amino acid residues, including approximately equal proportions of basic and acidic side chains. The predominance of charged residues in the N- and C-termini of TMC1 [1] was also observed for the N- and C-termini of TMC2 and TMC3 (Fig. 1). However, the proportions of charged residues in the N-termini of the latter four genes are lower, and the residues do not occur in the same pattern of regularly alternating clusters of similarly charged side chains observed in the N-termini of TMC1 [1].

Tissue distribution of expression

PCR amplification products corresponding to each of the *TMC/Tmc4*, *-5*, *-6*, *-7*, and *-8* genes were detected in each of the placenta, prostate, and testis cDNA libraries. Moreover, numerous EST clones corresponding to *TMC4*, *TMC5*, *TMC6*, *TMC7*, and *TMC8* have been isolated from a broad array of tissue sources from mice and humans (not shown). Whereas Unigene assemblies contained 14 to 80 ESTs each for *TMC4*, *Tmc4*, *TMC5*, *Tmc5*, *TMC7*, *Tmc7*, *TMC8*, and *Tmc8*, they had more than 200 each for *TMC6* and *Tmc6*. In contrast, there are only 5 or fewer ESTs for each of *TMC1*, *Tmc1*, *TMC3*, and *Tmc3*, and no ESTs have been deposited for *TMC2* or *Tmc2*. PCR amplification products corresponding to *TMC3/Tmc3* were detected only in brain and pituitary cDNA libraries. The full range of tissues expressing *TMC1* to *TMC3* may not have been adequately sampled if their mRNAs are expressed at very low levels.

Nonmammalian homologs

Expressed sequence tags with homology to the *TMC* genes were also identified from other mammals, including *Rattus norvegicus* (rat) and *Bos taurus* (cow), and nonmammalian species including *Gallus gallus* (chicken), *Xenopus laevis* (frog), *Danio rerio* (zebra fish), *Anopheles gambiae* (mosquito), *Ciona intestinalis* (sea squirt), *Strongyloides stercoralis* (nematode), and *Molgula tectiformis* (sea shell). A tBLASTn search of the *Fugu rubripes* (Japanese pufferfish) genomic DNA database identified eight distinct genomic loci homologous to mammalian TMC sequences (not shown).

tBLASTn searches of the BDGP (Berkeley *Drosophila* Genome Project) database identified a clone (GenBank Ac-

