RESEARCH ARTICLE

An Integrated Genetic and Functional Analysis of the Role of Type II Transmembrane Serine Proteases (TMPRSSs) in Hearing Loss

Michel Guipponi, 1 Min-Yen Toh, 1 Justin Tan, 2 Daeho Park, 3 Kelly Hanson, 4 Ester Ballana, 5 David Kwong, 1 Ping Z.F. Cannon, 1 Qingyu Wu, 6 Alex Gout, 7 Mauro Delorenzi, 7 Terence P. Speed, 7 Richard J.H. Smith, 8 Henrik H. Dahl, 9 Michael Petersen, 10 Rohan D. Teasdale, 4 Xavier Estivill, 5 Woo Jin Park, 3 Ping Z.F. Cannon, 1 Qingyu Wu, 6 Alex Gout, 7 Mauro Delorenzi, 7 Terence P. Speed, 7 Richard J.H. Smith, 8 Henrik H. Dahl, 9 Michael Petersen, 10 Rohan D. Teasdale, 4 Xavier Estivill, 5 Woo Jin Park, 3

1 Division of Molecular Medicine, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia; 2 The Bionic Ear Institute, Victoria, Australia; 3 Department of Life Sciences and National Research Laboratory of Proteolysis, Kwangju Institute of Science and Technology (K-JIST), Kwangju, Korea; 4 Institute for Molecular Bioscience and ARC Centre in Bioinformatics, University of Queensland, Queensland, Australia; 5 Genes and Disease Program, Center for Genomic Regulation, Barcelona, Spain; 6 Department of Molecular Cardiology, Lerner Research Institute, Cleveland, Ohio; 7 Division of Bioinformatics, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia; 8 Molecular Otolaryngology Research Laboratories, Department of Otolaryngology-Head and Neck Surgery, The University of Iowa, Iowa City, Iowa; 9 Murdoch Children’s Research Institute, Victoria, Australia; 10 Department of Genetics, Institute of Child Health, ‘Aghia Sophia’ Children’s Hospital, Athens, Greece

Communicated by Dvorah Abeliovich

Building on our discovery that mutations in the transmembrane serine protease, TMPRSS3, cause nonsyndromic deafness, we have investigated the contribution of other TMPRSS family members to the auditory function. To identify which of the 16 known TMPRSS genes had a strong likelihood of involvement in hearing function, three types of biological evidence were examined: 1) expression in inner ear tissues; 2) location in a genomic interval that contains a yet unidentified gene for deafness; and 3) evaluation of hearing status of any available Tmprss knockout mouse strains. This analysis demonstrated that, besides TMPRSS3, another TMPRSS gene was essential for hearing and, indeed, mice deficient for Hepsin (Hp) also known as Tmprss1 exhibited profound hearing loss. In addition, TMPRSS2, TMPRSS5, and CORIN, also named Tmprss10, showed strong likelihood of involvement based on their inner ear expression and mapping position within deafness loci PKS7, DFNB24, and DFNB25, respectively. These four TMPRSS genes were then screened for mutations in affected members of the DFNB24 and DFNB25 deafness families, and in a cohort of 362 sporadic deaf cases. This large mutation screen revealed numerous novel sequence variations including three potential pathogenic mutations in the TMPRSS5 gene. The mutant forms of TMPRSS5 showed reduced or absent proteolytic activity. Subsequently, TMPRSS genes with evidence of involvement in deafness were further characterized, and their sites of expression were determined. Tmprss1, 3, and 5 proteins were detected in spiral ganglion neurons. Tmprss3 was also present in the organ of Corti. Tmprss1 and 3 proteins appeared stably anchored to the endoplasmic reticulum membranes, whereas Tmprss5 was also detected at the plasma membrane. Collectively, these results provide evidence that Tmprss1 and Tmprss3 play and Tmprss5 may play important and specific roles in hearing. Hum Mutat 29(1), 130–141, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: deafness; serine proteases; Tmprss1; Hpn; Tmprss5

INTRODUCTION

Hearing loss occurs in approximately one in 1,000 children and more than half of these cases can be considered to have a genetic origin [Morton, 1991]. In addition, a great number of individuals will suffer with age-related hearing loss, with 50% of the

Grant sponsor: Garnett Passe and Rodney Williams Memorial Foundation; Nossal Leadership Award, The Walter and Eliza Hall Institute of Medical Research (WEHI); Grant sponsor: Medical Research and Technology Grant (Victoria, Australia); Grant sponsor: Marion and E.H. Flack Trust; Grant sponsor: Swiss National Science Foundation; Grant number: 3100A-114077-1; Grant sponsor: National Health and Medical Research Council (NHMRC), Australia; Grant numbers: Project No. 215305; and Program No. 25750L; Grant sponsor: National Institute on Deafness and Other Communication Disorders; Grant number: NO1-DC-3-1005.

Current address for Michel Guipponi: Division of Medical Genetics, University Hospital of Geneva, Hôpital Belle-Idée, 1225 Chêne-Bourg, Switzerland.

Current address for Mauro Delorenzi: Bioinformatics Core Facility, NCCR Molecular Oncology, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland.

DOI 10.1002/humu.20617

Published online 5 October 2007 in Wiley InterScience (www.interscience.wiley.com).

© 2007 WILEY-LISS, INC.
population having a significant hearing impairment by age 80 years [Seidman et al., 2002]. Most of the cases of early-onset inherited hearing loss are due to single gene defects. Identifying genes underlying hearing loss represents a major objective of current biomedical research. Indeed, the identification and characterization of these genes will lead to advances in our understanding of hearing loss, and, in turn, to improvements in diagnostic accuracy and therapeutic options. Significant progress has been made in the last few years in the localization and identification of genes for deafness. To date, 93 loci for autosomal and X-linked forms of nonsyndromic deafness have been reported and 39 causative genes identified [Van Camp and Smith, 2007].

