TMPRSS3, a type II transmembrane serine protease mutated in non-syndromic autosomal recessive deafness

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1. ABSTRACT

Recently, we and others have shown that mutations in *TMPRSS3* were responsible for autosomal recessive non-syndromic hearing loss. *TMPRSS3* is a member of the Type II Transmembrane Serine Protease (TTSP) family and encodes for a protease that also contains LDLRA (low-density lipoprotein receptor class A) and SRCR (scavenger receptor cysteine rich) domains. Fourteen pathogenic mutations, which occur not only in the catalytic domain but also in the LDLRA and SRCR domains, have been identified to date that cause the DFNB8/10 forms of deafness. *In vitro* experiments demonstrated that *TMPRSS3* mutants were proteolytically inactive indicating that *TMPRSS3* protease activity is critical for normal auditory function. However, how missense mutations in the LDLRA and SRCR domains affect the proteolytic activity of *TMPRSS3* remains to be elucidated. Although the role of *TMPRSS3* in the auditory system is currently not completely understood, it has been shown to regulate the activity of the ENaC sodium channel *in vitro* and could therefore participate in the regulation of sodium concentration in the cochlea. *TMPRSS3* mutations are not a common cause of hereditary deafness, the elucidation of its function is nevertheless important for better understanding of hearing, and provide biological targets for therapeutic interventions.

2. INTRODUCTION

The human genome contains over 560 protease genes which account for about 2% of the human genes. Proteases are essential for synthesis of all proteins, controlling protein composition, size, shape, turnover and degradation. Proteases are important in conception and birth, life, ageing, and death of all organisms. Most tissues use proteases in a sophisticated network of endogenous regulators of basic cellular functions. The challenge is to unravel the highly specific roles carried out by each protease and to determine their respective importance to life, health, ageing, disease, and ultimately their value to man in medical application.

TMPRSS3 is a member of the Type II Transmembrane Serine Protease family (TTSP), a class of membrane-bound proteolytic enzymes that are important mediators in a variety of biological processes (1). It was originally cloned and named Tumor Associated Differentially-expressed Gene-12 (TADG-12), as a new serine protease overexpressed in ovarian cancer specimens (2). Subsequently, several others studies have reported overexpression of *TMPRSS3* in pancreatic (3), ovarian (4) and breast (5) cancers. The authors of these studies proposed that *TMPRSS3* might play an important role in cancer development and progression and could serve as a

molecular target for therapy and/or a diagnostic marker. Besides its association with cancer biology, *TMPRSS3* is mainly recognized and studied for the important role it plays in the auditory function. Indeed, in 2001 Scott et al. showed that *TMPRSS3* was the gene mutated in non-syndromic autosomal recessive deafness (DFNB8/10) (6). This was the first description of a serine protease involved in hearing loss. Since this discovery, there has been an intense effort to determine the contribution of *TMPRSS3* to the genetics of deafness and to study its role in the hearing process.

Little is known about the molecular basis of normal auditory function. The auditory system has proven difficult to access and very small amount of tissue, containing highly specialized cells, are available for analysis. For these reasons, a genetic approach has been exceptionally useful for identifying the key components of auditory transduction, as it makes no assumptions about the nature or expression level of molecules essential for hearing. Approximately 1/900 children are born deaf and around 60% of these cases of congenital deafness are genetic. These genes underlying monogenic inherited hearing loss may also play a role in age-related hearing loss (presbyacusis), the most common cause of hearing impairment (7). Therefore, identifying the genes underlying hearing loss represents a major objective of current biomedical research.

From the identification and initial characterization of the crucial role played by *TMPRSS3* in the hearing process, researchers are moving towards the identification of the cellular and molecular processes controlled by TMPRSS3 proteolytic activity. This research should lead to a comprehensive understanding of the role of this serine protease in inner ear function and insights into the pathogenesis of congenital and age-related hearing loss, as well as strategies for their prevention and treatment.

3. LOCI FOR DEAFNESS DFNB10 AND DFNB8 ON CHROMOSOME 21Q22.3

A large consanguineous Palestinian family (BT117) was described with more than 40 deaf individuals segregating an autosomal recessive form of non-syndromic deafness (8). Hearing evaluation of affected and non-affected members by pure-tone audiometric tests showed severe deafness in the affected individuals. A genome wide linkage analysis resulted in the mapping of the causative gene to a region of 12 cM on chromosome 21q22.3 (8). This family defined DFNB10, an autosomal recessive, non-syndromic, congenital deafness.

A large consanguineous Pakistani family (1DF) was described that segregated recessive, non-syndromic childhood onset deafness (9). The age of onset of deafness was 10-12 years and hearing was completely lost within 4-5 years. Linkage analysis mapped this disease locus to a region of 15 cM on chromosome 21q22.3 (9). This family defined DFNB8, an autosomal recessive, non-syndromic, childhood onset deafness.

As the description of these two families occurred independently in the same year, two different locus numbers were attributed to them, but it was rather clear that the two large genomic regions of linkage on chromosome 21q22.3 were overlapping. However, as the phenotypes of these two pedigrees were not identical -childhood onset in DFNB8 versus congenital deafness in DFNB10- it was thought they were likely to define two different loci as opposed to allelic variants (10).

