Iterative, multiplexed CRISPR-mediated gene editing for functional analysis of complex protease gene clusters

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Elucidation of gene function by reverse genetics in animal models frequently is complicated by the functional redundancy of homologous genes. This obstacle often is compounded by the tight clustering of homologous genes, which precludes the generation of multigene-deficient animals through standard interbreeding of single-deficient animals. Here, we describe an iterative, multiplexed CRISPR-based approach for simultaneous gene editing in the complex seven-member human airway trypsin-like protease/differentially expressed in squamous cell carcinoma (HAT/DESC) subfamily of type II transmembrane serine proteases. Through four cycles of targeting, we generated a library of 18 unique congenic mouse strains lacking combinations of HAT/DESC proteases, including a mouse strain deficient in all seven proteases. Using this library, we demonstrate that HAT/DESC proteases are dispensable for term development, postnatal health, and fertility and that the recently described function of the HAT-like 4 protease in epidermal barrier formation is unique among all HAT/DESC proteases. The study demonstrates the potential of iterative, multiplexed CRISPR-mediated gene editing for functional analysis of multigene clusters, and it provides a large array of new congenic mouse strains for the study of HAT/DESC proteases in physiological and in pathophysiological processes.

Functional redundancy is a long-recognized obstacle to the elucidation of gene function by the use of reverse genetics (1–6). Because of the frequent generation of homologous genes through gene duplication, this obstacle is frequently exacerbated by the genetic linkage of homologous genes, which often makes the generation of multigene-deficient animals through standard interbreeding of animals with single gene deficiencies all but a practical impossibility. For example, in the genome of the mouse—one of the most frequently used model organisms for studying vertebrate gene function—within the family of proteolytic enzymes alone, there are more than 100 protease-encoding genes located within 10 gene clusters that range in size from 4 to 25 genes (Table S1).

The human airway trypsin-like protease/differentially expressed in squamous cell carcinoma (HAT/DESC)2 subfamily of type II transmembrane serine proteases is an example of such a protease gene cluster (7–13). This cluster contains seven genes, (Tmprss11a–g), that encode proteases with high amino acid identity and largely overlapping patterns of expression, which predicts a high level of functional redundancy between individual family members (7, 14). Consistent with this notion, mice with individual deficiencies in the HAT/DESC proteases, TMPRSS11A and HAT, were reported to display no deleterious phenotype (14), whereas individual deficiency in a third HAT/DESC protease, HAT-like 4, results in only a nonlethal defect in epidermal barrier function (15).

Here we show that the simplicity and unsurpassed efficiency of CRISPR-mediated gene disruption (16) can be used for rapid analysis of multigene protease clusters through the generation of high-complexity “libraries” of congenic mouse strains with single and combined gene deficiencies. Specifically, we show that by using four iterative cycles of multiplexed CRISPR-mediated gene disruption, we were able to generate 18 unique mouse strains with single or multiple deficiencies in HAT/DESC proteases, including a mouse strain lacking all seven proteases. Furthermore, we used this library of mouse strains to show that the loss of all HAT/DESC proteases is compatible with normal development, postnatal survival, and reproduction and that the function of the HAT-like 4 protease in epidermal barrier formation is unique to HAT-like 4, with no contribution from any of the other six HAT/DESC proteases.

The described approach should prove useful for the functional analysis of other protease gene clusters, as well as for the analysis of gene clusters encoding other classes of homologous genes with predicted functional redundancy and close physical proximity. Furthermore, the large library of congenic, passenger mutation-free mouse strains generated in the study may prove valuable in elucidating the function of HAT/DESC proteases in normal physiology, as well as in pathological processes such as cancer, skin disorders, obstructive airway disease, and viral infection.