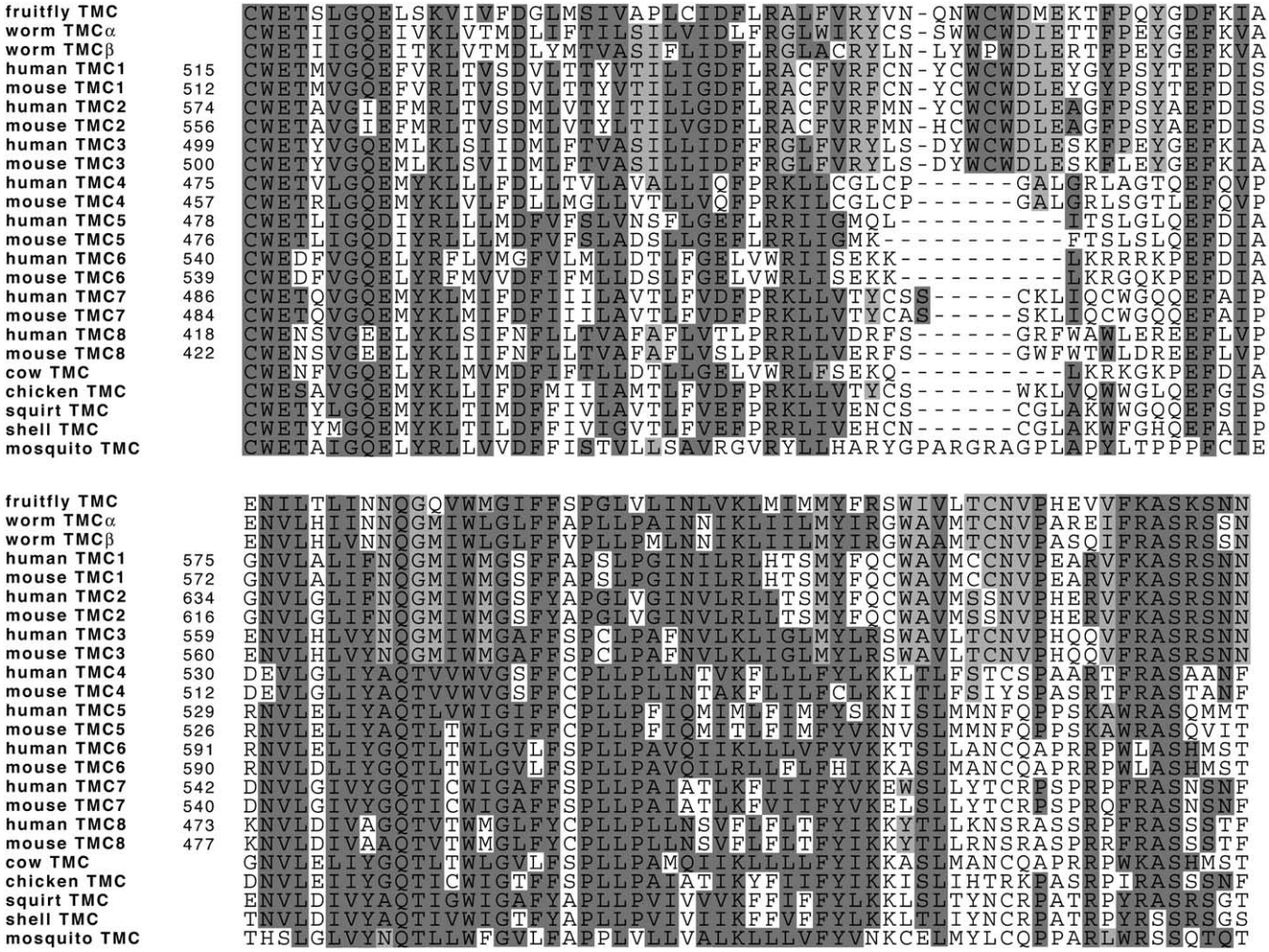

Fig. 2. ClustalW alignment of conserved TMC domains. Amino acid positions of the human and mouse sequences are indicated. Residues that are identical or conservatively substituted among more than 50% (>12/24) of the homologs are darkly shaded. Lightly shaded residues are additional residues that are conserved or conservatively substituted among seven or more of the following homologs: *Drosophila melanogaster* TMC (FlyBase CG3280), *Caenorhabditis elegans* TMCα and TMCβ (WormBase T13G4.3 (α) and B0416.1 (β)), and human and mouse TMC1, TMC2, and TMC3. The species origins of additional TMC sequences are indicated: cow, *Bos taurus* (GenBank Accession No. BM255599); chicken, *Gallus gallus* (BU440863); sea squirt, *Ciona intestinalis* (BW054501); shell, *Molgula tectiformis* (AU282683); mosquito, *Anopheles gambiae* (BM636384). Homologous ESTs from *Rattus norvegicus* (BQ199107), *Danio rerio* (AI353074), and *Xenopus laevis* (BI442346) did not fully span this region and were not included in the alignment.

cession No. AE003551) predicted to encode a 1937-amino-acid protein (FlyBase Annotation CG3280) with significant sequence similarity to mammalian TMC sequences, including the highly conserved TMC domain (Fig. 2). tBLASTn searches of *Drosophila melanogaster* genomic DNA confirmed the presence of just one genomic locus with homology to *TMC* genes. A tBLASTn search of WormBase (http://www.wormbase.org/db/searches/blast) identified at least two distinct *Caenorhabditis elegans* genes predicted to encode proteins with significant sequence similarity to the mammalian TMC domains. The deduced amino acid sequences of these two *C. elegans* homologs and the single *D. melanogaster* homolog have 14 to 38% pair-wise amino acid identity with mammalian TMC sequences, with the highest sequence similarities observed with TMC1 to TMC3 (Fig. 2). There were no mutant phenotypes colocalizing with the *Tmc* homologs in *C. elegans* or *D. melanogaster*. Due to the lack of complete genomic sequence information for the other homologs shown in Fig. 2 (cow, chicken, sea squirt, sea shell, and mosquito), the *TMC* sequences were derived from ESTs and probably do not represent the full complement of *Tmc* genes in those species.