However, specific difficulties have made the identification of mutant genes that are responsible for inherited forms of nonsyndromic deafness complicated. Different mutations in many genes can lead to the condition, and the lack of distinctive clinical features for the different gene defects and assortative mating that exists among deaf people, makes it difficult to track more than one gene through several generations of a family. Studies of large consanguineous families living in geographically isolated areas have cleared these hurdles in many studies to date [Guilford et al., 1994]. However, families that are large enough to accurately map genes for nonsyndromic deafness are becoming rare, and systematic mutation screening of candidate genes in a large, well-characterized cohort of patients can provide an alternative strategy for identifying relevant genes. Large-scale approaches, independent of mapping by genetic linkage, have successfully been used to identify numerous disease-causing mutations in families with mental retardation [de Brouwer et al., 2007; Tarpey et al., 2007]. In addition to uncovering mutant genes that give rise to monogenic disorders, this strategy also identifies novel polymorphisms that provide an opportunity to understand the genetic susceptibility to similar common polygenic phenotypes [Antonarakis and Beckmann, 2006; Peltonen et al., 2006]. Potentially, polymorphic variations that have a functional effect by giving rise to hypomorphs, or by affecting the level of expression of any of the numerous genes involved in cochlear function, could impact on an individual’s risk of age-related hearing loss, the most common form of hearing impairment [Liu and Yan, 2007].

Type II transmembrane serine proteases (TMPRSSs; summarized in Table 1) are a new class of membrane-bound proteolytic enzymes that are important in a variety of biological and pathological processes. These serine proteases have well-characterized roles in diverse cellular activities, including blood coagulation, wound healing, digestion, and immune responses, as well as tumor invasion and metastasis. They are ideally positioned to interact with surface proteins as well as soluble proteins, matrix components, and proteins on adjacent cells. In addition, these membrane-spanning proteins have cytoplasmic N-terminal domains, suggesting possible functions in intracellular signal transduction [Wu, 2003]. Recently, we and others have shown that mutations in TMPRSS3 are responsible for both familial and sporadic forms of nonsyndromic recessive deafness [Ben-Yosef et al., 2001; Masmoudi et al., 2001; Scott et al., 2001; Wattenhofer et al., 2002, 2005; Ahmed et al., 2004; Hutchin et al., 2005]. Genes involved in deafness can be grouped into functional categories (ion channels, transcription factors, motor molecules, extracellular matrix components, and cytoskeletal components). TMPRSS3 did not belong to any of the already existing groups and could represent the first member of a new functional group, tentatively named proteolysis components.

We addressed this hypothesis by investigating all known TMPRSS genes for three biological criteria: expression in inner ear tissues, localization within a genomic interval harboring a thus far unidentified deafness locus, and evaluation of the hearing status of any available Tmprss knockout mouse strains. TMPRSS genes were then classified in different groups based on their predicted likelihood of involvement in hearing loss. The best candidates were prioritized for a systematic mutation analysis in a large cohort of familial and sporadic deaf cases. The proteolytic activity of TMPRSS mutants was assessed to define their potential pathogenic mechanisms. TMPRSS genes showing evidence of implication in hearing loss were further characterized through defining their cellular and subcellular localizations.

**MATERIALS AND METHODS**

**Expression Profiling**

To explore the expression of Tmprss genes in the inner ear, RT-PCR was performed on total RNA extracted from 5-day old rat modiolus, stria vascularis, organ of Corti, and cultured spiral ganglia cells as described [Marzella et al., 1997]. To control for genomic DNA amplification, all primer pairs were designed to span an intron. PCR primer sequences and conditions are available upon request.

**Hearing Evaluation by Click-Evoked Auditory Brainstem Responses (ABRs)**

Mice with targeted disruption of the Tmprss1 gene were obtained from Wu et al. [1998]. Procedures for auditory brainstem response (ABR) measurements have been previously described [Shepherd et al., 2005]. Mice 7–8 weeks of age were anesthetized with intraperitoneal injections of ketamine (75 mg/kg body weight; Parnell Laboratories, Sydney, Australia) and xylazil (7.5 mg/kg body weight; Troy Laboratories, Sydney, Australia). Briefly, computer-generated rarefaction clicks were channeled to a loud speaker positioned 10 cm from the pinna of the measured ear. Stimuli were presented at a rate of 33 per second and responses were amplified 10^5-fold and bandpass filtered (150 Hz–3 kHz). Threshold was determined as the smallest stimulus level required to give peak-trough response amplitude of > 0.25 μV for wave II of the ABR. At each stimulus level, recording was repeated to ensure consistency.

**DNA Samples**

All patients included in this study were diagnosed with severe to profound sensorineural prelingual nonsyndromic deafness and were negative for the 35delG GJB2 mutation. DNA samples from sporadic patients were obtained from Australia (65 samples), Greece (100), and Spain (197). DNA samples from the DFNB24 and DFNB25 deafness families [Van Camp and Smith, 2007] were also included in this study. All control samples were obtained from normal hearing people matched with the patients for ethnic origin. All samples were obtained with informed consent, and their use was approved by the Institute for Human Research Ethics Committee (Institutional Review Board [IRB] number 02/02).

**Mutation Analysis**

All PCR products were designed to amplify the exons and splice sites of each candidate gene. PCR primers were designed using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3 www.cgi) (Supplementary Table S1TBL S1, available online at http://www.interscience.wiley.com/ijpages/1059-7794 suppmat). All amplicons were screened by DHPLC using the Helix™ system from Varian (Melbourne, Australia), coupled with
the 364 multichannel fluorescence detector according to supplier’s recommendations (www.varianinc.com). For each amplicon, four samples were analyzed: patient DNA alone, patient DNA mixed with control DNA sample, control DNA alone, and a “no template” control. The following bacterial artificial chromosome (BAC) clones were used as control DNA: RP11-60A22 for TMPRSS1, RP11-141J16 and 953H33 for TMPRSS10, RP11-121A4 for TMPRSS2, and RP11-150A24 for TMPRSS5. Melting temperatures were estimated using the Stanford DHPLC melt program (http://insertion.stanford.edu/melt.html) and experimentally confirmed using control DNA samples (BACs). For DHPLC multichannel fluorescence detection, up to four amplicons with identical predicted melt temperatures and retention times were organized into “batches” and their primers tagged with different fluorescent dyes [Guipponi et al., 2005]. PCR products showing an abnormal chromatographic profile were directly sequenced using the Big Dye terminator V3 and run on an ABI3730 sequencer (Applied Biosystems, Foster City, CA). Potential pathogenic sequence variants were eliminated by their presence in SNP databases (www.ncbi.nlm.nih.gov/SNP) or by screening a panel of 200 control chromosomes by either restriction analysis or DHPLC. Sequence variations identified in TMPRSS genes are listed according to the official nomenclature for the description of sequence variants (www.hgvs.org/mutnomen). The A of the initiation codon is considered as+1 (based on the GenBank reference sequences listed in Table 1).

