A role for *TENM1* mutations in Congenital General Anosmia

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Abstract

Congenital general anosmia (CGA) is a neurological disorder entailing a complete innate inability to sense odors. While the mechanisms underlying vertebrate olfaction have been studied in detail, there are still gaps in our understanding of the molecular genetic basis of innate olfactory disorders. Applying whole-exome sequencing to a family multiply affected with CGA, we identified three members with a rare X-linked missense mutation in the *TENM1* (*teneurin 1*) gene (ENST00000422452:c.C4829T). In *Drosophila melanogaster, TENM1* functions in synaptic-partner-matching between axons of olfactory sensory neurons and target projection neurons and is involved in synapse organization in the olfactory system. We used CRISPR-Cas9 system to generate a *Tenm1* disrupted mouse model. *Tenm1*$^{-/-}$ and point-mutated *Tenm1*$^{A/A}$ adult mice were shown to have an altered ability to locate a buried food pellet. *Tenm1*$^{A/A}$ mice also displayed an altered ability to sense aversive odors. Results of our study, that describes a new *Tenm1* mouse, agree with the hypothesis that *TENM1* has a role in olfaction. However, additional studies should be done in larger CGA cohorts, to provide statistical evidence that loss-of-function mutations in *TENM1* can solely cause the disease in our and other CGA cases.

Key Words

Teneurin, *ODZ1*, *TENM1,* CGA, Congenital General Anosmia, CRISPR-Cas9 system, olfaction.

Introduction

Pathological modifications in the general sensitivity to odorants have important health and well-being implications. The most extreme cases involve complete loss of olfactory sensory function (general anosmia), affecting as many as 5% of the general population (1). Only in a much smaller part of the population with olfactory deficit (0.1-3%), a complete loss of olfaction is present from birth - Congenital General Anosmia (CGA) (2-5). This may appear as an isolated deficit (OMIM#107200), or alternatively be syndromic, appearing in conjunction with other anomalies. This is exemplified by Kallmann syndrome (KS, OMIM#308700), where CGA appears together with hypogonadotropic hypogonadism (2, 6). Syndromic CGA was also diagnosed in patients suffering from indifference to pain (OMIM#243000) *(7, 8)* and CHARGE syndrome (OMIM#214800) (9, 10). Most of the reported CGA cases are sporadic (11), and only a few are familial with various modes of inheritance (2, 12-15). For some syndromic cases, causative genes have been identified (15-18), e.g. *ANOS1(6, 17)*, *FGF8(19)*, *FGFR1(18)*, *FGF17(20)*, *IL17RD(20)*, *DUSP6(20)*, *SPRY4(20)*, *FLRT3(20)*, *PROK2(21)*, *PROKR2(21)*, *CHD7(22)* for KS, and *SCN9A(7, 8)* for

indifference to pain. Despite an intensive search, only a few mutations underlying isolated CGA have been reported so far. Two of them were detected in genes previously associated with KS - *PROKR2* and *PROK2* (23). A third mutated gene is *CNGA2* identified in a single CGA family (12).

We report here a family with three siblings affected with CGA (Fig. 1). Whole exome sequencing revealed a candidate X-linked missense mutation in the *TENM1* gene, affecting a highly conserved proline residue (Fig 2). The variant has zero frequency in ~67600 control samples, and is predicted to be damaging by all available protein impact prediction sources. Knockout mice (*Tenm1*$^{-/-}$) and mice carrying the human mutation (*Tenm1*$^{A/A}$) were produced and their olfactory functions were evaluated in order to examine the role of *TENM1* in olfaction, and the relevance of *TENM1* mutations in CGA.

Materials and Methods

Human subjects

The study was conducted with the approval of the Institutional Review Board for human experiments in the Meir Hospital, Kfar Saba, Israel. The informed consent was signed by all participants. A Jewish Ashkenazi/Yemenite family 230 (Fig. 1) with three siblings (two of them were self-reported dizygotic twins) affected with CGA was recently recruited as part of larger collection of families with CGA (13) and phenotyped as described previously (13). Genomic DNA was extracted from whole blood or saliva (Oragene•DNA (OG-500), DNA Genotek, Ontario, Canada) by standard methods.