2 The abbreviation used is: HAT/DESC, human airway trypsin-like protease/differentially expressed in squamous cell carcinoma.
Results

Construction of a large congenic library of HAT/DESC protease-deficient mice through iterative, multiplexed CRISPR-mediated gene editing

The human genome contains five TMPRSS11 genes (TMPRSS11A, TMPRSS11B, TMPRSS11D, TMPRSS11E, and TMPRSS11F), all of which have orthologs in the mouse genome (Tmprss11a, Tmprss11b, Tmprss11d, Tmprss11e, and Tmprss11f). The mouse genome has two additional Tmprss11 genes (Tmprss11c and Tmprss11g) for which no functional orthologs have been found in the human genome. A list of the seven Tmprss11 genes with the commonly used name of the proteases they encode, as well as the NCBI reference sequences of their mRNA and protein is shown in Table 1. The seven mouse Tmprss11 genes are located within a 0.5-Mb region of chromosome 5E1, syntenic with human chromosome 4q13.3, which harbors the five human TMPRSS11 genes (7). All HAT/DESC proteases are type 2 transmembrane proteins that consist of a short N-terminal cytoplasmic tail, a signal anchor that forms a single-pass transmembrane domain, a SEA (sperm protein, enterokinase and agrin) domain, and a C-terminal serine protease domain (7).

To generate simultaneous null mutations in Tmprss11 genes in the mouse germ line, we used CRISPRs designed to eliminate DNA sequences encoding all or part of the signal anchor to prevent insertion of the mutant protein into the endoplasmic reticulum membrane. We furthermore selected for mice carrying mutations introducing a shift in the reading frame to prevent the translation of any Tmprss11 coding regions downstream from the deleted signal anchor. Founders were screened for CRISPR-induced mutations, and founders with desired mutations were tested for germ-line transmission through breeding to WT mice. For founders carrying mutations in more than one Tmprss11 gene, the locations of these mutations on the same or opposite chromosomes were determined by the absence or presence of segregation of the mutant alleles within the F1 offspring. Established lines carrying desired mutant Tmprss11 alleles in cis were then used as donors for successive rounds of CRISPR-mediated gene targeting, until a mouse line carrying mutations in all seven Tmprss11 genes was generated. A list of the 18 congenic Tmprss11 mutant mouse strains generated through this effort, including the specific mutation introduced into each Tmprss11 gene, is shown in Table 2.

Because antibodies specific for each of the seven HAT/DESC proteases are not available and are unlikely to be successfully produced because of the high amino acid identity of these proteases, we asserted enhanced vigilance in ensuring that the mutations introduced in each of the seven Tmprss11 genes generated a null allele. First, we generated synthetic DNA corresponding to the predicted cDNA sequence of each of the seven WT and mutant Tmprss11 alleles. These were inserted into an expression plasmid fused to a C-terminal c-Myc tag to allow for immunological detection of the recombinant proteins using mouse c-Myc antibodies. These plasmids then were transfected into HEK293T cells, and cell lysates from the transfected cells were analyzed by Western blotting. A protein exhibiting the expected molecular weight was detected in the cell lysate from cells transfected with plasmids expressing the WT cDNA (Fig. 1, lanes 1, 3, 5, 7, 9, 11, and 13). As expected, no protein product was detected in cell lysate or supernatant from cells transfected with plasmids expressing the mutant cDNAs (Fig. 1, lanes 2, 4, 6, 8, 10, 12, and 14). We also used RT-PCR of mRNA isolated from tongue and testes of mice bred to homozygosity for all seven mutant Tmprss11 alleles (hereafter referred to as Tmprss11a,b,c,d,e,f,g−/−) followed by DNA sequencing to search for alternatively spliced mRNAs for each of the mutant Tmprss11 alleles that would restore an ORF and thereby be capable of producing truncated HAT/DESC protease products (albeit these would be unable to translocate to the extracellular/pericellular space or form disulfide bridges essential for protein folding and catalysis). However, no such mRNAs were identified (Fig. S1). Note that no Tmprss11c transcripts could be amplified, consistent with previous data (14). Taken together, these studies demonstrate that the CRISPR-mediated gene disruptions resulted in the introduction of combinations of null mutations into each of the seven Tmprss11 genes. Furthermore, it demonstrates the feasibility of using iterative, multiplexed CRISPR-mediated gene editing for simultaneous targeting of multiple homologous genes.