Evolution of TMC genes

The *C. elegans* and *D. melanogaster* homologs were used to root a phylogenetic tree of the human and mouse *TMC* genes (Fig. 4). The results indicate that *TMC1, -2,* and *-3* comprise an evolutionarily distinct subfamily, while *TMC4, -5, -6, -7,* and *-8* form another subfamily. *TMC5* and *TMC7* are separated by approximately 400 kb in a tandem

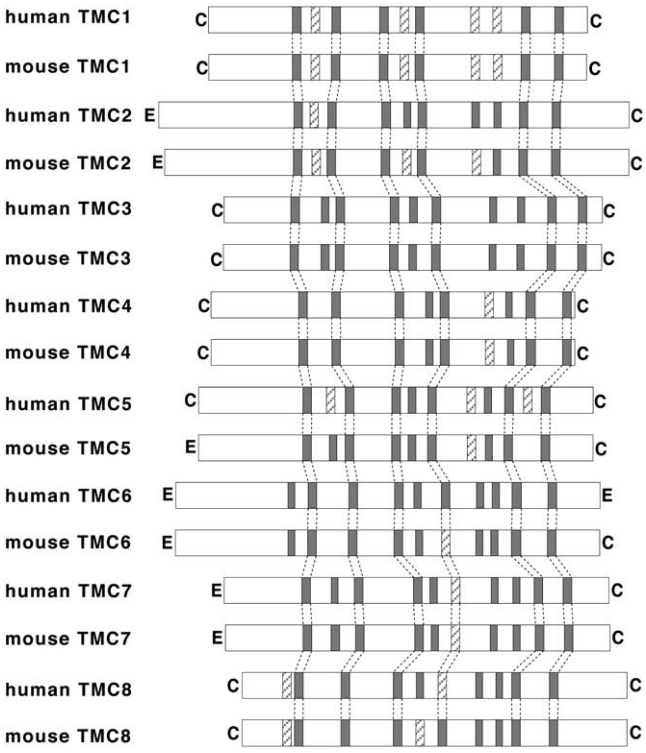

Fig. 3. Transmembrane topologies of TMC proteins predicted by TMHMM2.0. Shaded regions are predicted to span membranes, and striped regions have a lower, subthreshold posterior probability of spanning membranes. Sequences homologous to the six transmembrane domains of TMC1 are aligned with dotted lines. Predicted topologic orientations of N- and C-termini are indicated with E (extracellular) or C (cytoplasmic).

head-to-tail configuration with at least two intervening genes on chromosome 16p12, while *TMC6* and *TMC8* are arranged in a head-to-head configuration separated by only 4 kb on chromosome 17q25.3. The mouse orthologs *Tmc5* and *Tmc7* are oriented head-to-head and separated by 50 kb on chromosome 7, whereas *Tmc6* and *Tmc8* are separated by only 2 kb in a head-to-head configuration on chromosome 11 (not shown). Given the high sequence similarity between *TMC5* and *TMC6* and between *TMC7* and *TMC8* (Table 1), these four paralogs likely arose from a duplication of two tandemly arranged ancestral homologs. However, we could not detect shared linkage of these two gene pairs with paralogs of other gene families to confirm this hypothesis. Moreover, the TMC sequences have diverged to an extent that precludes inference of previous evolutionary events.

TMC6/TMC8 mutant phenotype

Four different recessive, truncating mutations of *EVER1* or *EVER2* have recently been reported to cause epidermodysplasia verruciformis (EV; MIM 226400), characterized by susceptibility to cutaneous human papilloma virus (HPV) infections and associated nonmelanoma skin cancers [6]. The long splice isoforms of *EVER1* and *EVER2* are identical in sequence and genomic location to *TMC6* and *TMC8*, respectively. EV-based nomenclature is not applicable to most of the *TMC* family members, and it is unknown if *TMC3* to *TMC8* are specifically expressed in cochlea. Therefore, according to HUGO gene nomenclature guidelines [7], the *TMC* locus prefix is retained for all of these genes but their name has been changed to transmembrane channel-like genes.