Olfactory sensitivity screening of human subjects

Potential anosmic individuals and their healthy family members were tested by the olfactory sensitivity screening test (13) that was carried out using a 3-way forced-choice bottle test with 2 odorants (isoamyl acetate and eugenol, 30 µl/ml of mineral oil (light, Sigma-Aldrich). For each odorant, 15 trials were conducted. In each trial, subjects were presented with one stimulus and two solvent blanks. Subjects were asked to sniff and choose/guess the different odor among the 3 samples. University of Pennsylvania Smell Identification Test (Sensonics, Inc., Haddon Heights, NJ) (24) was also administered to the female affected sibling. Subjects performing at chance level in these tests were considered anosmic. Test criteria for CGA was a) complete reported lack of the sense of smell for an entire lifespan; b) null performance in a three-way forced choice olfactory sensitivity tests; c) the lack of any alternative medical explanation for the general anosmia. The results were scored as the number of odorants recognized correctly. The acetic acid (1:30 aqueous solution), that is known to have a strong trigeminal component, was used to study the trigeminal perception of all subjects by a

forced-choice 3-way bottle test as described for isoamyl acetate and eugenol. 8 acetic acid trials were conducted.

Whole exome sequencing and data analysis

Next-generation sequencing was performed at Macrogen Inc, (Seoul, Korea). Paired-end sequencing was performed using Agilent SureSelect Human V5 Target Enrichment System (Agilent Technologies, Santa Clara, CA). The resulting reads were aligned to the reference genome (build 37) using the Burrows-Wheeler Alignment (BWA) tool (25). The mean depth of target regions was ~120 reads. Variations relative to the reference genome were identified by the SAMtools (26) and GATK (27). The ANOVAR software (28) was used for variant annotation. Phenotype-based gene prioritization was aided by VarElect tool (29) (29)(https://ve.genecards.org/). For population frequency controls we used 1000 genomes project, NHLBI exome sequencing database, Complete Genomics whole genome data and our own exome database with 408 Jewish exomes (181 females and 227 males). The IBD (identity by descent) check was done by using Plink (30) and KING (31) software.

X-inactivation skewing

We used a highly polymorphic trinucleotide repeat within the first exon of the human androgen receptor gene and the methylation-sensitive restriction enzyme *HpaII* to distinguish between two different alleles and to determine their methylation status. We calculated the ratio of inactivation between the two alleles that reflects whether a female has a random or nonrandom pattern of X chromosome inactivation.

RFLP analysis

The mutation in *Tenm1* gene was verified in all family members by using a *BstNI* restriction enzyme (New England Biolabs, Ipswich, MA). The PCR product was digested by a *BstNI* restriction enzyme under standard conditions in a volume of 20 µl, and products were analyzed on 2% agarose gel electrophoresis. The same technique was used to verify the frequency of the mutation in additional 103 individuals (45 females and 58 males) with CGA.

Mutant mice

All mouse experiments were approved by the Weizmann Institute's IACUC committee and were carried out in accordance with their approved guidelines.

Mutant mice were generated by CRISPR/Cas9 gene targeting technology as described by Wang et al 2013 (32). To generate hemizygous (males) or homozygous (females) *Tenm1* knockout mice (collectively referred to as *Tenm1-/-* mice, irrespective of the sex of the animals), a guide RNA, sgRNA-1 was designed to target the 5th out of 32 coding exons (ENSMUSE00000327581) of the mouse *Tenm1* gene, to create indels leading to frameshift mutations and premature stop codons. To introduce the human mutation (*Tenm1*$^{A/A}$) into mice via homology directed repair (HDR), a second sgRNA, sgRNA-2 was designed to target the region of the *TENM1* human mutation (exon 25, chrX:42,576,470) cutting close to the site of mutation. A corresponding ssDNA oligonucleotide with the modified nucleotide of interest (variant G to A, chrX:42,576,470) (Table S1) and 140 bp of homologous sequence flanking the target site was ordered from Applied Biosystems. In vitro transcribed Cas9 RNA (100ng/ul), sg RNA (50ng/ul), together with repair oilgo (200ng/ul) where relevant, were injected into one cell fertilized embryos isolated from superovulated CB6F1 hybrid mice (F1 of BALB/cAnNHsd inbred female x C57BL/6NHsd inbred male) mated with CB6F1 males Harlan Biotech Israel Ltd. (Rehovot, Israel). Injected embryos were transferred into the oviducts of pseudopregnant ICR females. 241 pups were obtained in total. The target site in all pups was examined by High Resolution Melt (HRM) analysis and Sanger sequencing using tail tip DNAs.