Mutagenesis and Protease Activity

The extracellular region of TMPRSS5 was amplified by PCR and mutations F369L, A317S, and Y438X were introduced by PCR-based mutagenesis (QuickChange II Site-Directed Mutagenesis Kits, Stratagene, La Jolla, CA). These were fused to the truncated luminal region of the yeast Golgi membrane protein STE13, yielding protease vectors pADH-Ste13-TM5 and pADH-Ste13-mut-TM5. Note that mutant pD31V could not be analyzed because of its location upstream of the transmembrane domain. Proteolytic activity of TMPRSS5 wild-type and mutants were assayed using a yeast based protease assay called “secretory Genetic Assay for Site-specific Proteolysis” (sGASP) [Yun Kim et al., 2002]. Briefly, a kex2 mutant strain, KSY01 (MAT’a leu2 ur3 his3 trp1 lys2 suc2-89 kex2::HIS3), was transformed with both protease and substrate vectors. Leu+ and Trp+ transformants were selected on minimal medium containing 2% glucose but lacking leucine and tryptophan. Transformants were replica plated on Yeast Extract/Peptone/Dextrose (YPD) medium containing 2% sucrose and 10 μg/ml antimycin A. Growth of the transformants on the sucrose plates depended on cleavage of the substrate sequence by TMPRSS5.

Immunohistological Analysis

Cochleae from 7- to 8-week-old mice were fixed in 3% paraformaldehyde (PFA) in PBS, decalcified in 10% ethylene diamine tetra-acetic acid (BDH Laboratory Supplies, Dorset, UK) in PBS, and incubated overnight (O/N) in 25% sucrose in PBS. Cochleae were cryosectioned in 12-μm thicknesses and mounted on SuperFrost Plus (Menzel Gmbh & Co KG, Braunschweig, Germany) microscopic slides. Sections were permeabilized for 10 min at room temperature (RT) with 0.1% Triton-X, blocked with 1% BSA (Sigma-Aldrich, St. Louis, MO) in 0.1% Triton-X for 30 min and incubated O/N at 4°C with primary antibodies. Sections were then washed 3 x with PBS for 10 min, before incubating for 2 hr at RT with the appropriate secondary antibodies. Sections were subsequently rinsed 3 x in PBS for 20 min, before mounting in Vectorshield (Vector Laboratories, Burlingame, CA) containing the nuclear stain, 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI). All sections were viewed with a Zeiss Axiosplan 2 microscope (Zeiss, Oberkochen, Germany). Affinity-purified goat polyclonal antibody against TMPRSS1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA; sc-23776) and used in 1:25 dilution. Rabbit polyclonal antibody against TMPRSS3 [Guipponi et al., 2002] was used in a 1:200 dilution. Affinity-purified goat polyclonal antibody against TMPRSS5 (Abcam, Cambridge, United Kingdom: ab2245) was used in 1:50 dilution. To control for the specificity of these antibodies, we used blocking peptides recommended by the manufacturer for TMPRSS1 (sc-23776P), or naïve rabbit serum for TMPRSS3, or affinity-purified control immunoglobulin G (IgG) from naïve goats from Santa Cruz Biotechnology (sc-2028) for TMPRSS5 (Supplementary Fig. S1). Purified monoclonal antibody against the 200-kDa neurofilament (Chemicon, Temecula, CA; MAB5266, 1:500) was used in colocalization experiments. The following fluorescein-conjugated secondary antibodies from Invitrogen (Carlsbad, CA) were used at a 1:500 dilution: highly cross-adsorbed Alexa Fluor 488 goat anti-mouse IgG (A-11029), highly cross-adsorbed Alexa Fluor 594 goat anti-rabbit IgG (A-11037), and Alexa Fluor 594 chicken anti-goat (A-21468).

Subcellular Localization Analysis

N- and C-terminally tagged V5-epitope constructs were generated using the Gateway cloning system (Invitrogen). Initially, attB-PCR products of the coding sequences of TMPRSS1, 3, and 5 were cloned into the pDONR221 donor vector, which includes attP sites (Invitrogen) via a recombination reaction between the attB and the attP sites to create entry clones. Expression clones were generated via a recombination reaction between the attL sites of the entry clone and the attR sites of the destination vector, pcDNA3.2/V5-DEST (Invitrogen) for C-terminal V5 tagging, and pcDNA3.2/nV5DEST (Invitrogen) for N-terminal tagging. Monoclonal mouse antibodies were used to detect the V5 epitope tag, and were in turn detected by Cy3-conjugated goat anti-mouse antibodies (Invitrogen). Plasmid ICAT-GFP (pcCMT-GFP) has been described previously [Dai et al., 1998] and Rab5Q79L-GFP was obtained from Timothy Evans, Institute for Molecular Bioscience, University of Queensland, St. Lucia, 4072, Queensland, Australia (IMB). Texas Red-X Phalloidin (Invitrogen) was used according to the manufacturer’s instructions.