4. POSITIONAL CLONING OF THE *TMPRSS3* GENE

4.1. A *TMPRSS3* mutation causes DFNB10

The group of N. Shimizu and J. Kudoh at Keio University in Japan, and S.E. Antonarakis and H.S. Scott at the University of Geneva, were active in physical and transcription mapping of chromosome 21q22.3, as well as compiling the genomic sequence of the region. The advances of the physical map and sequencing of chromosome 21 allowed us to narrow down the DFNB10 locus to a critical region (CR) of approximately 1 Mb (11). By assuming DFNB8 and DFNB10 were in fact caused by the same gene, the CR could be refined to approximately 740-kb. Detailed analysis of the genomic sequence of the CR revealed 13 genes, including TMPRSS3. Interestingly, the exon 11 of the *TMPRSS3* gene was abnormally large in all affected individuals of family BT117 and characterized by the insertion of 18 complete ß-satellite repeat monomers. This would result in a frameshift mutation from G393 within the protease domain of TMPRSS3 and termination at 404 amino acids after the addition of 11 unrelated amino acids. It was concluded that this mutation in *TMPRSS3* causes DFNB10 (6).

4.2. A *TMPRSS3* mutation also causes DFNB8

We subsequently tested for *TMPRSS3* mutations in the Pakistani family 1DF with DFNB8. A mutation G to A in position −6 of IVS4, possibly creating a novel acceptor splice site, was found in the affected members of this family. *In vitro* splicing analyses revealed the use of the putative splice acceptor site created by IVS4-6G>A. The use of this alternative splice site would result in a frameshift from C107, and termination at 132 amino acids after the addition of 25 unrelated amino acids. Thus IVS4-6G>A can be considered to be a pathogenic mutation (6).

5. THE *TMPRSS3* GENE, TRANSCRIPT AND EXPRESSION

The *TMPRSS3* gene has 13 exons spanning 24 kb of genomic sequence (Figure 1a) and expresses a main transcript of 2468 bp (*TMPRSS3A*, NM_024022; Figure 1b) encoding a 454-residue protein (NP_076927; Figure 1c). Four rare alternative transcripts, *TMPRSS3b* to *e* encoding putative polypeptides of 327, 327, 344 and 538 amino acids respectively, were also detected. The *TMPRSS3b* and *TMPRSS3c* transcripts encode for the same peptide that does not contain a TM domain (data not shown) (6, 12).

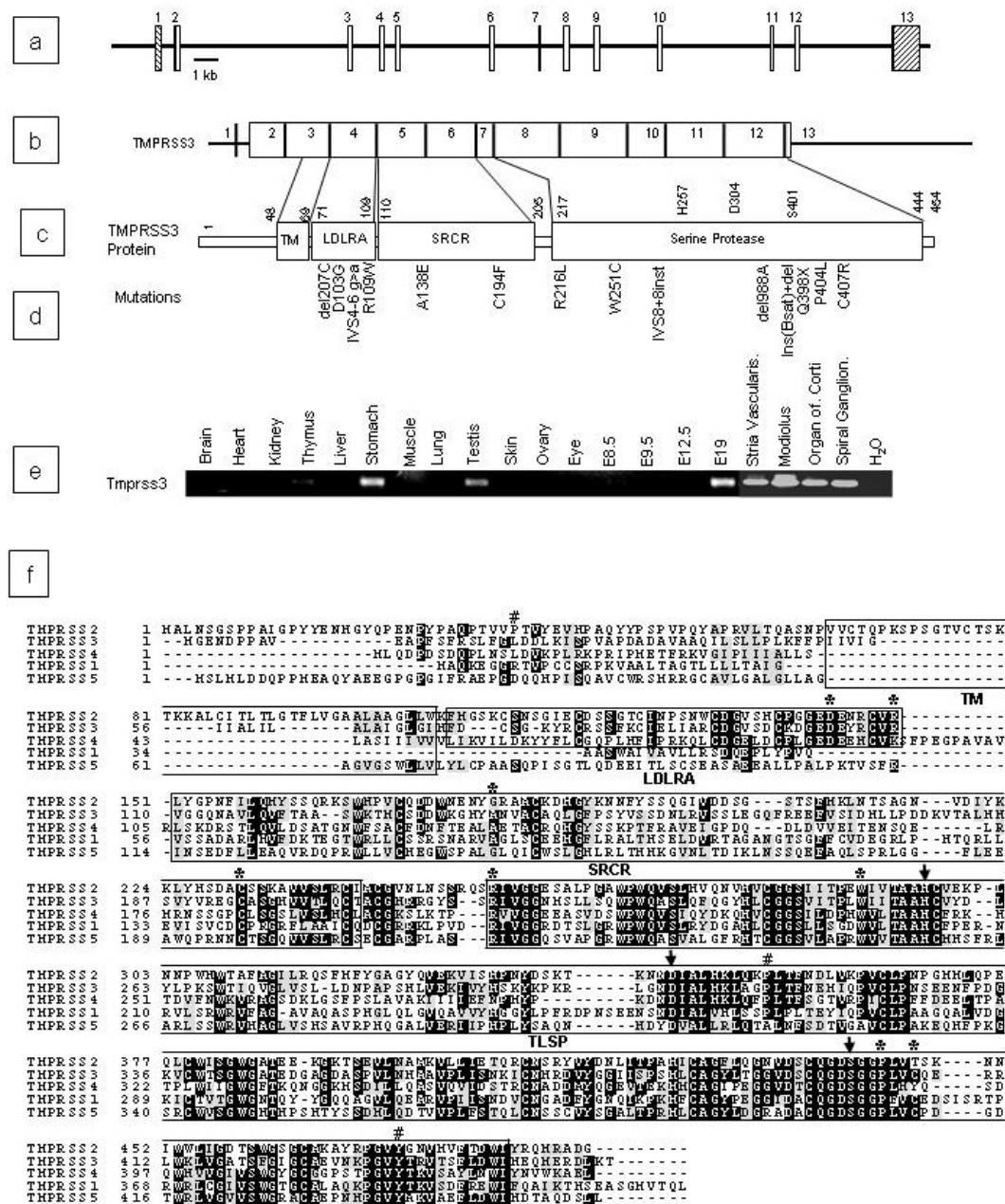Northern blot analysis showed very weak expression of the *TMPRSS3a* transcript. Semi-quantitative