We identified all potential off-target genomic sites for sgRNAs targeting exons 4 and 25 of the *Tenm1* gene. We checked by Sanger sequencing three highly potential off-target sequences in F1 mice for each sgRNA, all of them containing three or four base pair mismatches compared to the 20 bp sgRNA target sequence. We examined the off-target sequences in three best sites all of them containing four base pair mismatches, in all the (*Tenm1* $^{-/-}$) pups, that were used in behavioral experiments, and detected no off-target modifications in two of the examined sites (Table S2). However some of the pups were found to have modifications in an intronic off-target site on chromosome 15 (tggtggcaGCTTCCTGGCAC_TGGC; chr15:73026135-73026158, GRCm38). This genomic interval, which is located ~50bp from the closest exon, is not evolutionary conserved, and does not show any indication of being a regulatory region as reflected from browsing the mouse and human ENCODE data. We also examined off-targets in three best sites in (*Tenm1*$^{A/A}$) mice, all of them containing three base pair mismatches, and detected no off-target modifications in any of the examined sites (Table S2).

Phenotyping of *Tenm1*$^{-/-}$ and *Tenm1*$^{A/A}$ mice

Housing

Mice were housed in standard cages in same sex mixed genotype groups in the WIS (Weizmann Institute of Science) mouse facility; they were maintained on a 12 h light/dark

cycle with food and water *ad lib*. All behavioral experiments were conducted during the dark/active phase; mice were 8–20 weeks old when the different behavioral assessments were carried out. All protocols were approved by the WIS Institutional Animal Care and Use Committee. The *Tenm1*$^{+/+}$ littermates were used in all the behavioral tests.

Behavioral tests

Mice underwent Homing Test (33), Buried Food Test (34), Odor Preference Test (35), Open Field Test (36) and Home - Cage Locomotion (37). The behavior of mice in all the tests were recorded by either video or built-in tracking systems and subsequently scored and analyzed. The detailed description of all behavioral tests can be found in the Supplementary material.

Morphological examination

Macroscopic examination of brains was carried out to analyze the olfactory bulb/brain length ratio as previously described for other mouse models of anosmia (38, 39). Mice were treated with a lethal dose of pentobarbital and brains were perfused via the ascending aorta with ice-cold PBS followed by cold 4% paraformaldehyde in PBS. Tissues were frozen in dry ice and sectioned. Measurements were taken of the brain, and olfactory bulb length.

Statistics

Data are expressed as mean ± standard error of the mean (sem). Data were analyzed using SPSS 20.0. All data sets were tested for departures from normality with Shapiro-Wilks test. Student's t-test or Mann-Whitney was used for all comparisons between two groups. ANOVA or Kruskal-Wallis H test were used for comparing multiple groups. Dunn's pairwise comparisons or Bonferroni comparsions were used as post-hocs. * indicates $p<0.05$, ** indicates <0.01 and *** indicates $p<0.001$.

Results

Human subjects

We recruited a family of a Ashkenazi-Yemenite origin with adult twin-brothers and a sister affected by CGA (Fig. 1). They didn't report a failure to start puberty. One of the twin brothers has a slight intellectual disability. The siblings were tested for olfactory sensitivity and were found to perform at chance level, while their parents, aunt and grandmother were found to have a normal sense of smell (Table S3). We applied whole exome sequencing to screen the exonic regions of the 3 affected siblings and their parents and identified 844 rare (<0.5%) variants shared by all the 3 siblings. An IBD analysis of the exome data (PLINK, KING) (30, 31) showed that the twin brothers are

monozygotic (Fig. S1). The data was analyzed using several genetic models: recessive, X-linked and dominant with partial penetrance (which is widely accepted in cases with syndromic congenital anosmia). No homozygous rare variant shared by the three siblings and heterozygote in both parents were found. Compound heterozygous variants were detected in *FBF1* and *RECQL5* genes. These variants were not predicted to have an effect on the protein and according to their function; they are not obvious candidates. A list of all the variants, shared by all affected siblings and predicted to be deleterious, is presented in Table S4.