Subconfluent human hepatocellular liver carcinoma (HepG2), human cervical carcinoma (HeLa), and HEK293 cells were transiently transfected with N- or C-terminal V5-epitope tagged PCR expression constructs using Lipofectamine2000 (Invitrogen) and OptiMEM (Invitrogen) as per the manufacturer’s instructions. Cells harvested 16–24 hr posttransfection were fixed in 4% PFA in PBS for 30–90 min prior to permeabilization in 0.1% Triton-X-100 for 5 min, washed 3 x in a blocking solution of 0.8% gelatin (Sigma-Aldrich) and 5% fetal calf serum (Invitrogen) in PBS prior to incubation with the primary antibody for 30–90 min. The cells were subsequently washed 3 x prior to incubation with the secondary antibody for 30–60 min. The cells were then washed 3 x with blocking solution prior to three PBS washes and subsequent mounting on slides with MO-WIOL (Calbiochem, San Diego, CA). All colocalization data were captured on a Zeiss Axiovert 200M SP LSM 510 META inverted laser scanning confocal microscope with appropriate band pass filter settings. Data were analyzed using the LSM 510 META (Zeiss) software.
<table>
<thead>
<tr>
<th>TMPRSS gene (OMIM No.)</th>
<th>Organism</th>
<th>Other name(s)</th>
<th>% Identity to Hs</th>
<th>Accession No.</th>
<th>Chromosomal location</th>
<th>Deafness locus SV</th>
<th>Mod</th>
<th>OoC</th>
<th>SG</th>
<th>Brain</th>
<th>Kidney</th>
<th>Thymus</th>
<th>Liver</th>
<th>Stomach</th>
<th>Muscle</th>
<th>Lung</th>
<th>Testis</th>
<th>Skin</th>
<th>Ovary</th>
<th>Eye</th>
<th>E8.5</th>
<th>E9.5</th>
<th>E12.5</th>
<th>E19</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMPRSS1 Human</td>
<td>Mouse</td>
<td>Hepsin, HPN</td>
<td>100</td>
<td>M18930.1</td>
<td>19q13.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(H24440)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS2 Human</td>
<td>Mouse</td>
<td>Epitheliasin</td>
<td>88</td>
<td>AF030065</td>
<td>U75329/AF329454.1</td>
<td>7 21q22.3</td>
<td>PKSR7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(602060)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS3 Human</td>
<td>Mouse</td>
<td>Echos</td>
<td>100</td>
<td>NM_005656</td>
<td>21q22.3</td>
<td>DFNB8/10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(605511)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS4 Human</td>
<td>Mouse</td>
<td>TMPRSS3, UNQ76, PRO1570</td>
<td>88</td>
<td>AF19224</td>
<td>11q11-13</td>
<td>DFNB5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(606565)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS5 Human</td>
<td>Mouse</td>
<td>Spinesin</td>
<td>77</td>
<td>not deposited</td>
<td>NM_010701</td>
<td>11q</td>
<td>DFNB24</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(606750)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS6 Human</td>
<td>Mouse</td>
<td>UNQ354, PRO618, MT-SP2</td>
<td>79</td>
<td>NM_010009</td>
<td>11q11-13</td>
<td>DFNB5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(609862)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS7 Human</td>
<td>Mouse</td>
<td>matriptase-3</td>
<td>90</td>
<td>NM_027902</td>
<td>15q13.1</td>
<td>3q13.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(60477)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS9 Human</td>
<td>Mouse</td>
<td>polyserase-I</td>
<td>96</td>
<td>NM_124555</td>
<td>10q13</td>
<td>NM_152973</td>
<td>19p13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(605236)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS11A Human</td>
<td>Mouse</td>
<td>HATL1, HESP</td>
<td>82</td>
<td>AB013874</td>
<td>4q11</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(605211)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS11D Human</td>
<td>Mouse</td>
<td>AK142820 HAT</td>
<td>73</td>
<td>NM_00103323/3</td>
<td>5q35.3</td>
<td>4q31</td>
<td>4q11.3</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(605111)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS11E Human</td>
<td>Mouse</td>
<td>DESC1, UNQ742, PRO H61</td>
<td>81</td>
<td>NM_155561</td>
<td>5q13.1</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(605236)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS11F Human</td>
<td>Mouse</td>
<td>HATL1, HESP</td>
<td>88</td>
<td>NM_124555</td>
<td>4q11.13</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(605211)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMPRSS13 Human</td>
<td>Mouse</td>
<td>MSPL</td>
<td>93</td>
<td>NM_172930.2</td>
<td>11q23</td>
<td>4q11.12</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(608058)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enteropeptidase</td>
<td>Human</td>
<td>Enterokinase, PRSS7</td>
<td>89</td>
<td>NM_001013373</td>
<td>3q13.3</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(226200)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT-SP1 Human</td>
<td>Mouse</td>
<td>Matriptase, SNC19, ST14, PRSS4, Epithin</td>
<td>85</td>
<td>NM_124555</td>
<td>4q11.13</td>
<td>DFNA27</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(606797)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HGNC-approved gene symbols are in bold.

SV, stria vascularis; Mod, modiolus; OoC, organ of Corti; SG, spiral ganglion.
RESULTS

Candidate Gene-Mining Approach

To identify the existence of unidentified TMPRSSs, human (April 2002 hg12 assembly) and mouse (February 2002 mm2 assembly) genomic sequences (http://genome.ucsc.edu/index.html) were analyzed for the presence of known and potentially novel TMPRSSs. All 12 TMPRSSs known at the time were detected. In addition, a further 10 potential TMPRSSs were found and expression profiled (not shown). Three were found expressed in the inner ear (EnsGene8514, EnsGene7682, and SGP16547) and confirmed by sequencing to exist as their predicted forms. EnsGene8514, SGP16547, and GenScan4441 have been subsequently described as bona fide TMPRSS genes and named Tmprss6 (NM_153609), Tmprss7 (AK131211), and Tmprss9 (NM_182973), respectively. The 16 TMPRSS genes, alternative names, human mouse percentage homology, and chromosomal localization are given in Table 1. We subsequently classified the TMPRSS genes for their likelihood of involvement in hearing based on two or three different types of biological evidence, as described below.