Figure 1. The *TMPRSS3* gene, transcript, protein, mutations and expression. a. The *TMPRSS3* gene contains 13 exons (boxes) spanning 24kb of genomic sequence on human chromosome 21q22.3. b. *TMPRSS3* main transcript (coding regions in boxes, non-coding regions indicated by lines). c. A schematic of the TMPRSS3 protein showing the transmembrane (TM), low-density lipoprotein receptor class A domain (LDLRA), scavenger receptor cysteine-rich domain (SRCR) and protease domains and their position in the 454 amino acid peptide. The active site residues His257, Asp304, and Ser401 are indicated. d. The positions of the 14 *TMPRSS3* pathogenic mutations relative to the protein are indicated. e. RT-PCR analysis of the expression of *TMPRSS3* on cDNA from 20 tissues. f. Representative protein homologies with other human type II transmembrane serine proteases are shown. They are TMPRSS1 (CAA30558), TMPRSS2 (O15393), TMPRSS4 (AAF74526), and TMPRSS5 (AB028140). Domains, as detected in *TMPRSS3* (NP_076927), are boxed according to their position in TMPRSS3 and labeled underneath with the active site residues His257, Asp304, and Ser401 indicated by arrows above the alignment. TMPRSS2 and 4 share exactly the same domain structure as TMPRSS3 while TMPRSS1 and TMPRSS5 lack an LDLRA domain. *TMPRSS3* missense mutations are indicated above the sequence alignment with red asterisks (*). Potential TMPRSS5 mutations are shown above the sequence alignment with a hash (#).

RT-PCR analysis revealed that all five transcripts show distinct pattern of expression (6), but TMPRSS3a was the most abundant, with significant expression found in the thymus, stomach, testis and E19 embryos (12, 13). In the auditory system, strong TMPRSS3 expression was detected in the stria vascularis, spiral ganglion neurons, modiolus and organ of Corti by RT-PCR (Figure 1e). RNA in situ hybridization further showed evidence of specific TMPRSS3 expression in inner ear tissues. TMPRSS3 was present in the otocyst of embryonic day 10.5 and 14.5 mice (http://www.tigem.it/ch21exp/body/bodyTMPRSS3.html). In postnatal day 5 mice, when inner ear development is almost complete, TMPRSS3 expression was found in the spiral ganglion neurons and in the epithelium, which supports cells of the organ of Corti. Weak expression was also observed in the stria vascularis (13).

6. THE TMPRSS3 PROTEIN

The *TMPRSS3a* transcript encodes a putative 454 amino acid peptide that contains in order a cytoplasmic tail, a transmembrane (TM), a low density lipoprotein receptor A (LDLRA), a scavenger receptor cysteine-rich (SRCR) domain and a serine protease domain (Figure 1c). This domain structure has been observed in other proteases including the human transmembrane serine protease TMPRSS2 which shows the highest homology to TMPRSS3. *TMPRSS2* also maps on 21q just centromeric of the DFNB10 critical region. The domain structure of the TMPRSS3 protein is reflected in the gene structure with the TM domain encoded by exon 3, the LDLRA domain by exon 4 and the SRCR domain by exons 5 and 6 (Figure 1b, c).

The serine protease domain of TMPRSS3 (residues 217 to 444) showed between 38 and 45% identity with other transmembrane serine proteases. The TMPRSS3 protease domain is compatible with the S1 family of the SA clan of serine-type peptidases for which the prototype is chymotrypsin (14, 15). The serine protease active site residues (H257, D304, and S401) are conserved and TMPRSS3 is predicted to cleave after K or R residues as it contains D395 at the base of the specificity pocket (S1 subsite) that binds to the substrate. The N-terminus of the protease domain is immediately preceded by the peptide sequence, RIVGG. Proteolytic cleavage between R and I would result in protease activation similar to other serine protease zymogens (14, 15), converting TMPRSS3 to a non-catalytic and catalytic subunit linked by a disulfide bond (probably C207 to C324). The TMPRSS3 serine protease domain contains 6 conserved cysteine residues which by homology to other proteases and 3D modeling are likely to form the following intrasubunit disulfide bonds: C242-C258; C370-C386; C397-C425.

As no recognizable leader sequence precedes the predicted hydrophobic TM domain (residues 48-69), TMPRSS3 is likely to be a type II integral membrane protein. Where the subcellular localization is described, the TTSPs are anchored to the plasma membrane with a cytosolic N-terminus and extracellular protease domain (1,

16, 17). Similarly, TMPRSS3 is predicted to have its N-terminus on the inside of a membrane and the protease domain on the outside of a membrane.