The X-linked and heterozygous variants were further prioritized using a score that combines the population frequency and a functional deleteriousness score calculated by in-house scripts. The variant with the highest score was found in the *TENM1* gene (chrX:123554314, G/A, ENST00000422452:c.C4829T:p.P1610L). The variant has a zero frequency in the population (1000G, NHLBI, CG, ExAC and internal database of 400 Jewish exomes), and was not found in any of the 103 CGA cases in our repositories. Further, phenotype-based gene prioritization with VarElect (29) did not reveal any known CGA genes. The identified variation is predicted to be damaging by 8 different prediction programs available through the dbNSFP database (40). *TENM1* encodes a 2725 amino acid long protein. TENM1 protein is involved in neuronal development, regulating the establishment of proper connectivity within the nervous system (41-44).

Sanger sequencing provided segregation analysis in the extended family whereby the unaffected mother and maternal grandmother were found to be carriers of the variant, while the maternal aunt was not (Fig. 1b). This is consistent with X-linked mode of inheritance with partial penetrance in females. We measured X-inactivation skewing in both the affected female and her mother, showing X inactivation ratios of 63:37 in the mother and 28:72 in the daughter. We note that these numbers do not pass the clinically acceptable extreme skewing cutoff (90:10).

Expression and conservation of *TENM1*

Teneurin-1 belongs to the teneurin protein family. These are type II transmembrane proteins with a single transmembrane domain. The intracellular N-terminal part contains 314 amino acids, leaving the remaining of the protein outside the cell. The extracellular part contains eight conserved EGF-like repeats, two six-bladed beta-propeller TolB-like motifs, 3 YD motifs and an inactive version of the GHH domain (Fig S2). The P1610L mutation is located at the second six-bladed beta-propeller TolB-like motif (IPR011042) which consists of six 4-stranded beta-sheet. Teneurin-1 is highly conserved across vertebrates, with a sequence similarity of 97% with its mouse ortholog. The in-silico gene expression profile of *TENM1* in the GTEX project data (45) indicated an enhanced

expression in Cerebellar Hemisphere. The examination of the expression profile of *Tenm1* in The Mouse Gene Expression Database (http://www.informatics.jax.org/marker/MGI:1345185) revealed that *Tenm1* is expressed in the embryonic nervous system and sensory organs, including a weak expression in the olfactory epithelium and olfactory bulbs between E13.5 and E15 (E=embryonic day).

Crispr/Cas9 mutation modeling

To test the functional role of *TENM1* in olfaction we employed the CRISPR/Cas9 system (32) to generate homozygous/hemizygous knockout mice (*Tenm1*$^{-/-}$) and homozygous/hemizygous mouse knock-in (*Tenm1*$^{A/A}$) (collectively referred to as *Tenm1*$^{-/-}$ or *Tenm1*$^{A/A}$ mice, irrespective of the sex of the animals) (Fig. 3). We generated 60 pups with indels in the exon 5 target site and 16 pups with the desired homozygous or heterozygous missense single base modifications. All the behavioral experiments were done with the F1 generation male animals verified not to have off-target CRISPR modifications in suspected functional regions.

Phenotyping

All *Tenm1*$^{-/-}$ and *Tenm1* $^{A/A}$ mice were normal at birth and showed no visible phenotype during early and adult development.

Motor function and anxiety: The adult mice were subjected to a locomotor activity test, and the *Tenm1*$^{-/-}$ animals did not show a significant difference in their activity levels as compared to *Tenm1*$^{+/+}$ (Fig. S3). Additional open field test assessed both locomotor activity and anxiety-like behavior and indicated no significant differences between the *Tenm1*$^{-/-}$ and *Tenm1*$^{+/+}$ mice in any of the locomotor indices (total distance traveled and speed, Fig. S4, a–b) as well as in anxiety indices (number of visits in center, latency to first visit in center, and time in center; Fig. S4, c–e).

Macroscopic examination of brains of F0 *Tenm1* modified mice indicated no significant change in olfactory bulb length and in the length ratio olfactory-bulb/brain as compared to *Tenm1*$^{+/+}$ (Fig. S5). The result was reproduced in F1 *Tenm1* modified mice. However, additional more sensitive assays should be done to provide insight into how loss of *Tenm1* gives rise to olfactory behavior.

Olfactory testing

Homing Test (PD13): The latency to reach the home litter sawdust did not differ significantly between the genotypes (Fig. 4a and 4b).

Buried food test (adulthood). *Tenm1*$^{-/-}$ and *Tenm1*$^{A/A}$ mice took significantly longer to locate the buried pellet compared to *Tenm1*$^{+/+}$ (Fig. 4c).