Discussion

There are 10 published mutant alleles of *TMC1/Tmc1* that include genomic deletions, frameshift, nonsense, and splice site mutations and three different missense substitutions: D572N in dominant DFNA36 hearing loss, M654V in recessive DFNB7/B11 deafness [1], and M412K in the dominant hearing loss mutant Beethoven [2]. All three substituted residues are conserved between human and mouse TMC1, whereas only D572 and M412K are also conserved in TMC2, and none of them are uniformly conserved among all of the remaining family members. It is possible that one or more of the missense substitutions are nonpathogenic, rare polymorphisms in linkage disequilibrium with undetected mutations elsewhere in *TMC1* or a closely linked gene. Alternatively, these residues may underlie a critical function or structure that is unique to *TMC1* and, possibly, *TMC2*.

The amino acid sequences of the predicted transmembrane domains and intervening polypeptide segments are generally the most conserved among the TMC proteins (Fig. 1), including the 120-amino-acid TMC domain, which immediately precedes the fifth transmembrane domain (Fig.

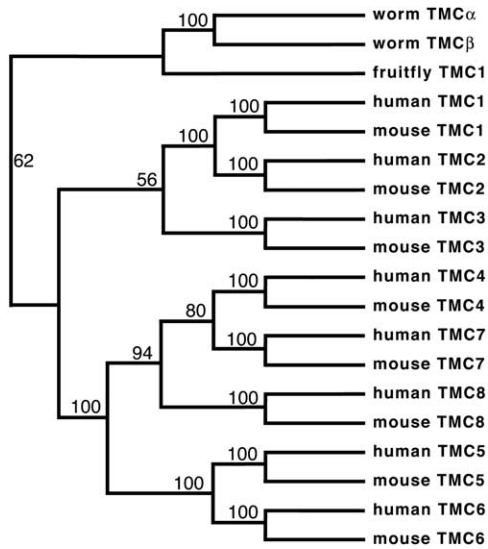

Fig. 4. Phylogeny of human, mouse, *C. elegans* (worm), and *D. melanogaster* (fruitfly) *TMC* genes predicted by PAUP* 4.0. Bootstrap values for 100 replicates of each parsimony analysis are indicated at nodes of the phylogenetic tree, which was rooted with the three invertebrate homologs.

2). However, the amino acid sequences between the fourth transmembrane domain and the TMC domain are not conserved, with several different gaps and insertions among the family members (Fig. 1). Similarly, the N- and C-termini of the TMC proteins are highly divergent, which is observed in some receptor and ion channel gene families such as the Kir3 (GIRK) inwardly rectifying potassium channel family [8]. By analogy, the conserved transmembrane domains and intervening polypeptide regions of TMC proteins may mediate potential core molecular properties shared among some or all of the TMC family members, such as signal transduction, transmembrane ion conductance, or homotypic or heterotypic assembly into higher order multimers.

The recent description of truncating mutations of *TMC6* and *TMC8* that cause EV [6] identifies new experimental models for investigating their function. A typical feature of EV is decreased cell-mediated immunity [9,10], and the identification of *TMC6* and *TMC8* ESTs and cDNA from lymphoid tissue (T lymphocytes and palatine tonsil) is consistent with a model in which EV mutations exert their direct effects in the immune system. Perhaps *TMC6*, *TMC8*, or both underlie signal transduction or ion channel activities in the immune system whose genes have not been cloned, such as Ca^{2+} release-activated Ca^{2+} channels or Ca^{2+} release-activated nonselective cation channels in T lymphocytes [11,12]. The localization of transiently expressed TMC6 and TMC8 proteins to the endoplasmic reticulum of a human keratinocyte cell line [6] may not reflect their subcellular distribution in situ, although it does provide experimental evidence that *TMC* genes encode integral membrane proteins. A molecular pathogenesis similar to that in EV may also underlie the observed association of HPV with oropharyngeal (tonsillar) and cervical carcinomas [13,14].