Expression profiling in inner ear tissues. The first biological criterion was to identify TMPRSS genes that were expressed in inner ear tissues. Expression profiling of all 16 known TMPRSS genes across a variety of inner ear tissues, including stria vascularis, modiolus, organ of Corti, and spiral ganglion, was determined by RT-PCR. Eight TMPRSS genes were found to be expressed in inner ear tissues (Fig. 1; Table 1). Tmprss1, Tmprss3, Tmprss5, and MT-SP1 expression was detected in all tissues tested. Tmprss2 and Tmprss10 expression was not detected in the spiral ganglion but was present in the other tissues tested. Similarly, Tmprss6 was detected in three tissues tested but not in the organ of Corti. Finally, Tmprss7 expression was restricted to the modiolus and the spiral ganglion. Tmprss9, 11A, 11D, 11E, 11F, 13, and Enteropontidase expression was not detected in any inner ear tissues tested (data not shown). Expression of the 16 known TMPRSS genes was also tested in 12 adult tissues including brain, heart, kidney, thymus, liver, stomach, muscle, lung, testis, skin, ovary, and eye, and in four embryonic stages E8.5, E9.5, E12.5, and E19. All TMPRSS genes showed expression in multiple tissues. Tmprss1 was the more widely expressed with expression in all tissues tested except ovary and thymus, whereas Tmprss10 expression appeared restricted to the heart and testis (Table 1).

Genomic location. At least 93 different loci for nonsyndromic deafness have been mapped on the human genome, and the genes responsible for more than half of these are still unknown [Van Camp and Smith, 2007]. A total of seven TMPRSS genes mapped within a genomic interval that contains a yet uncharacterized deafness gene. The PKSR7 family showed potential linkage to the DFNB8/10 locus but no mutations in the TMPRSS3 gene, suggesting that this family might carry a mutation in a different gene located in the same region [Ben-Yosef et al., 2001]. Tmprss2 mapped within the PKSR7 critical interval, a 1.3-Mb genomic region on human chromosome 21q defined by markers D21S1260 and D21S1411. Unfortunately, DNA samples from the PKSR7 family were not available for analysis. Tmprss5 localized within the DFNB24 locus, a 4.4-Mb region on human chromosome 11 delimited by markers D11S1986 and D11S1992. Tmprss10 mapped within the DFNB25 genomic interval, a 13-Mb region on human chromosome 4 defined by markers D4S405 and D4S428. Four TMPRSS genes, Tmprss11A, D, E, and F, which are all clustered on a 1.5-Mb region on human chromosome 4q12, mapped within the critical interval for DFNA27, a 15-Mb region defined by markers D4S428 and D4S392 [Van Camp and Smith, 2007].

Evaluation of hearing in Tmprss deficient mice by ABR analysis. At the time of the study, the Tmprss1 knockout mouse was the only Tmprss knockout line available and viable for hearing evaluation. Mice deficient for Mt-sp1 failed to develop to term, uniformly dying within 48 hr of birth, and therefore could not be subjected to auditory-evoked brainstem response (ABR) measurement [List et al., 2002]. The ABR test records brain wave activity in response to a series of clicks, and indirectly estimates the level of hearing. Both wild-type and Tmprss1/-/ mice showed similar click-evoked ABR thresholds, whereas Tmprss1/-/ animals (Fig. 2A) produced normal click-evoked waveforms suggesting a functional auditory system. However, Tmprss1/-/ mice showed poorly defined or no ABR response when attenuation was set at 0 or 20 dB (decibel attenuation), respectively, whereas a clearly defined auditory waveform could be measured at 20 dB.A in wild-type mice (Fig. 2A). The average ABR thresholds to clicks for wild-type and Tmprss1/-/ mice were 53 ± 3.92 and 56 ± 3.66 dB sound pressure level (SPL), respectively, whereas the ABR threshold for the Tmprss1 null mice was 95.5 ± 2.68 dB SPL (Fig. 2B). Wild-type and Tmprss1/-/ mice showed similar click-evoked ABR thresholds, whereas Tmprss1/-/ mice had a 42 dB SPL threshold increase, indicating severe hearing impairment (Fig. 2B) [Guipponi et al., 2007].

This candidate gene-mining approach identified, besides Tmprss3, at least seven additional TMPRSS genes that may be playing or are likely to play a role in hearing, based on their expression patterns in the inner ear. We discovered that Tmprss1 is expressed in inner tissues and is essential for hearing, as Tmprss1 knockout mice showed severe hearing loss. The other TMPRSS genes were analyzed for only two biological criteria: inner ear...
expression and genomic location coincident with a deafness locus, as knockout mice were not available for ABR analysis. Three genes, TMPRSS2, TMPRSS5, and TMPRSS10, were classified as candidates on the basis of these two lines of evidence. A total of seven other genes were classified as candidates based on one line of evidence; MT-SPI, TMPRSS6, and TMPRSS7 for expression in inner ear tissues and TMPRSS11A, D, E, and F for mapping within a deafness locus. Recently, a mutation in MT-SPI has been identified in a consanguineous Israeli-Arab family with autosomal recessive ichthyosis with hypotrichosis syndrome. No signs of hearing impairment have been reported in affected members of this family, suggesting that MT-SPI is unlikely to play an important role in hearing [Basel-Vanagaite et al., 2007].

This ongoing candidate gene-mining approach allowed the identification of TMPRSS1 as a gene essential for hearing and Tmprss1 knockout mice were subjected to click-evoked auditory brainstem response (ABR) measurements. The amplitude of the response is measured in microseconds (ms). Click stimulus intensity is indicated in decibel attenuation (dBA). Maximum stimulus intensity corresponds to 0 dBA. The green portion of the graph highlights the waveform chosen to determine threshold. A: Representative ABR response of wild-type, Tmprss1 heterozygote +/-, and Tmprss1 null—/- mice. Adult wild-type, Tmprss1 heterozygous, and Tmprss1 knockout mice were subjected to click-evoked auditory brainstem response (ABR) measurements. The amplitude of the response is measured in millivolts (mV). The latency is shown in milliseconds (ms). The p.Y438X nonsense mutation was predicted to result in a truncated protein with normal hearing. Haplotype analysis showed that Patient 85 carried the p.A317S missense mutation and the p.F369L polymorphism, in heterozygosity. P .F369L is a common variant that has been assayed in the HapMap-CEU European population and p.F369L homozygote accounts for 48.1% of people, presumably with normal hearing. Haplotype analysis showed that these two variants were present on different chromosomes p.A317 is located a few nucleotides downstream of the D308 residue of the H258-D308-S405 catalytic triad. Interestingly, p.A317S showed no detectable proteolytic activity when tested by yeast-based protease assay because of its location upstream of the transmembrane domain [Yun Kim et al., 2002].