Simultaneous loss of all HAT/DESC proteases is compatible with normal development and postnatal survival in mice

Tmprss11a,b,c,d,e,f,g−/− mice (with mutant alleles all located on the same chromosome; see Construction of a large congenic library of HAT/DESC protease-deficient mice through iterative, multiplexed CRISPR-mediated gene editing) were interbred to generate Tmprss11a,b,c,d,e,f,g−/− mice lacking all HAT/DESC proteases and WT littermates for comparison.
Surprisingly, genotyping of offspring from these crosses at birth (Fig. 2A) or at weaning (Fig. 2B) showed a Mendelian inheritance of the Tmprss11a,b,c,d,e,f,g⁺/⁻ allele, demonstrating that the simultaneous loss of all HAT/DESC proteases is compatible with both embryonic and postnatal development. To study the impact of loss of all HAT/DESC proteases on long-term health and survival, we next set up separate cohorts of female and male Tmprss11a,b,c,d,e,f,g⁻/⁻ mice and their associated Tmprss11a,b,c,d,e,f,g⁺/⁻ and WT littermates. The health and survival of mice in these cohorts were prospectively monitored by weekly outward inspection and weight measurement. Interestingly, neither female nor male Tmprss11a,b,c,d,e,f,g⁻/⁻ mice that could be followed for at least 6 months after weaning displayed obviously abnormal appearance, abnormal weight gain or weight loss (Fig. 2, C and D), or increased mortality (Fig. 2, E and F).
We next collected organs from Tmprss11a,b,c,d,e,f,g/H11002/H11002 mice and WT littermates and prepared hematoxylin and eosin-stained sections for histological analysis (Fig. 3). Consistent with their normal development, weight gain, normal outward appearance, and survival, no histological abnormalities were observed in skin, tongue, colon, small intestine, testis/uterus, liver, kidney, lungs, or trachea of Tmprss11a,b,c,d,e,f,g/H11002/H11002 mice when compared with their WT littermates.

**Fertility of mice lacking all HAT/DESC proteases**

Several Tmprss11 genes are co-expressed in reproductive organs, including uterus and testis (14), suggesting possible unique or shared functions of HAT/DESC proteases in reproduction. To test this, we examined the litter frequency (Fig. 4A) and litter size (Fig. 4B) of intercrossed Tmprss11a,b,c,d,e,f,g/−/− mice as compared with intercrossed Tmprss11a,b,c,d,e,f,g+/− mice. This analysis showed no indication of impaired fertility in mice lacking all HAT/DESC proteases when compared with their HAT/DESC-sufficient littermates.

**Mice lacking all HAT/DESC proteases display impaired epidermal barrier function, which is solely attributable to the loss of HAT-like 4**

Mice with a single deficiency in Tmprss11f, encoding HAT-like 4, were recently reported to display a defect in epidermal barrier function, which manifests at birth and persists throughout adulthood (15). This would predict that Tmprss11a,b,c,d,e,f,g/−/− mice, which in addition to lacking HAT-like 4, are deficient in all other epidermally expressed HAT/DESC proteases, would display a similar or more pronounced loss of epidermal barrier function. Indeed, transepidermal water loss was significantly increased in newborn Tmprss11a,b,c,d,e,f,g/−/− mice when compared with their Tmprss11a,b,c,d,e,f,g+/− and WT littermates (Fig. 5A).