The ~40 amino acid long LDLRA domain, which contains 6 disulfide-bound cysteines (C72, C79, C85, C92, C98, and C107), was originally found in the low density lipoprotein receptor as the binding sites for LDL (18) and calcium (19, 20). It has subsequently been described in numerous extracellular and membrane proteins (PDOC00929; http://www.expasy.ch/cgi-bin/get-prodoc-entry?PDOC00929).

An ~100 residue long putative adhesive extracellular SRCR domain was also identified in TMPRSS3. SRCR domains linked to serine protease domains have been reported in secreted or membrane-bound molecules with diverse biological roles in development and immunity (21) (PDOC00929; http://www.expasy.ch/cgi-bin/get-prodoc-entry?PDOC00348). The LDLRA and SRCR domains of TMPRSS3 are potentially involved in binding with extracellular molecules and/or the cell surface.

When expressed in *Xenopus* oocytes, the *TMPRSS3a* transcript was detected as a three protein species by Western blot analysis: a predominant doublet of 50 and 54 kDa and a faint 30 kDa fragment suggesting that a significant fraction of TMPRSS3 is proteolytically processed (Figure 2c). The faint 30 kDa fragment migrated at the same position as the catalytic serine protease domain (SP domain, lane 11) consistent with an auto-proteolytic cleavage at the activation site between R216 and I217, as occurs on other TTSPs (22). The unprocessed isoform of TMPRSS3 was detected for all TMPRSS3 deafness mutants indicating absence of cleavage. These results indicate that the LDLRA and SRCR domains and the serine protease domain are all implicated in the processing of TMPRSS3 (Figure 2c) (13).

7. *TMPRSS3* MUTATION SPECTRUM IN DEAFNESS

Subsequent to the discovery that *TMPRSS3* was mutated in the non-syndromic autosomal recessive deafness DNFB10 and DFNB8, ourselves and other investigators examined the DNA of additional patients in both familial and sporadic cases of deafness. The 14 pathogenic changes detected to date are summarized in Table 1 and Figure 1d and 1f. In addition to the evidence detailed below, all pathogenic changes were excluded as being polymorphisms by examining a large number of control chromosomes from relevant populations.

The 207delC mutation appeared to be the most common of the *TMPRSS3* mutant alleles. This sequence variant has been found in affected individuals of Spanish, Greek, Newfoundland and Pakistani origins. This deletion of 1 cytosine is predicted to result in a frameshift after amino acid I69 just beyond the transmembrane domain, addition of 18 novel amino acids and premature termination of TMPRSS3 (23).

Table 1. Summary of all pathogenic *TMPRSS3* sequence variants and age of onset of hearing loss

Mutation	Domain	AA Level	Activity[1]	Hearing phenotype	Origin	Ref.
del207C		Frameshift +88 STOP		Prelingual, severe to profound	1 Spanish patient	22
				Prelingual, severe to profound	1 Greek patient[2]	22
				Prelingual, severe to profound	1 Newfoundland family[3]	12
				Congenital, profound	2 Pakistani families	12
308A>G	LDLRA	D103G	2%	Prelingual, severe to profound	1 Greek patient[2]	22
IVS4-6G>A		Frameshift +132 STOP		Childhood onset, progressive, severe to profound	1 Pakistani family (DFNB8)	6
325C>T	SRCR	R109W	0%	Congenital, profound	1 Pakistani family	23
413 C>A	SRCR	A138E	N.D	Childhood onset, progressive, moderate to severe	1 UK family	24
581G>T	SRCR	C194F	1%	Congenital, profound	2 Pakistani family	23, 12
647 C>T	Activation site	R216L	N.D	Congenital, severe to profound	1 Turkey family	25
753G>C	Protease	W251C	0%	Congenital, severe to profound	1 Tunisian family	26
IVS8+8insT		Frameshift +272 STOP		Prelingual, severe to profound	1 Newfoundland family[3]	12
del988A	Protease	Frameshift +352 STOP		Prelingual, severe to profound	1 Palestinian family	27
Ins(B-sat)+del	Protease			Congenital, profound	1 Palestinian family (DFNB10)	6
1192 C>T	Protease	Q398X	N.D	Congenital, severe to profound	1 Turkey family	25
1211C>T	Protease	P404L	2%	Congenital, severe to profound	1 Tunisian family	26
				Childhood onset, severe to profound	1 Turkey family	25
1219T>C	Protease	C407R	0.5%	Congenital, profound	3 Pakistani families	23, 12

[1] Proteolytic activity of *TMPRSS3* mutants using a yeast-based protease assay. N.D: not determined (31). [2] This greek patient is compound heterozygote for the del207C and D103G mutations (23). [3] The majority of the individual of this family are homozygous for the del207C mutation, while two of the hearing impaired subjects are compound heterozygotes for the del207C and IVS8+8insT mutations (12).

The D103G missense mutation was found in heterozygosity in a Greek pedigree. The mutation affects an Asp residue of the LDLRA domain that is well conserved. 3D modeling of the mutation suggested that this substitution impairs the calcium binding site of the LDLRA domain (23).

The missense mutation, R109W, was detected in homozygosity in one Pakistani family. This substitution is in the last amino acid of the LDLRA domain, which is potentially involved in binding of TMPRSS3 with extracellular molecules and/or the cell surface. Two out of the other three most closely related TTSPs have either Arg or the similar positively charged Lys at this position (24).