Patient 85 carried the p.A317S missense mutation and the p.F369L polymorphism, in heterozygosity. P .F369L is a common variant that has been assayed in the HapMap-CEU European population and p.F369L homozygote accounts for 48.1% of people, presumably with normal hearing. Haplotype analysis showed that these two variants were present on different chromosomes p.A317 is located a few nucleotides downstream of the D308 residue of the H258-D308-S405 catalytic triad. Interestingly, p.A317S showed no detectable proteolytic activity when tested by yeast-based protease assay, indicating potential pathogenic properties. The enzymatic activity of p.F369L was reduced to 45% in comparison with TMPRSS5 WT, suggesting a hypomorphic nature (Table 3). This functional analysis indicated that the p.A317S and p.F369L allelic combination yielded a markedly reduced enzymatic activity compared to the wild-type protein, suggesting that a TMPRSS5 activity below threshold might cause hearing impairment.

Patient 7 was heterozygous for both the potential pathogenic mutation p.Y438X and the hypomorphic polymorphism p.F369L. The p.Y438X nonsense mutation was predicted to result in a prematurely truncated protein that would be deleted of two highly conserved motifs of its protease domain. Haplotype analysis showed that Patient 7 carried both mutations on the same chromosome. Direct sequencing of the coding sequence and promoter region of TMPRSS5 and search for deletions using long-range PCR did not reveal any other pathogenic changes (data not shown). Three genetic hypotheses compatible with an autosomal dominant mutation have been considered. First, we demonstrated, using our yeast-based protease assay, that the p.Y438X mutant

**Figure 2.** Quantiﬁcation of the average ABR thresholds of wild-type, Tmprss1 heterozygote +/-, and Tmprss1 null—/- mice. A: Representative ABR response of wild-type and Tmprss1 heterozygote +/- mice at 0 and 20 dBA. B: Quantiﬁcation of the average ABR thresholds of wild-type, Tmprss1 heterozygote, and Tmprss1 null mice. Graph indicates mean ± standard error of the mean (SEM). No statistical signiﬁcance was found between wild-type and Tmprss1 heterozygote +/- mice. (P > 0.05). Statistical diﬀerence was signiﬁcant between wild-type and Tmprss1 null mice (*P = 4.7 x 10^-1), and between heterozygote and null mice (**P = 8.6 x 10^-13).
showed total absence of proteolytic activity (Table 3). Therefore, it is possible that the disease results from haploinsufficient reductions in TMPRSS5 proteolytic activity toward native substrates. This hypothesis is rather unlikely, because carrying two p.F369L hypomorphic alleles, which provide a similar amount of enzymatic activity as one WT allele (Table 3), is not associated with hearing loss. Indeed, several hearing individuals carried two copies of this polymorphic allele. Second, the mutant enzymes could gain a novel toxic function. Cytotoxicity is somewhat unlikely because the mutant enzymes did not appear to be toxic to yeast cells. Indeed, an equivalent number of yeast cells transformed with novel toxic function. Cytotoxicity is somewhat unlikely because the mutant enzymes did not appear to be toxic to yeast cells. Indeed, an equivalent number of yeast cells transformed with an empty expression vector (Table 3). Although we cannot formally exclude that expression of the mutant protein decreased the expression of either the wild-type or the mutant enzymes grew, indicating that, compared to the wild-type, the mutant enzyme did not induce significant apoptosis (data not shown). At least under the simple culture conditions employed in this assay, there is no evidence for a toxic gain of function activity in the mutant protease. Third, the mutant enzyme may have a dominant negative effect on the wild-type protease. We addressed this hypothesis through coexpression of both wild-type and mutant enzymes. Coexpression of the mutant protease appeared to inhibit the activity of the wild-type enzyme, when compared with cotransfection with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3). Therefore, when cotransfected with an empty expression vector (Table 3)
**TMPRSS1, 3, and 5 Proteins Have Different Subcellular Localizations and Spatial Expression in the Inner Ear**

To explore the function of TMPRSS1, 3, and 5 proteins in hearing, we determined their respective expression profiles in the inner ear using immunohistochemistry assays. TMPRSS1 and 5 proteins were found to share a similar expression pattern, whereas TMPRSS3 was expressed in different parts of the cochlea. TMPRSS1 and 5 expression was detected in the neuron bodies and nerve fibers of the spiral ganglion (Fig. 3). No expression was detected in the organ of Corti (Fig. 4). TMPRSS3 displayed a different pattern characterized by expression in the neuron bodies but not in the nerve fibers of the spiral ganglion (Fig. 3) and in the inner hair cells of the organ of Corti by immunohistochemistry, although expression was detected in this tissue by RT-PCR. This discrepancy may simply result from the greater sensitivity of RT-PCR vs. immunohistochemistry.

**Differential Subcellular Localization of TMPRSS1, 3, and 5 Proteins**

To further explore where these three TMPRSS proteins might exert their function, N- or C-terminally V5 epitope-tagged proteases were transiently transfected into three different cell lines, HepG2, HeLa, and human embryonic kidney (HEK293T) cells. These cell lines were selected because they endogenously express one of the three TMPRSSs studied (data not shown). TMPRSS1 and TMPRSS3 proteins colocalized to some extent with ICAT, an endoplasmic reticulum (ER) marker, indicating that these proteins appeared to be stably anchored to the ER membranes (Fig. 5A and B). Absence of colocalization with Rab5Q79L, an endosomal marker and phalloidin (phln), a marker of the periphery of the cell (not shown), support the ER localization of these two proteins. Interestingly, TMPRSS5 showed a different subcellular localization. As observed in Fig. 5C (panel i), expression of TMPRSS5 can clearly be seen at the plasma membrane where it colocalized with phln, a stain for F-actin that highlights the periphery of the cell. Each of the three TMPRSSs was tested in all cell lines and results obtained were similar whatever the cell line used (data not shown). In addition, cells were treated with 1 mM of the protein synthesis inhibitor, cyclohexamide, for 2–6 hr before immunodetection. Expression of TMPRSS1, 3, and 5 was still visible, even after 6 hr of treatment, indicating that these proteases are stably anchored in the endoplasmic reticulum (ER) membrane (data not shown).