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**Figure 2. Simultaneous loss of all HAT/DESC proteases is compatible with mouse development and postnatal survival.** A and B, genotype distribution of 94 offspring at birth (A) and of 166 offspring at weaning (B) from intercrossed Tmprss11a,b,c,d,e,f,g+/−/− mice. C and D, body weights of male (C) and female (D) Tmprss11a,b,c,d,e,f,g/−/− (blue circles), Tmprss11a,b,c,d,e,f,g+/−/− (red squares), and Tmprss11a,b,c,d,e,f,g/−/− (green triangles) mice followed for up to 26 weeks after birth. The numbers in parentheses indicate the number of mice initially enrolled in the cohort. The data are shown as means ± S.E. E and F, Kaplan–Meier plots of the survival of male (E) and female (F) Tmprss11a,b,c,d,e,f,g/−/− (blue lines), Tmprss11a,b,c,d,e,f,g+/−/− (red lines), and Tmprss11a,b,c,d,e,f,g/−/− (green lines) mice followed from weaning and up to 26 weeks after birth. The numbers in parentheses indicate the number of mice initially enrolled in the cohort.
Moreover, in mice with a single deficiency in \textit{Tmpress11f} (generated in this study; Table 2), an increase in transepidermal water loss very similar to the published rate of water loss (15) was observed (Fig. 5B). Among the 18 congenic mouse strains generated in this study were mice that are deficient in all HAT/DESC proteases, except HAT-like 4 (\textit{Tmpress11a,b,c,d,e,g} \textsuperscript{−/−} mice; Table 2), providing an opportunity to query the role of each of these genes in epidermal barrier function. Surprisingly,
transepidermal water loss in Tmprss11a,b,c,d,e,g\(^{-/-}\) mice was identical to their Tmprss11a,b,c,d,e,g\(^{-/-}\) and WT littermates (Fig. 5C), revealing that HAT-like 4 is solely responsible among HAT/DESC proteases for maintaining epidermal barrier function with no compensation by any of the six other HAT/DESC proteases. Six Tmprss11 genes (14) have been shown to be expressed in mouse skin (Tmprss11a, Tmprss11b, Tmprss11d, Tmprss11e, Tmprss11f, and Tmprss11g), making the failure of any of the other five Tmprss11 genes to compensate for the loss of Tmprss11f somewhat unexpected. To further investigate this, we performed an RNAseq analysis of skin from newborn mice to obtain quantitative estimates of the level of expression of each of the seven Tmprss11 genes (Fig. 5, D and F). Tongue, an organ also rich in keratinized tissue and expressing the same six Tmprss11 genes (14), was included in the analysis for comparison. This analysis confirmed that Tmprss11a, Tmprss11b,
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Tmprss11d, Tmprss11e, Tmprss11f, and Tmprss11g were expressed in the two organs (Fig. 5, D–F; GEO accession number GSE133070). Interestingly, however, in the skin, more than 90% of all Tmprss11 transcripts were derived from the Tmprss11f gene. This expression pattern was strikingly different in the tongue, where three Tmprss11 genes (Tmprss11a, Tmprss11b, and Tmprss11d) were all prominently expressed. This indicates that the lack of functional compensation for the loss of Tmprss11f by other Tmprss11 genes as regards epidermal barrier function is likely due to the preponderant expression of Tmprss11f in skin rather than to a unique functional property of the HAT-like 4 protease.

Absence of compensatory gene expression in mice lacking all HAT/DESC proteases

The lack of robust developmental or postnatal phenotypes in animals in response to targeted gene disruptions is often attributed to compensatory changes in gene expression. We therefore next performed a global transcriptomic analysis by comparing gene expression levels in skin and tongue of newborn Tmprss11a,b,c,d,e,f,g−/− mice and their WT littermates by RNAseq analysis. The complete data set was deposited in the GEO database (accession number GSE133070). A total of 23,420 genes were compared, and gene expression differences were analyzed. Surprisingly, no genes were found to be significantly up- or down-regulated in response to the simultaneous loss of all Tmprss11 genes using an adjusted p value of <0.05. Furthermore, no trend was observed toward enhanced or decreased expression of genes encoding other membrane-anchored serine proteases (St14, Prss8, and Tmprss13) or membrane-anchored serine protease inhibitors (Spint1 and Spint2) with known importance for epidermal barrier function (17–22). Likewise, mRNAs for desquamation-associated kallikreins (Klk5, Klk7, and Klk14) and their principal inhibitor (Spink5) were expressed at similar levels in Tmprss11a,b,c,d,e,f,g−/− mice and WT littermates.