The A138E missense mutation has been identified in homozygosity in a non-consanguineous Caucasian family. This change alters a highly conserved residue of the scavenger domain (25).

The missense mutation, C194F, was detected in homozygosity in affected members of one Pakistani pedigree. The mutation is within the SRCR domain and affects a highly conserved Cys residue (24).

The R216L missense mutation was identified in homozygosity in a consanguineous Turkish family. This mutation affects a well-conserved residue among the serine proteases and leads to a mutant form that fails to undergo activating proteolytic cleavage, thereby resulting in an inactive protein (26).

The W251C missense mutation was found in homozygosity in a consanguineous Tunisian family. This mutation lies in the serine protease domain and affects a Trp residue that is highly conserved among serine proteases of the S1 type. Examination of the predicted 3D-structure suggested that the W251C mutation might lead to a destabilization of TMPRSS3, as the large side chain of the Trp residue occupies a large hydrophobic pocket on the exterior of the protein. Structural rearrangements caused by

substituting the smaller Cys would likely affect the nearby active site H257 residue and thus the activity of the enzyme (27).

The IVS8+insT splice site mutation was identified in heterozygosity in a family from Labrador, a Canadian island whose small population lives in isolated villages. This insertion of a thymine residue after the splice donor site of exon 8 is predicted to result in the skipping of exon nine and premature termination of TMPRSS3 (12).

The del988 mutation was found in homozygosity in a Palestinian family. This mutation is predicted to lead to a prematurely truncated protein lacking the last residue of the HDS catalytic triad leading to an inactive mutant (28).

The Q398X nonsense mutation was identified in homozygosity in a consanguineous family from Turkey. This mutation is predicted to result in a prematurely truncated protein lacking the last residue of the HDS catalytic triad leading to an inactive mutant (26).

The P404L missense mutation was observed in homozygosity in two consanguineous families from Tunisia and Turkey. This mutation is located within the sequence signature characteristic of serine protease active sites, separated from the catalytic Ser by two Gly residues (-Ser-Gly-Gly-Pro-Leu-). P404 is well conserved among the members of the S1 chymotrypsin family of proteases (Figure 3f). For an exchange of Pro with Leu at position 404 we would expect a significant alteration of the geometry of the active site loop affecting the catalytic activity. The P404L mutation did not appear to have a single origin, as haplotypes around the *TMPRSS3* gene in the two families are different favouring an independent origin of the mutation (26, 27).

The C407R missense mutation was found in homozygosity in two Pakistani pedigrees. This substitution is within the serine protease domain only a few amino acids from the active site residue S401 within the substrate pocket. Although C407 is not highly conserved, the non-
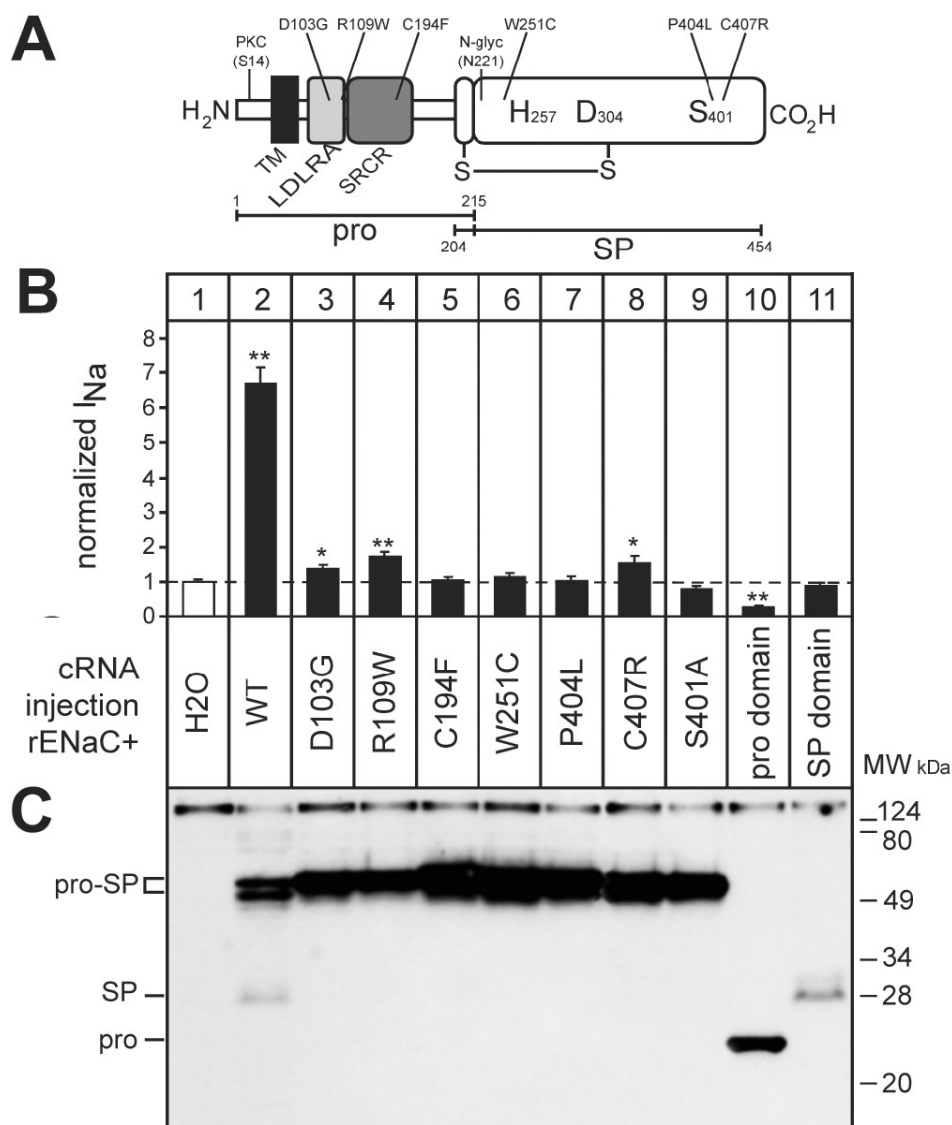
Figure 2. Functional expression of *TMPRSS3* in *Xenopus* oocytes. A. Predicted structure of TMPRSS3. Numbers indicate the amino acid positions. The serine protease catalytic triad residues (H257, D304 and S401), the predicted disulfide bridge which links the pro- and catalytic domains of TMPRSS3, and the tested human deafness associated *TMPRSS3* missense mutations D103G, R109W, C194F, W251C, P404L and C407R are shown. pro, pro-domain; SP, serine protease domain. B. Comparison of the effect of WT-*TMPRSS3* and mutants on I_{Na} in *Xenopus* oocytes. Oocytes were injected with rat ENaC subunits in the presence of either water (lane 1, white column), WT-*TMPRSS3* (lane 2), *TMPRSS3* missense deafness associated mutants (lanes 3-8), serine catalytic mutant (lane 9), pro domain construct (lane 10) or serine protease domain construct (SP) of *TMPRSS3* (black columns). ** = p< 0.01, * = p< 0.05 vs lane 1. Lanes 1-9 and 11, [n ≥ 25]; lane 10, [n=16]. C. Biochemical analysis of WT and mutants *TMPRSS3* by Western blot on protein extracts from pools of 10 injected oocytes. Pro-SP, pro-serine protease; pro, pro-domain; SP, serine protease domain. Same conditions as described under B.