TMC2 is a positional candidate for Harboyan syndrome (CDPD1; MIM 217400; Table 3), an autosomal recessive disorder comprising corneal dystrophy and postlingual progressive sensorineural hearing loss [15], and *Tmc2* is located within the critical interval for the Tailchaser mutation (*Tlc*), which causes progressive hearing loss, vestibular dysfunction, and abnormal hair cell stereocilia development in affected *Tlc*/+ mice [16]. However, no mutations have been detected in *Tmc2* exons and adjacent splice sites in Tailchaser genomic DNA (personal communication, Ronna Hertzano and Karen Avraham, Tel Aviv University, March 5, 2003). *Tmc2* is also located in the region of a quantitative trait locus for susceptibility to neuroadapted Sindbis virus replication with paralysis and mortality in female mice (*Nsv1*) [17].

TMC5 and *TMC7* are located on chromosome 16p12, where nonsyndromic dominant hearing loss DFNA40 is reported to be located (Hereditary Hearing Loss Home page), although there are no published genetic map data for this locus. *TMC5* and *TMC7* are also located within the critical interval for autosomal dominant medullary cystic kidney disease 2 (MCKD2; MIM 603860) on chromosome 16p12.3 [18], and both of these genes or their mouse orthologs have ESTs derived from kidney. The phenotypically similar polycystic kidney disease is known to be caused by mutations in polycystin-1 or -2 [19,20], which comprise a nonselective cation channel implicated in intracellular release of Ca^{2+} [21] and mechanosensation of fluid flow by primary cilia of renal tubule cells [22]. The latter phenomenon is notable in its similarity to mechanotransduction of auditory stimuli by cochlear hair cell stereocilia [23]. Another potential link to cilia function may be provided by *TMC4*, which is located within the interval for primary ciliary dyskinesia 2 (CILD2; MIM 606763) [24]. Interestingly, mice that are homozygous for a functional null allele of polycystin-2 have lateralization defects [25], a phenotypic hallmark of primary ciliary dyskinesia. Our study now provides a theoretical foundation and experimental tools to address the roles of *TMC* genes in these and other disorders.

Materials and methods

In silico analyses

TMC1 and TMC2 amino acid sequences (GenBank Accession Nos. AAL86399, AAL86400, AAL86401, and AAL86402 for human and mouse TMC1 and human and mouse TMC2, respectively) were used as query sequences for tBLASTn analyses with the Celera Discovery System, NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), and Ensembl Genome Browser (http://www.ensembl.org/). ClustalW (MacVector 7.0, Genetics Computer Group, Madison, WI, USA) was used to align human and mouse amino acid sequences.

Deduced amino acid sequences of human and mouse TMC1 to TMC8, two *C. elegans* TMC homologs (T13G4.3 and B0416.1), and a *D. melanogaster* homolog (CG3280) were aligned with ClustalX version 1.8 [26] using Gonnet Protein Matrix with a gap opening penalty of 5.0 and gap extension penalty of 0.1. Nonhomologous regions, including the N- and C-termini as well as sequences with gaps in one or more homologs, were deleted for analysis by the parsimony method with PAUP* 4.0 (Sinauer Associates, Inc, Sunderland, MA, USA). The phylogenetic tree was rooted with the three nonmammalian homologs. Transmembrane topology and domain search analyses of mammalian TMC1 to TMC8 were performed with PROSITE (http://us.expasy.org/cgi-bin/prosite-list.pl), PSORT II (http://psort.ims.u-tokyo.ac.jp/), TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

cDNA cloning and sequence analysis.

Genomic DNA, Unigene, and EST sequence information was used to construct composite predicted sequences and design PCR primers for amplification of *TMC/Tmc3*, *-4*, *-5*,

-6, *-7*, and *-8* from placenta, prostate, testis, brain, or pituitary Marathon-Ready cDNA libraries (Clontech, Palo Alto, CA, USA). PCRs were performed in 25 μl with 2.5 μl cDNA library, 20 pmol forward and reverse primers, 200 mM each dNTP, 1× PCR buffer, and 2.5 U of Takara LA-*Taq* polymerase (PanVera LLC, Madison, WI, USA). Cycling conditions were 95°C for 1 min followed by 35 cycles of 96°C for 10 s and 68°C for 3 min. 5′- and 3′-RACE were also performed as needed to amplify cDNA ends. Nucleotide sequence analysis of amplification products was performed as described [27]. Primer sequences are available from the authors upon request.

Acknowledgments

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