Discussion

Functional redundancies of homologous genes, compounded by their frequent genetic linkage, remain serious impediments to the use of reverse genetics to elucidate physiological and pathophysiological gene functions in model organisms. This includes the functional study of the more than 600 genes in the mouse genome that encode proteolytic enzymes (23). Here, we describe the application of an iterative, CRISPR-based approach for multiplexed gene disruption and its use for functional analysis of the seven-member gene cluster encoding the mouse HAT/DESC subfamily of type II transmembrane serine proteases (7–13). By using an iterative approach, through four cycles of targeting, we were able to generate a total of 18 unique congenic mouse strains with single or multiple gene deficiencies, including a mouse strain that lacked all seven HAT/DESC proteases. The described approach should be readily amenable to functional studies of other protease gene clusters and to the functional study of homologous, genetically linked genes in general.

HAT/DESC proteases have mainly been studied as modulators of human diseases that afflict the skin, airways, and upper digestive tract, which represent main sites of their expression. In the context of cancer, individual HAT/DESC proteases (DESC1, HAT, and HAT-like 5) have been identified as either promoters of human tumorigenesis (24–26) or as putative tumor suppressors (DESC1, HAT, HAT-like 5, and Tmprss11a) (27–30). HAT has been proposed to promote chronic airway disease through its ability to induce inflammatory cytokine and mucin production (31, 32) but also to ameliorate bleomycin-induced pulmonary fibrosis by suppressing several pro-inflammatory pathways (33). Additionally, HAT was reported to exacerbate psoriasis vulgaris symptoms via induction of cytokine release (34).

As of yet, few validated substrates for HAT/DESC proteases have been reported. Like many membrane-anchored serine proteases (35), HAT can activate protease-activated receptor-2 in cell-based assays (36) and can cleave macrophage-stimulating protein (37), fibrinogen (38), and the urokinase plasminogen activator receptor (39). Most recently, HAT like-5 was reported to cleave basigin, a chaperone of the lactate monocarboxylate transporter, MCT4, to promote lung cancer progression (25). Interestingly, DESC1, HAT, and Tmprss11A also have emerged as essential processing proteases for maturation of envelope glycoproteins of highly pathogenic viruses, including several influenza A viruses and severe acute respiratory syndrome coronavirus (40–48).

Because of the relative paucity of knowledge regarding the physiological functions of HAT/DESC proteases, in this study, we undertook a systematic genetic analysis of these proteases in mice. Because the high amino acid identity and substantial overlap in tissue expression of HAT/DESC proteases predicted a considerable level of functional redundancy, we hypothesized that a robust phenotype might only manifest after simultaneous removal of several HAT/DESC proteases. Remarkably, however, even mice having all seven Tmprss11 genes inactivated displayed no overt developmental or postnatal phenotype, when assayed for Mendelian inheritance, pre- and post-weaning survival, outward health, weight gain, fertility, and histological appearance of major organs. Furthermore, mice lacking all HAT/DESC proteases showed no trend toward compensatory gene expression in whole-transcriptome analysis of two tissues with high Tmprss11 gene expression. Mice carrying mutations in all Tmprss11 genes, however, did display a defect in epidermal barrier function, as was recently described for mice with a single deficiency in Tmprss11f (15). Contrary to our prediction of functional redundancy between HAT/DESC proteases, however, by using the library of Tmprss11 mutants generated in this study, we were able to unequivocally demonstrate that the barrier defect in mice lacking all Tmprss11 genes is solely attributable to the loss of Tmprss11f. This was evident by the comparative transepidermal water loss rates in mice lacking all Tmprss11 genes and mice lacking only Tmprss11f and, more importantly, by the absence of a barrier defect in mice lacking all Tmprss11 genes, except Tmprss11f. In summary, we have demonstrated the feasibility of using iterative, multiplexed CRISPR-mediated gene targeting to construct large libraries of congenic, passenger mutation-free mouse strains for the functional analysis of multigene clusters. Furthermore, we believe that the 18 mouse strains generated in
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this study will prove to be a valuable community resource facilitating the elucidation of the physiological functions of HAT/DESC proteases, as well as determining the contribution of this relatively unexplored protease subfamily to cancer, inflammatory diseases, fibrosis, viral infection, and other diseases.