conservative substitution of a small polar uncharged Cys to a large positively charged Arg so close to the S401 active site residue is expected to alter the geometry of the active site loop and therefore affect the serine protease activity (24).

Pathogenic mutations in the *TMPRSS3* gene have been identified in two Palestinian, nine Pakistani, two Tunisian, three Turkish, one United Kingdom and one Newfoundland families as well as two sporadic Caucasian

patients from Spain and Greece. Collectively, these analyses reported low to very low percentages of *TMPRSS3* mutations in the studied populations. The frequency of *TMPRSS3* mutations in a European childhood deaf population is approximately 0.5 % (3/543) after exclusion of the common 35delG GJB2 mutation. However, the estimate in the Pakistani population is approximately 1.8% (8/449), and 5% (2/39) in Tunisian families. In both these populations, GJB2 mutations were not excluded and thus
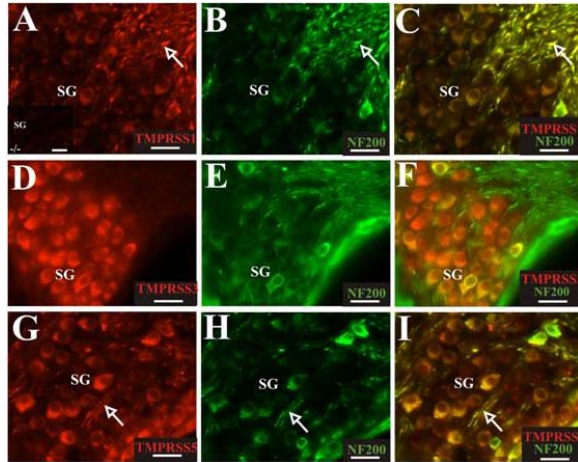
Figure 3. Immunohistochemistry analysis of TMPRSS1, 3 and 5 expression in spiral ganglion neurons of mouse cochlea. (A) TMPRSS1 expression was detected strongly in soma (SG) and fibres (arrow) of spiral ganglion neurons in wild type mice but not in *Tmprss1* deficient mice (inset). Considerable overlap 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 fibres was weaker and co-localisation between NF200 (E) and TMPRSS3 was notably restricted to the soma (F). Like TMPRSS1, TMPRSS5 expression was found in the soma (SG) and fibres (arrow) of spiral ganglion neurons (G) and co-localisation was observed between TMPRSS5 and NF200 (H) and merged signal is shown in (I). Scale bar = 20 µm.
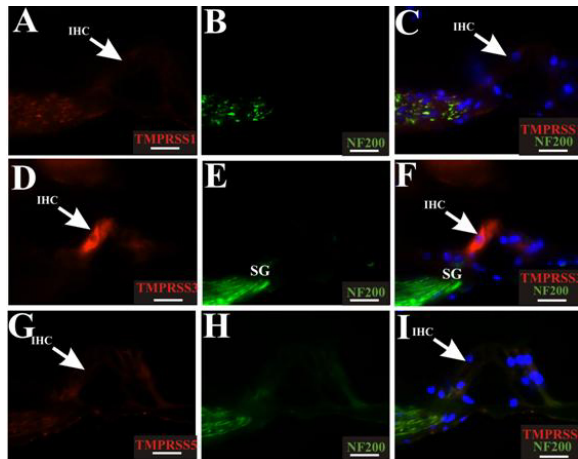

Figure 4. Immunohistochemistry analysis of TMPRSS1, 3 and 5 expression in the organ of Corti. (A) TMPRSS1 protein was not detected in inner hair cells (arrow). Location of inner hair cell was confirmed using NF200 to trace afferent projection to the organ of Corti (B) and DAPI staining of nuclei (C). In contrast, TMPRSS3 was found to be expressed strongly in inner hair cells (D and F, arrow). (G, I) TMPRSS5 was not detected in inner hair cells (arrow) while afferent projection could be identified using NF200 antibody (H). Scale bar = 20 µm.