Odor Preference test (adulthood): No inter-genotype differences were found in latency to sniff the filter paper with water (Fig. 4e). However, *Tenm1*$^{A/A}$ mice took significantly less time to a first sniff of the filter paper with 2-methyl butyric acid compared with *Tenm1*$^{+/+}$ mice (Fig. 4d), compatible with the lack of olfactory-mediated aversion. The latency of the *Tenm1*$^{-/-}$ modified mice to sniff the same filter paper was not significantly different from that of *Tenm1*$^{+/+}$ mice (Fig. 4d).

Discussion

To investigate the possible contribution of inherited rare variants to CGA we performed whole exome sequencing in a family with three affected siblings and identified a very rare missense variant in the *TENM1* gene which, to the best of our knowledge, has not been associated with any human disease yet. The mutation was maternally inherited. The X-inactivation skewing could not explain the absence of the CGA phenotype in the mother and grandmother, therefore, this phenotype seems to have an X-linked dominant inheritance with incomplete penetrance. It was previously shown that some X-linked disorders have a highly variable penetrance in females (46).

TENM1 and its three human paralogs (*TENM 2–4*) belong to *Tenm/Odz* gene family of transmembrane proteins. Two orthologs of this human gene family (*ten-a* and *ten-m*) were initially identified in *Drosophila melanogaster* (47, 48). In non-human vertebrates there are four members called *Teneurins* (*Tenm1-4*). *Teneurins* were shown to have an important role in the development of the nervous system in *Drosophila melanogaster*, *Caenorhabditis elegans* (*C. elegans*), chicken and mouse, but the relevant molecular mechanisms are only partially deciphered [33]. The *Drosophila melanogaster Ten-m* was shown to have a role in motor neuron axon routing (49), and to be highly expressed in the embryonic central nervous system (48). In chicken and *Drosophila melanogaster* it was also shown that *teneurin-1/ten-m* is highly expressed in neurons of the developing visual system (41, 42). *Ten-m* was further shown to be involved in regulation of early embryonic development and to have a role in later stages of development of nervous system of *Drosophila melanogaster* (47, 48). It was suggested that *teneurin-1* has a function in directing neuronal connectivity (41, 42) and its ortholog *ten-m* was reported to control sensory neuronal synaptic-partner-matching in the *Drosophila melanogaster* olfactory lobe (43) and to be required for neuromuscular synapse organization and target selection (44). Most relevant to the present report, *Ten-m* was shown to be involved in synapse organization in the *Drosophila melanogaster* antennal lobe that mediates olfaction (50). In *C. elegans* this protein was shown to be essential for early embryogenesis, neuronal migration and pathfinding, germ cell development, gonad migration and epidermal

morphogenesis (51). Studies in chick detected an expression of *teneurin-1* in different brain regions involved in general sensation, olfaction, the processing of auditory information, and the coordination of complex motor behavior (52). In chicken at embryonic day 17, the highest expression of *teneurin-1* was found in the mitral cells of the olfactory bulb (52). The expression analysis of the *Tenm1* in mice detected the first expression of *Tenm1* only at E15.5 in different brain regions (53). In adult brain Tenm1 protein was found in cortex, thalamus, hippocampus, and in the granular layer of the cerebellum, and was suggested to be a "neuronally expressed and axonally transported molecule"(53). The examination of the expression profile of *Tenm1* in The Mouse Gene Expression Database revealed that *Tenm1* is also expressed in the embryonic olfactory epithelium and bulb. All the above are in accordance with a role for *TENM1* in the development and wiring in the human olfactory pathway.

