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Biallelic variants in *TMEM222* cause a new autosomal recessive neurodevelopmental disorder

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS DECLARATION

Individuals were identified in different centers worldwide in diagnostic or research settings approved by the respective institutional review boards: the Institutional Review Board Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen, the Netherlands; Institutional Review Board at University of Maryland School of Medicine, Baltimore, Maryland, USA; Institutional Review Board, Allama Iqbal Medical College, University of Health Sciences, Lahore, Pakistan; Institution Board of Jordan University Hospital, Amman, Jordan; Bioethics Committee of the University Hospitals and the Canton of Geneva, Switzerland; Ethics Committee of the Research Centre for Medical Genetics, Moscow, Russia; Persian BayanGene Research and Training Center Ethics Committee, Shiraz, Iran; institutional review boards of the Hanover Medical School the Children's and Youth Hospital Auf der Bul, Hannover, Germany. Written informed consents were obtained from all the participating adults and guardians of affected individuals. Specific written informed consent was obtained for publishing the patients' photographs. This study adhered to the World Medical Association Declaration of Helsinki (2013).

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Abstract

PURPOSE: To elucidate the novel molecular cause in families with a new autosomal recessive neurodevelopmental disorder.

METHODS: A combination of exome sequencing and gene matching tools was used to identify pathogenic variants in 17 individuals. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) and subcellular localization studies were used to characterize gene expression profile and localization.

RESULTS: Biallelic variants in the *TMEM222* gene were identified in 17 individuals from nine unrelated families, presenting with intellectual disability and variable other features, such as aggressive behavior, shy character, body tremors, decreased muscle mass in the lower extremities, and mild hypotonia. We found relatively high *TMEM222* expression levels in the human brain, especially in the parietal and occipital cortex. Additionally, subcellular localization analysis in human neurons derived from induced pluripotent stem cells (iPSCs) revealed that *TMEM222* localizes to early endosomes in the synapses of mature iPSC-derived neurons.

CONCLUSION: Our findings support a role for *TMEM222* in brain development and function and adds variants in the gene *TMEM222* as a novel underlying cause of an autosomal recessive neurodevelopmental disorder.

INTRODUCTION

Intellectual disability (ID) is a clinically and genetically heterogeneous group of developmental disorders, characterized by significant limitations in both intellectual functioning and adaptive behavior¹. ID has an estimated prevalence of approximately 1–3% in the general population, although a higher prevalence is observed in less-developed countries and in populations with a high frequency of consanguineous marriages^{2–4}. Considering that 30–50% of the known human protein coding genes are expressed in the brain^{5,6}, and calculations based on the 125 known X-linked ID genes and the 821 genes in the X chromosome^{7–9}, it is estimated that variants in the range of 2,000–3,000 genes may be involved in the genetic basis of Mendelian ID, of which half have not yet been identified^{8,10,11}. To date, more than 1,300 ID genes are listed in the SysID database (<https://sysid.cmbi.umcn.nl>)¹². It has been estimated that most of the unknown gene–disease associations are caused by pathogenic variants in genes resulting in autosomal recessive modes of inheritance^{13,14}. The discovery of genetic autosomal recessive causes of ID has been facilitated by the study of consanguineous families because of large genomic region in homozygosity¹⁵.

TMEMs are a heterogeneous group of transmembrane proteins that span the lipid bilayer of various cell membranes, such as lysosomes, mitochondrial membranes, Golgi apparatus, and endoplasmic reticulum^{16,17}. A variety of different biological functions have been assigned to TMEM proteins, such as neurite modulation (SLITRK2)¹⁸, transmembrane calcium influx