Experimental procedures
Animal work
All experiments involving mice were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited vivarium following Institutional Guidelines and Standard Operating Procedures as approved by the NIDCR Institutional Animal Care and Use Committee.

Generation of Tmprss11-deficient mouse strains
Two guide RNAs that target the signal anchor domain–encoding exons of each of the seven mouse Tmprss11 genes (Table S2) were designed by using the CRISPR guide RNA design tool developed by Dr. Feng Zhang’s lab at MIT (49). All 14 guide RNAs were cloned into the SpCas9 and chimeric guide RNA expression plasmid pX330 (Addgene catalog no. 42230) (50). Successful cloning of each of the guides was confirmed by DNA sequencing. To generate the Tmprss11–targeted mouse strains, plasmids containing guide RNA were mixed equimolarly and diluted to a final concentration of 5–10 ng/μl in 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA. The pDNA mix was then microinjected into the male pronucleus of FVB/NJ zygotes, which were implanted into pseudopregnant mice. All founders were screened for any changes in the targeted regions of any of the seven Tmprss11 genes by PCR amplification followed by DNA sequencing (Table S3 for sequences of PCR primers used for founder screening). Mice carrying gene-inactivating mutations were bred to FVB/NJ WT mice to test the germ-line transmission and, in case of multiple mutations present in a single founder, for genetic linkage. Four rounds of CRISPR/Cas9-mediated targeting was used to generate a strain carrying mutations in all seven Tmprss11 genes and 17 mouse strains with mutations in one or more Tmprss11 gene (see “Results” and Table 2 for details). Depending on the strain, offspring were genotyped by PCR using either primers used in the original screening or designed to specifically detect knockout and WT alleles of the targeted gene or genes, followed by DNA sequencing when necessary (Table S3).

Cell culture and transfection
HEK293T cells were grown in 6-well plates in high glucose Dulbecco’s modified Eagle’s medium (Gibco) containing penicillin, streptomycin and 10% fetal bovine serum until reaching 90% confluence. pCMV6-AC-Myc expression plasmids (catalog no. PS100003; OriGene Technologies, Rockville, MD) carrying C-terminal Myc-tagged WT and mutant variants of mouse Tmprss11 cDNA were synthesized by Blue Heron Biotech (Seattle, WA). 2500 ng of plasmid was transfected into cells using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells were grown in serum-free, high-glucose Dulbecco’s modified Eagle’s medium.

Western blotting
Two days after transfection, the cells were lysed in 2% SDS, 10% glycerol, and 6.25 mM Tris/HCl, pH 6.8. 100 μg of each sample was deglycosylated with an enzymatic protein deglycosylation kit (Thermo Fisher) according to the manufacturer’s protocol. For Western blotting analysis, 40 μg of each deglycosylated sample was reduced in NuPAGE sample buffer (Thermo Fisher) containing 7% β-mercaptoethanol by boiling for 5 min. The samples were loaded into a 4–12% Bis-Tris gel (Thermo Fisher) and run in NuPAGE MOPS running buffer (Thermo Fisher) at 150 V for 90 min. Samples were transferred to a polyvinylidene difluoride membrane (0.2-μm pore size; Thermo Fisher) in NuPAGE transfer buffer (Thermo Fisher) for 1 h at 30 V. The membrane was blocked for 30 min using TBST (Fisher Scientific) containing 0.05% Tween 20 (Sigma–Aldrich) with 5% dry milk. To detect protein, the membrane was incubated overnight at 4°C with 1 μg/μl mouse anti-c-Myc primary antibody (9B11; Cell Signaling Technology, Danvers, MA). Subsequently, three 5-min washes with TBST were performed before incubating the membrane for 1 h with 1 μg/μl goat anti-mouse secondary antibody conjugated with alkaline phosphatase (D0487; Agilent, Santa Clara, CA). After incubation, three 5-min washes with TBST were performed before developing the membrane in nitro blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (Thermo Fisher). Development was stopped by washing the membrane in water.