To check the putative role of *TENM1* gene in congenital general anosmia we generated knockout ($Tenm1^{-/-}$) and knock-in ($Tenm1^{A/A}$) mice. $Tenm1^{-/-}$ and $Tenm1^{A/A}$ mice look normal at birth and showed no visible phenotype during early and adult development. A homing test that was performed at PD13 did not show any difference between $Tenm1^{+/+}$ and $Tenm1^{-/-}$ *or* $Tenm1^{A/A}$. This finding can be explained by the fact, that mice mostly use their vomeronasal organ (an accessory olfactory system) to discriminate between urinary odors (54) and thus their performance in this test does not depend solely on the main olfactory system. However in the buried food test, $Tenm1^{-/-}$ and $Tenm1^{A/A}$ adult mice took significantly longer to locate the buried pellet, as compared with $Tenm1^{+/+}$ mice, suggesting an olfactory deficit in the $Tenm1^{-/-}$ *and* $Tenm1^{A/A}$. The home-cage locomotion assessment and Open Field test showed that the failure to find the buried food was not caused by anxiety or locomotor deficit of these mice. Finally, in the odor preference test, $Tenm1^{-/-}$ *and* $Tenm1^{A/A}$ mice showed impaired odor discrimination ability for the aversive odorant 2-methyl butyric acid, and this was especially noticeable in $Tenm1^{A/A}$ mice. The lack of significantly impaired odor discrimination ability in $Tenm1^{-/-}$ mice can be possibly explained by the CB6F1 genetic background. In general, these results are in agreement with our hypothesis that the *TENM1* gene is involved in mammalian olfactory system development or function. However, the genetically modified mice seem to be hyposmic and not anosmic like human subjects carrying the mutation. It was previously shown that mouse models do not always fully repeat the hallmark characteristics of human disease phenotypes (55-57). Further, it was demonstrated that the interpretation of a mouse phenotype can be influenced by genetic background (58). In this respect, it is possible that the inheritance model is oligogenic, resembling two previously deciphered CGA cases (23). Also it is possible that the abnormal phenotype will only become obvious under conditions that have not yet been tested (59).

We did not perform MRI in all affected human siblings to assess the presence of olfactory bulbs. Therefore additional experiments still should be done to compare the presence of normal size olfactory bulbs in our genetically modified mice to prospective MRI measurements of the olfactory bulbs in anosmic patients.

Unfortunately we do not have a large enough sample of unrelated CGA cases to provide statistical evidence that *TENM1* gene is more frequently mutated in affected individuals than in the general population. However, there are other reported in the literature disease causing variants that were detected only in a single family implicating the specific gene in a disease (12, 60). This is exemplified by a disease causing mutation in the papalog of *TENM1* (*TENM3)* that was reported only in a single family (60). 1-bp insertion (2083dupA) in exon 12 of the *TENM3* gene was detected in two siblings with nonsyndromic bilateral colobomatous microphthalmia (OMIM#615145). The reported pathogenic variant was shown to have a zero frequency in ~5000 control samples. Moreover, up to date only one variant was reported to cause CGA in a gene non-related to Kallmann syndrome. Mutation in this gene was also reported only in a single family (12). Karstensen et al. (2015) performed whole-exome sequencing in the 2 siblings with CGA and found that both of them were hemizygous for a c.634C-T transition in the *CNGA2* gene. The mutation was shown to have a zero frequency in thousands of control samples. No pathogenic variants were also found by a sequencing of the *CNGA2* gene in 31 additional CGA patients. Previously, we also failed to find pathogenic variants in this gene by screening of *CNGA2* in 64 our CGA patients (13). Karstensen et al. (2015) (12) concluded that mutations in *CNGA2* are a rare cause of CGA. Similar to this case, mutations in *Tenm1* gene also seem to cause CGA very rarely. The candidate variant in the *TENM1* gene was found to have a zero frequency in 400 in-house exomes, 1000G, NHLBI, CG and ExAC databases and was not found in any of the 103 CGA cases in our repositories. We also failed to find pathogenic variants in this gene by screening *TENM1* in additional ten unrelated CGA Israeli families.

Together, our results may suggest that *TENM1* has a role in olfaction and that human isolated CGA in our patients could be caused by the discovered rare mutation in this gene. However *TENM1* is likely to be responsible for only a small fraction of CGA. Future studies should elucidate the exact mode of inheritance, as well as the more detailed role of *TENM1* in human olfactory function. Also, additional studies in more anosmic samples are required to address the generalizability of the findings.