**DISCUSSION**

Type II transmembrane serine proteases (TMPRSSs) are an emerging class of proteolytic enzymes that are involved in various physiological and pathological processes [Wu, 2003]. TMPRSS3, a member of the TMPRSS family, causes nonsyndromic recessive deafness when inactivated by pathogenic mutations [Ben-Yosef et al., 2001; Masmoudi et al., 2001; Scott et al., 2001; Guipponi et al., 2002; Wattenhofer et al., 2002, 2005; Lee et al., 2003; Hutchin et al., 2005]. The involvement of TMPRSS3 in hearing loss suggests the possible implication of other TMPRSS members, as shown for members of the myosin superfamily [Gibson et al., 1995; Weil et al., 1995; Donaudy et al., 2003]. To identify which TMPRSS members are likely to be involved in the hearing process,

**FIGURE 3.** Immunohistochemistry analysis of TMPRSS1, 3, and 5 expression in spiral ganglion neurons of mouse cochlea. A: TMPRSS1 expression was detected strongly in soma (SG) and fibers (arrow) of spiral ganglion neurons in wild-type mice but not in TMPRSS1 gene-deleted mice (inset). Considerable overlap of TMPRSS1 immunoreactivity was observed with NF200 (B), indicating neuronal expression of TMPRSS1 (C, arrow); whereas TMPRSS3 expression was similarly detected in soma of spiral ganglion neurons (D, SG), its expression in the projecting fibers was weaker and colocalization between NF200 (E) and TMPRSS3 was notably restricted to the soma (F). Like TMPRSS1, TMPRSS3 expression was found in the soma (SG) and fibers (arrow) of spiral ganglion neurons (G) and colocalization was observed between TMPRSS5 and NF200 (H) as merged signal is shown in (I). Scale bar = 20 μm.

Human Mutation DOI 10.1002/humu
we used a candidate gene-mining approach. We examined each TMPRSS gene for a variety of criteria relevant for involvement in hearing function. We hypothesized that candidates would demonstrate involvement of their mouse orthologs in deafness through evaluation of the auditory function of the corresponding mouse knockout line and/or would show expression in inner ear tissues and mapping to a genomic interval that contains a yet unidentified deafness gene.

At the time of the study, only four Tmprss null mice had been generated. Matriptase deficient mice develop to term but die within 2 days of birth, well before the onset of hearing function [List et al., 2002]. Tmprss10 knockout mice develop normally and survive to postnatal life. They have spontaneous hypertension and exhibit cardiac hypertrophy [Chan et al., 2005]. Tmprss2 deficient mice develop normally and survive to adulthood with no obvious phenotype [Kim et al., 2006]. Tmprss1 knockout mice were the only line available for analysis. These mice are viable and fertile and do not exhibit obvious defects in growth, liver function, or blood coagulation [Wu et al., 1998]. Evaluation of their hearing status showed severe hearing impairment. TMPRSS1 is ubiquitously expressed, yet no other overt dysfunctions were detected in TMPRSS1 deficient mice. This suggests that in most tissues, other proteases substitute for the function normally performed by TMPRSS1, whereas it has a unique contribution to the auditory function. As expected, TMPRSS1 expression was detected in different structures of the mouse cochlea. The fact that TMPRSS1 protein was not observed in cochlea tissues that were shown to express Tmprss1 mRNA suggests absence of translation in these tissues or difference in detection thresholds between protein and mRNA molecules. The human TMPRSS1 gene mapping position did not coincide with any known deafness locus. These data convincingly demonstrate that the Tmprss1 gene plays an important role in the auditory system in mice and strongly support the selection of its human ortholog for mutation search. This mutation analysis showed that the TMPRSS1 gene is not contributing to the genetics of deafness in our large cohort of deaf individuals. It does not exclude a role for TMPRSS1 in deaf populations of different origins. Indeed, it has been shown that the prevalence of mutations in the connexin 26 gene, a major cause of nonsyndromic autosomal recessive deafness, varies in different ethnic groups [Rabionet et al., 2000]. The absence of mutations in TMPRSS1 was rather surprising based on the severe hearing loss observed in Tmprss1 null mice. We could not exclude, however, that Tmprss1 null mice suffer from syndromic deafness characterized by severe hearing loss and other clinical features that are mild or impossible to detect if not specifically investigated. If this is the case, we have not screened the clinically relevant cohort of individuals with hearing loss.

For about half of the 93 described forms of hereditary nonsyndromic deafness, the causal genes remain to be identified, although they have been mapped to more-or-less well-delimited regions in the human genome [Van Camp and Smith, 2007]. The genes encoding for TMPRSS proteins are dispersed on various chromosomes, except for a cluster of four genes on the long arm of chromosome 4. We identified seven TMPRSS genes, which mapped within four distinct genomic intervals that harbor a deafness gene that is still unidentified. Among them, TMPRSS2, 5, and 10, which mapped within the PKSR7, DFNB24, and DFNB25 deafness loci, respectively, had previously been found expressed in different structures of the mouse cochlea. In general, TMPRSS members demonstrate remarkably restricted expression patterns, with the exception of TMPRSS1 and TMPRSS2, which are present...
in many different tissues. It was not surprising to detect TMPRSS5 in inner ear tissues based on its expression in neurons and epithelial tissues. It is surprising however, to find the presence of TMPRSS10 in the inner ear, given its specific expression and function in the heart [Wu, 2003]. These four genes were selected for mutation search in the relevant familial cases, when available for analysis, and in our large cohort of deaf cases.