(ORAI1)¹⁹, protein glycosylation (TMEM165)²⁰, and regulation of lysosomal trafficking (TMEM106B)²¹. However, the function of the majority of TMEM proteins remains unclear. This is mainly due to difficulties in extraction and purification of these transmembrane proteins. Pathogenic biallelic variants in TMEM genes have been found in patients with autosomal recessive neurodevelopmental disorders, for example Joubert syndrome 6 (OMIM 610688, *TMEM67*), Joubert syndrome 14 (OMIM 614424, *TMEM237*), Meckel syndrome 2 (OMIM 603194, *TMEM216*), syndromic ID (OMIM 618316, *TMEM94*), and muscular dystrophy–dystroglycanopathy (OMIM 615041, *RXYLTI*). Transmembrane protein 222, encoded by *TMEM222* at 1p36.11, is a 208–amino acid protein of unknown function, comprising three transmembrane (TM) domains and a domain of unknown function (DUF778)²².

Here, we report nine unrelated families with a total of 17 affected individuals, presenting with autosomal recessive ID and variable associated phenotypes using exome sequencing and a gene matchmaking approach²³, with deleterious variants in *TMEM222* (NM_032125.2) as the likely cause of this syndrome.

MATERIALS AND METHODS

Family recruitment

Individuals were identified in different centers worldwide in diagnostic or research settings (Table 1). *TMEM222* variants were identified by exome sequencing (ES) and confirmed by Sanger sequencing (Supplemental data, Table 1).

Gene expression profile

For *TMEM222* expression analysis, total RNA from 13 brain areas, 9 fetal tissues, and 10 adult tissues was obtained from Stratagene (La Jolla, CA, USA), BioChain (Newark, CA, USA), and Clontech (Mountain View, CA, USA) (Fig. 2). The concentration of the extracted total RNA was estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For quantitative reverse transcription polymerase chain reaction (RT-PCR), 2 µg total RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript VILO Master Mix (catalog number 11755050, Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions. Amplifications were performed in duplicates of 25 µl reactions in the presence of SYBR green (Applied Biosystems, Foster City, CA, USA), using the absolute quantification setting on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), using the Ct method to quantify differences in *TMEM222* gene expression compared with housekeeping genes *GUSB*, *PPIB*, and *CLK2*²⁴, as described previously²⁵. Primer sequences are provided upon request.

TMEM222 expression vector

To amplify the genomic region encompassing the coding sequence of the *TMEM222* gene, we designed PCR primers using Clontech infusion primers program selecting EcoR1 as digestion enzyme. The PCR reaction mixtures (50 µl) contained 25 µM of each primer pair, a high-fidelity (HF) Taq polymerase Phusion® High-Fidelity DNA Polymerase (catalog number M0530L; New England BioLabs, Ipswich, MA, USA), 1× PCR HF buffer, 10 mM

of dNTP mix, 1× Q solution (catalog number 1005485, Qiagen, Hilden, Germany), and 5 ng cDNA library of small intestine by Clontech. Amplifications were performed using a two-phase touchdown PCR program described previously. The resulting PCR products were run on a 0.8% agarose gel and purified with the NucleoSpin Gel and PCR cleanup kit (catalog number 740609.250; Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. Then, 150 ng of each purified insert was cloned into the linearized C2-GFP vector by using infusion Enzyme Mix (catalog number 011614; Clontech). The C2-GFP-TMEM222 cloned construct was validated by Sanger sequencing. Additionally, 150 ng of the purified insert was cloned into the pDONR201 vector by using the Gateway BP Clonase Enzyme Mix (catalog number 11789021; Thermo Fisher Scientific). The different pDONR cloned constructs were validated by Sanger sequencing, and 150 ng was cloned into the destination vector pcDNA3native/DEST by using the Gateway LR Clonase enzyme mix (catalog number 11791043; Thermo Fisher Scientific). Primer sequences are provided upon request.