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Legends

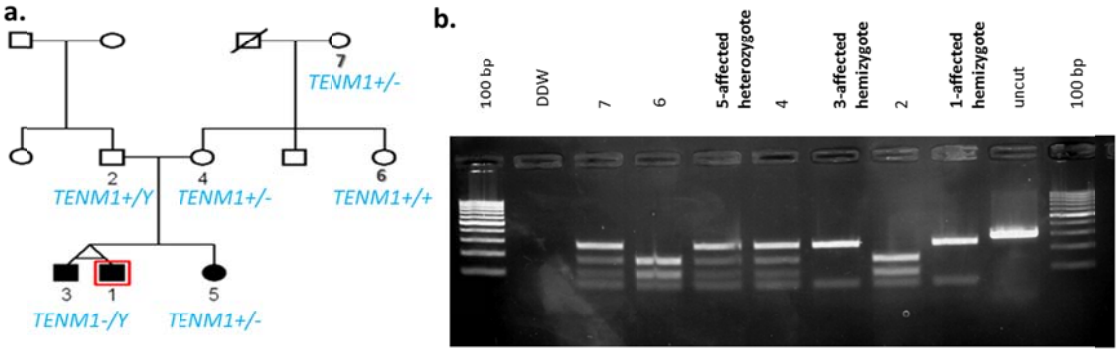

Figure 1. a. Family 230. Red square indicates proband. Filled-in symbols indicate affected family members. Whole exome DNA sequencing was applied to both parents and to three affected children (1,2,3,4,5). b. RFLP segregation analysis. The restriction enzyme digests WT allele.

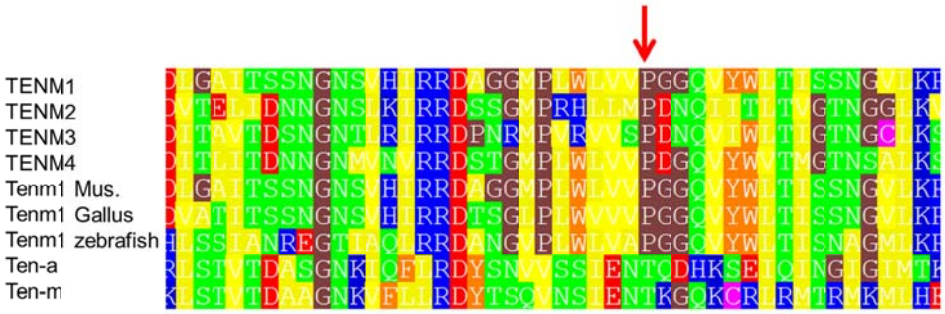

Figure 2. Conservation of the P1610L position.