TMPRSS3 mutations are still a significant cause of deafness. In the Turkish population, a prevalence of 11% of *TMPRSS3* mutations among patients with childhood hearing loss and negative for GJB2 mutations was observed indicating that this locus plays a role in about 8% of the total childhood deaf Turkish population (26).

8. CLINICAL PRESENTATION OF DEAFNESS CAUSED BY MUTATIONS IN THE *TMPRSS3* GENE

TMPRSS3-related deafness is generally characterized by congenital, bilateral and severe to profound sensorineural hearing loss (SNHL). However, variations in the expression of the phenotype, mainly the age at onset and severity of hearing deficits, have been reported. Indeed, two siblings from UK, carrying the A138E mutation in homozygosity, were affected by progressive, moderate to severe, sloping hearing loss first noticed at about 5 years of age (25). Based on the molecular and clinical data available to date, it seems that there is no relationship between the expression of phenotype and the severity, nature or location of the mutation. Even the same mutation may relate to variable severity of the phenotype. The P404L missense mutation has been identified in two different families from Turkey and Tunisia. The hearing loss in the Tunisian family was congenital, whereas in the Turkish family the onset of hearing impairment was at age 6-7 years. The variable phenotypic expression of the P404L mutation suggests that other factors, including environmental and/or genetic modifiers, influence the clinical presentation of hearing loss caused by mutations in the *TMPRSS3* gene. Another alternative would be that the age of onset was not well determined in one of the two families.

Therefore, patients with non-syndromic SNHL due to mutations in the *TMPRSS3* gene exhibit a variable disease phenotype in terms of age of onset, severity and progression. The diagnosis of deafness resulting from a mutation in *TMPRSS3* is therefore based on both clinical and molecular evaluation.

9. MOLECULAR PATHOPHYSIOLOGY OF *TMPRSS3* RELATED DEAFNESS

TMPRSS3 function is required for normal inner ear function. At least seven other TTSP genes are expressed in the inner ear and could theoretically compensate for the loss of *TMPRSS3* function but obviously do not (29). This may be explained by a cell specific expression and/or substrate specificity for *TMPRSS3* in the inner ear. To address this issue, we investigated the role of other TTSP family members to the auditory function and discovered that besides *TMPRSS3*, at least two other TTSP genes were also involved in hearing. Indeed, mice deficient for *Tmprss1* exhibited profound hearing loss characterized by various structural, cellular and molecular abnormalities that are likely to affect different cochlear processes (30). In addition, a large mutation screen revealed potential pathogenic mutations in the *TMPRSS5* gene that are associated with reduction or absence of proteolytic activity *in vitro*.

Interestingly, these three *TTSP* genes showed overlapping but distinct expression patterns in adult
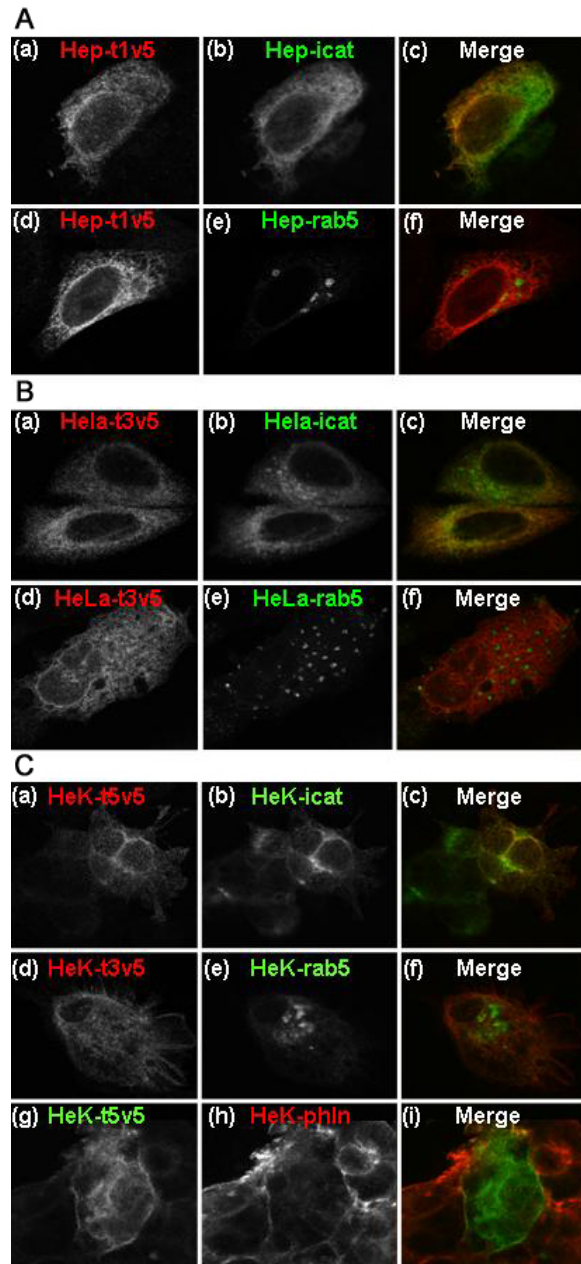
Figure 5. Intracellular localization of TMPRSS1, 3 and 5 proteins. (A) TMPRSS1-V5 C-terminus proteins were co-expressed in HepG2 cells (a, d) with either ICAT-GFP (b) or rab5-GFP (e). Merge panels c and f show superimposition of (a) and (b), and (d) and (e), respectively. (B) TMPRSS3-V5 C-terminus proteins were co-expressed in HeLa cells (a, d) with either ICAT-GFP (b) or Rab-5-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 co-expressed in HEK 293 cells (a, d, 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.