HEK293T cells were transfected with wild-type *TMEM222* expression gateway construct with FuGene HD Transfection Reagent (Promega, Madison, WI, USA). Transfected and control lines were lysed using standard lysis buffer with protease inhibitor cocktail (catalog number 11697498001, Roche Diagnostics, Rotkreuz, Switzerland). Proteins were separated using standard PAGE separation, transferred to a nitrocellulose membrane and detected by western blotting. The following antibodies were used: antitubulin (dilution 1:2,000; catalog number T5326, Sigma Aldrich, St. Louis, MO, USA) and anti-TMEM222 (dilution 1:2,000; catalog number NBP2-49295, Novus Biologicals, Centennial, CO, USA), and secondary antibodies antirabbit IRDye 800 (catalog number 9263221, LI-COR Biosciences, Bad Homburg vor der Höhe, Germany) and antimouse HRP (catalog number 115035062, Jackson ImmunoResearch Laboratories, Ely, UK) diluted 1:10,000.

Cell lines and culture maintenance

HEK293T cells (ATCC CRL-3216; American Type Culture Collection [ATCC], Wesel, Germany) were grown in DMEM supplemented with 10% (volume/volume) fetal calf serum (catalog number F7524; Sigma Aldrich), 1% (volume/volume) Na pyruvate (catalog number S8636; Sigma Aldrich), and penicillin/streptomycin (catalog number P4333; Sigma Aldrich).

Human induced pluripotent stem cells (iPSCs) 409B2 were obtained from Riken BRC—Cell engineering division (cell number HPS0076:409B2, Koyadai, Japan). Cells were maintained in E8 Flex media (STEMCELL Technologies, Vancouver, Canada) in 5% CO₂. Medium was changed every two days and at appropriate confluency; cells were split 1:8 or 1:10 using ReLeSR (STEMCELL Technologies, Vancouver, Canada), an enzyme-free reagent for dissociation.

Induced pluripotent stem cell differentiation into excitatory neurons (iNeurons)

We used a rapid and reliable protocol to produce hiPSC-derived cortical glutamatergic neurons as previously described²⁶. In brief, for the generation of *rtTA/Ngn2*-positive hiPSCs, two lentiviral vectors were used to stably integrate the *rtTA* and *Ngn2* transgenes

into the genome of the hiPSCs using the transfer vectors pLVX-EF1 α -(Tet-On-Advanced)-IRES-G418 (R) and pLVX-(TRE-thight)-(MOUSE)Ngn2-PGK-Puromycin(R), respectively. Selection of rtTA/Ngn2-positive colonies was performed with puromycin and G418. The differentiation of rtTA/Ngn2-positive hiPSCs to neurons overexpressing the transcription factor neurogenin-2 is carried out for four weeks in coculture with rat astrocytes, where the hiPSC-derived neurons show a neuronal-like morphology.

Subcellular localization

iNeurons were cultured on glass coverslips in a 24-well plate. After washing with cold phosphate-buffered saline (PBS) buffer, cells were fixed with 2 mL 4% paraformaldehyde for 20 minutes. Cells were then washed three times with cold PBS and permeabilized with 2 mL PBS with 0.1% Triton X-100 for 15 minutes and washed four times with cold PBS. For the colocalization experiments, the coverslips were incubated in anti-TMEM222 polyclonal antibody (catalog number NBP2-49295; Novus Biologicals) diluted 1:500 in 2% BSA with: anti-GM130 (catalog number 610822, BD Biosciences), anti-PSD95 (MA1-045, Thermo Fisher Scientific), anti-Synapsin 1 (catalog number 106001; Synaptic Systems, Göttingen, Germany), anti-MAP2 (catalog number 188004; Synaptic Systems), anti-EEA1 (catalog number ab70521, Abcam, Cambridge, UK), anti-Rab5 (catalog number ab66746, Abcam), anti-KIF16B (catalog number SC-390309, Santa Cruz Biotechnology, Dallas, TX, USA), anti-Sec31A (catalog number 6036944, BD Biosciences), anti-SAR1A (catalog number ab77029, Abcam), anti-NKHC1 (catalog number SC-398759, Santa Cruz Biotechnology) and anti-LMAN1 (catalog number NBP2-03381, Novus Biologicals) diluted 1:500 in 2% bovine serum albumin (BSA). Coverslips were incubated for 3 hours at room temperature and washed three times with PBS and then incubated with the secondary antibodies, goat antirabbit Alexa Fluor 488 (catalog number A11008, Life Technologies, Carlsbad, CA, USA), goat antimouse 568 (catalog number A11031, Life Technologies), and goat anti-guinea pig (catalog number A21450, Life Technologies) diluted 1:1,000 for 1 hour at room temperature. The coverslips were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (catalog number H-1200, Vector Laboratories, Burlingame, CA, USA). Images were capture using ZEISS Apotome.2 microscope and processed with Adobe Photoshop CC 2019 (Adobe Systems, Mountain View, CA, USA).