cDNA sequencing
Tongue and tests were collected from Tmprss11ahbcdefg−/− mice and their WT littermates aged 6–7 weeks. RNA was isolated from tissues using TRIzol reagent (Thermo Fisher) according to the manufacturer’s protocol. cDNA was synthesized using 1000 ng of RNA, using the RevertAid H minus cDNA synthesis kit (Thermo Fisher) and sequenced. The PCRs included denaturation at 95°C followed by 40 cycles with 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of elongation at 72°C before a final extension at 72°C for 5 min. Sequencing data were compared with expected sequences for Tmprss11 WT genes and were examined for alternative splice variants that could produce protein products.

Transepidermal fluid loss
Fluid loss in newborn mice was measured as a percentage of decrease in body weight, with measurements taken every 30 min for 5 h.

RNAseq and analysis
Skin and tongue were collected from newborn mice. Tissues were frozen on dry ice and stored at −80°C before further processing. Epidermis was manually separated from dermis after incubation for 5 min at 60°C in 5 mM EDTA. Tissues were homogenized in TRIzol reagent, and RNA was extracted according to the manufacturer’s protocol (Thermo Fisher). Double-stranded cDNA was prepared using the SMARTer V4 ultra-low input kit (Clontech). Libraries were prepared using a TruSeq v2 kit (Illumina, San Diego, CA), individually barcoded,
pooled to a 2 nM final concentration, and sequenced on a NextSeq500 sequencer (Illumina) using 37 × 37 paired-end mode. The base-called demultiplexed (fastq) read qualities were determined using FastQC (v0.11.2) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/),3 aligned to the GENCODE M11 mouse genome (GRCh38.p4), and gene counts were generated using STAR (v2.5.2a) (51). Post-alignment qualities were generated with QoRTS (version 1.1.6) (52). An expression matrix of raw gene counts was generated using R and filtered to remove low counts genes (defined as those with fewer than five reads in all samples). The filtered expression matrix was used to generate a list of differentially expressed genes between the sample groups using DESeq2 (53).

**Assessment of long-term health**

Littermate Tmprss11a,b,c,d,e,f,g−/−, Tmprss11a,b,c,d,e,f,g+/−, and WT mice were kept in HEPA-filtered, mixed genotype cages of up to five mice. The mice were weighed weekly, and outward health was assessed. The mice were euthanized after 6 months, and organs (dorsal skin, tongue, colon, small intestine, liver, kidney, uterus/testis, trachea, and lungs) were collected. Organs were fixed overnight in Z-fix (Anatech LTD), paraffin-embedded, sectioned, and hematoxylin- and eosin-stained. Examination of tissues was performed by light microscopy.

**Analysis of fertility**

Breeding pairs of female and male Tmprss11a,b,c,d,e,f,g−/− mice and their female and male Tmprss11a,b,c,d,e,f,g+/− littermates were established after weaning and genotyping. The breeding pairs were observed daily, and if pups were found, litter size and date of birth were recorded. Fertility was measured in terms of litter size and frequency. Litter frequency was measured in terms of litter size and date of birth. Fertility was measured in terms of litter size and frequency. Litter frequency was measured in terms of litter size and date of birth. Fertility was measured in terms of litter size and date of birth.


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**References**


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