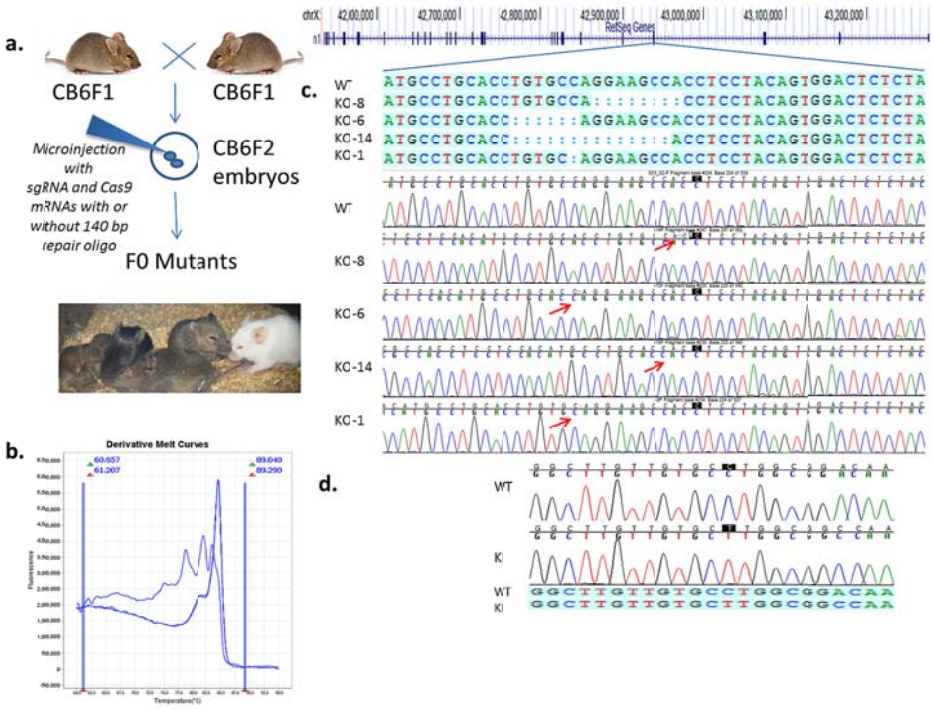

Figure 3. a. sgRNAs, the template and Cas9 mRNA were injected into fertilized eggs derived from CB6F1s crosses. b. High Resolution Melt (HRM) analysis of F0 pups. c. DNA sequence of PCR products amplified from the *Tenm1* gene of a F1 *Tenm1*$^{+/+}$ mouse and a mutant mouse carrying indel gene modifications after zygote-injection of Cas9 mRNA and sgRNA. d. DNA sequence of PCR products amplified from the *Tenm1* gene of a F1 *Tenm1*$^{+/+}$ mouse and a mutant mouse carrying the mutated allele induced by HDR after zygote-injection of Cas9 mRNA , sgRNA and oligo.

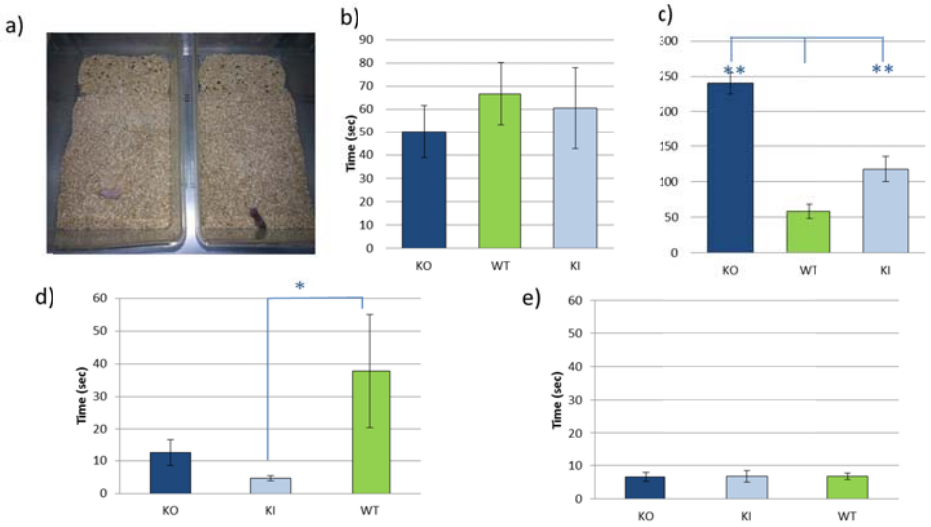

Figure 4. Olfactory testing. Homing test: (a) apparatus. (b) no differences were found in latency to reach the home litter sawdust among *Tenm1*$^{-/-}$ (n = 22), *Tenm1*$^{A/A}$ (n = 12) and *Tenm1*$^{+/+}$ (n = 17) mice [$X^2_{(2)}$=2.2; p=0.3]. (c) Buried food test: significant differences were evident between the groups in latency to find the buried food pellet [$X^2_{(2)}$=27.8; p=0.0] with the following mean ranks: *Tenm1*$^{+/+}$=10.8; *Tenm1*$^{A/A}$=20.0; *Tenm1*$^{-/-}$=33.7; Dunn's corrected pair-wise comparisons indicated *Tenm1*$^{-/-}$>*Tenm1*$^{+/+}$ p=0.0; *Tenm1*$^{-/-}$>*Tenm1*$^{A/A}$ p=0.02; Sample size: *Tenm1*$^{-/-}$ (n = 17), *Tenm1*$^{A/A}$ (n=10) and *Tenm1*$^{+/+}$ (n = 16). The odor preference test: (d) significant differences were evident between the groups in latency to first sniff the filter paper with 2-methyl butyric acid [$X^2_{(2)}$=7.32; p=0.03] with the following mean ranks: *Tenm1*$^{+/+}$=19.5; *Tenm1*$^{-/-}$=13.9; *Tenm1*$^{A/A}$=9.0; Dunn's corrected pair-wise comparisons indicated *Tenm1*$^{+/+}$>*Tenm1*$^{A/A}$ p=0.02; (e) No differences were evident between the groups in latency to first sniff the filter paper with water [$X^2_{(2)}$=0.37; p=0.83]; Sample size (d-e): *Tenm1*$^{-/-}$ (n = 10), *Tenm1*$^{A/A}$ (n=8) and *Tenm1*$^{+/+}$ (n = 10). Data are shown as means ± S.E.M in all panels; */**= significant difference p<0.05/0.01.