Among the 362 deaf patients analyzed, we identified three different mutations in the TMPRSS5 gene in three different sporadic cases. Patient 88 was found to be a compound heterozygote for the inactive mutant p.A317S and the common hypomorphic allele p.F369L. We hypothesized that this allelic combination might cause the disorder, since the partial activity of the L369-TMPRSS5 protein may not be sufficient to compensate fully for the enzymatically inactive S317-TMPRSS5 protein. This hypothesis also predicts that the L369 homozygotes do not suffer from hearing loss because two hypomorphic alleles achieve the minimal required activity for proper hearing. Biochemical characterization of the mutants suggests that a TMPRSS5 activity below threshold might cause hearing impairment.

Patient 7 was carrying the p.Y438X nonsense mutation and the p.F369L hypomorphic allele on the same chromosome. This patient inherited a normal allele from his hearing mother and a mutated allele from his father, unless the p.Y438X is a de novo mutation. Unfortunately, the father was not available for analysis and we did not have any audiological examinations of the parents. We consequently sought to define the biochemical consequences of the mutations occurring in this patient. Three possible hypotheses were examined. Haploinsufficient reduction in proteolytic activity did not gain much support because half reduction of TMPRSS5 enzymatic activity did not appear to be sufficient to cause hearing loss. We similarly did not find much evidence for gain of toxic function. We tested one potential novel function. Although our simple cell culture model cannot fully reproduce events in the inner ear, expression of wild-type or mutant forms of TMPRSS5 appeared to have no toxic effects on the cells. Another potential novel gain of function activity not addressed in this report could involve change of proteolytic activity, given that the p.Y438X mutation would not affect the catalytic site but delete two highly conserved motifs of the protease domain. In contrast, we found experimental support for the dominant negative inhibition hypothesis. When the wild-type and mutant proteases were coexpressed, the total enzymatic activity appeared inhibited by the mutant. Such a dominant negative effect has already been described in severe congenital neutropenia which results from mutations in ELA2, encoding a chymotryptic serine protease [Li and Horwitz, 2001]. Additionally, auto- and heterocatalytic requirements for each transmembrane protease are a necessity in the Xenopus oocyte expression system, supporting either a dominant negative effect of a potential digenic disease [Wattenhofer et al., 2005]. It is concluded that, despite functional evidence suggesting a dominant negative effect, we could not formally rule out that Patient 7 carried another TMPRSS5 pathogenic mutation in the gene’s unexplored noncoding regions or the possible implication of another TMPRSS gene for a digenic origin of the hearing loss [del Castillo et al., 2002; FIGURE 5. Intracellular localization of TMPRSS1, 3, and 5 proteins. A: TMPRSS1-V5 C-terminus proteins were coexpressed in HepG2 cells (a, d) with either ICAT-GFP (b) or Rab5-GFP (e). Merge panels (c) and (f) show superimposition of (a) and (b), and (d) and (e), respectively. B: TMPRSS3-V5 C-terminus proteins were coexpressed in HeLa cells (a, d) with either ICAT-GFP (b) or Rab5-GFP (e). Merge panels (c) and (f) show superimposition of (a) and (b), and (d) and (e), respectively. C: TMPRSS5-V5 C-terminus proteins were coexpressed in HEK 293 cells (a, d, and g) with either ICAT-GFP (b) or Rab5-GFP (e) or stained with the Texas Red-X Phalloidin probe (h). Merge panels (c, f, and i) show superimposition of (a) and (b), (d) and (e), and (g) and (h), respectively. Human Mutation DOI 10.1002/humu
Pallares-Ruiz et al., 2002; Zheng et al., 2005]. However, the latter hypothesis did not receive any experimental support as we did not find any potential pathogenic mutations in the other TMPRSS genes analyzed in Patient 7. Digenic inheritance might include defects not only in serine proteases but also in protein classes with very different functions. Indeed, the proteins encoded by the Usher genes, referred to as the Usher protein complex, include motor, scaffold, cell adhesion, and signaling proteins [Kremer et al., 2006]. It is also of interest to note that a recent study concluded that 53% of rare missense alleles are deleterious in humans by combining analysis of mutations causing human Mendelian diseases, of human-chimpanzee divergence, and of systematic data on human genetic variation and found that ~20% of new missense mutations in humans result in a loss of function, whereas ~27% are effectively neutral. Thus, the remaining 53% of new missense mutations have mildly deleterious effects [Kryukov et al., 2007].

Finally, we did not find any mutations in TMPRSS5 and TMPRSS10 genes in affected members of DFNB24 and DFNB25 deafness families, respectively, indicating that these genes are presumably not responsible. This has been confirmed for TMPRSS5, as mutations in the RDX gene have been shown to cause nonsyndromic hearing loss at the DFNB24 locus [Khan et al., 2007]. However, we did not formally exclude mutations in intronic or promoter regions and major changes such as exon deletions. In addition, the absence of mutations in TMPRSS2 and TMPRSS10 in our large cohort of sporadic deaf cases suggests that these two genes are unlikely to contribute significantly to the genetics of deafness.

The function of these TMPRSSs in the inner ear remains unknown. Determination of their expression pattern and subcellular localization suggests different roles in hearing. Expression of TMPRSS1, 3, and 5 in spiral ganglion neurons suggests a role in transmission of signals from sensory hair cells to the auditory centers of the brain. TMPRSS3 expression in the inner hair cells of the organ of Corti strongly supports a role in mechanotransduction. While TMPRSS1 and 3 functions seem to be restricted to the ER compartment, TMPRSS5 appears to be also present at the cell surface. The differences in the cellular and subcellular localization of these TMPRSS proteins may explain the absence of functional redundancy, their unique contribution to inner ear function, and suggest interaction with different substrates.

These results also validate that, similar to other candidate gene analyses, the systematic high-throughput analyses of candidate genes in large numbers of patient samples is an efficient approach in identifying human disease genes.

**ACKNOWLEDGMENTS**

We thank M. Fornito for her excellent technical assistance. This work was supported by a Garnett Passe and Rodney Williams Memorial Foundation Research Training Fellowship (to M.G.); and by the National Health and Medical Research Council (NHMRC) fellowship (to H.S.S.).

**REFERENCES**