cochlea tissues. 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 fibres of the spiral ganglion (Figure 3). *TMPRSS3* displayed a different pattern characterized by expression in the neuron bodies but not in the nerve fibres of spiral ganglion, and in the inner hair cells of the organ of Corti (Figure 3 and 4). At the subcellular level, *in vitro* analyses showed that TMPRSS1 and 3 proteins appeared to be primarily localized to the endoplasmic reticulum membranes (Figure 5). Interestingly, TMPRSS5 showed a different cellular trafficking as it can clearly be seen at the plasma membrane (Figure 5) (29).

The function of these *TTSPs* in the inner ear remains unknown. Expression of *TMPRSS1*, *3* and *5* in spiral ganglion neurons suggests a role in transmission of signals from sensory hair cells to the auditory centres 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 endoplasmic reticulum compartment, TMPRSS5 appears to be also present at the cell surface. These differences in the cellular and subcellular localization of these TTSP proteins could explain the absence of functional redundancy, their unique contribution to inner ear function and suggest interaction with different substrates.

Two independent studies have investigated the pathogenic effect of seven missense *TMPRSS3* mutations (D103G, R109W, C194F, R216L, W251C, P404L and C407R) causing deafness. Both studies have shown that the proteolytic activity of the mutated TMPRSS3 protein was significantly affected indicating that disruption of proteolytic activity of *TMPRSS3* correlates with the pathogenesis of DFNB8/10 deafness (13, 26, 31). Interestingly, missense mutations lie not only in the serine protease domain but also in the LDLRA and SRCR domains. *In vitro* studies have shown that missense mutations do not seem to affect mutant protein stability (13). It remains to understand how mutations in the LDLRA and SRCR domains affect the proteolytic activity of TMPRSS3. These domains may be required for proper folding or assembly of the catalytic domain or protease substrate recognition and binding. Interestingly, the mutant harboring the R216L missense mutation in the zymogen activation site failed to undergo proteolytic cleavage, thus providing evidence that auto-catalytic cleavage of TMPRSS3 is mandatory for normal function (26). Lastly, deletions/insertions, nonsense and splice site mutations that create premature stop codon are likely to be degraded by nonsense-mediated mRNA decay (32) or produce truncated protein, which are expected to act as null alleles.

Elucidating the substrate repertoire of TMPRSS3 in the inner ear will certainly facilitate our understanding on its role in pathology. To date, the epithelial amiloride-sensitive sodium channel (ENaC) is the only candidate substrate of TMPRSS3 in the inner ear. *In vitro*

experiments have shown that proteolytic processing of TMPRSS3 is associated with increased ENaC mediated currents. In contrast, seven *TMPRSS3* mutants causing deafness all failed to activate ENaC (Figure 2a and b) (13). These data suggest that the loss of ability to activate ENaC indicates that TMPRSS3 works its effect on the sodium channel through cleavage either directly on one or more ENaC subunits or indirectly on a protein or proteins that are involved in a cascade that leads to ENaC activation. However, the TMPRSS3/ENaC hypothesis has been challenged by the report that pseudohypoaldosteronism type 1 patients, which are homozygous for mutations in the α subunit of the ENaC channel, have normal hearing (33). To establish the physiological relevance of these data, it would be interesting to study the activity of the ENaC channel *in vivo* in animal model deficient for TMPRSS3. To this end, we have generated a mouse line that carries a nonsense mutation Y260X in the *TMPRSS3* gene. This mutation is predicted to result in a prematurely truncated protein lacking most of the protease domain. These mice deficient for TMPRSS3 proteolytic function are likely to represent an excellent animal model to study the DFNB8/10 related deafness.

10. PERSPECTIVES

At present, little is known about the spatial and temporal expression of *TMPRSS3*, its biosynthesis, its cellular trafficking and its mechanisms of activation, inhibition and the signaling pathway(s) controlled by this protease and therefore the molecular pathophysiology of the TMPRSS3-related deafness. The generation of a targeted disruption of *TMPRSS3* in mice will provide an outstanding animal model to study the molecular pathology of this particular recessive deafness and understand the underlying pathological processes. In addition, functional analysis aiming at identifying the critical substrates of TMPRSS3, the role of its different domains and its interacting partners would be of great biological importance and may allow the introduction of therapeutic possibilities.

11. ACKNOWLEDGEMENTS

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