RESULTS

Genetic findings

Genetic studies were performed in nine families with one or more individuals affected with moderate-severe ID, speech delay, and nonspecific facial dysmorphisms. Additionally, some individuals presented with behavioral problems such as aggressiveness and shy character, body tremors, decreased muscle mass in lower extremities, seizures, and hypotonia. Detailed clinical descriptions are provided in Table 1 and in the Supplemental data.

Exome sequencing was performed for the proband/index of each family (Supplemental data). All *TMEM222* (GenBank: NM_032125.2) variants were confirmed and their familial segregation was validated by Sanger sequencing. In family 1, a homozygous c.535_537del deletion (p.[Val179del]) was identified in *TMEM222* (Fig. 1a, Table 1). In family 2, we

found a homozygous c.214G>A (p.[Gly72Ser]) missense variant, in family 3 we identified a homozygous c.526G>A (p.[Gly176Arg]) missense variant, and in family 6 we found the homozygous c.442G>A (p.[Val148Met]) variant, all predicted to be deleterious (CADD score >29)²⁷. In family 4 we found a homozygous c.447_468del (p.[Leu150Thrfs*10]) frameshift deletion; in family 5, we identified the compound heterozygous splice-site variants c.279 + 2T>C and c.280–2A>T, affecting the strictly conserved nucleotides at the splice donor and acceptor site, respectively. In family 7 we found the homozygous splice variant c.539 + 2T>C affecting the strictly conserved nucleotides at the splice donor site. In family 8 we identified an insertion in exon 2 of *TMEM222* (c.207dup), which creates a frameshift starting at codon Ile70 resulting in termination after 47 amino acids (p.[Ile70Hisfs*47]). In family 9 we found the homozygous c.334C>T (p.[Gln112*]) variant leading to a premature stop codon. Predictions indicate messenger RNA (mRNA) could be degraded via nonsense-mediated mRNA decay (NMD), resulting in absence or an abnormally sized protein lacking the C-terminal part of the DUF778 domain and downstream amino acids.

Sanger sequencing showed that these variants were present in a recessive manner in the affected individuals and inherited from heterozygous parents. Unaffected siblings had either two wild-type alleles or were heterozygous for the familial *TMEM222* variant (Fig. 1b). None of these variants were present in the Genome Aggregation Database in a homozygous state (gnomAD; <https://gnomAD.broadinstitute.org/>)²⁸.

Clinical spectrum

All affected individuals presented with moderate to severe ID, motor delay, and no speech/speech delay (Table 1). Variable clinical manifestations include nonspecific facial abnormalities ($N=10/17$, 59%; Fig. 1b), hypotonia ($N=10/15$, 67%), broad gait ($N=5/12$, 42%), seizures ($N=7/17$, 41%), abnormalities in the magnetic resonance image (MRI) ($N=5/8$, 62%; Supplemental Fig. S2), and neuropsychiatric problems characterized by aggressive behavior ($N=6/17$, 35%). Five patients presented with body tremors, three patients had decreased muscle mass in lower extremities, and two patients presented with disorder of the motor neurons. Patients in family 3 presented with macrocephaly (IV:6 + 1.32 SD, IV:7 + 2.54 SD). Patients from family 2 (IV:2 –2.09 SD, IV:3 –3.31 SD, IV:13, –3.31 SD, IV:14 –3.32 SD), family 4 (IV:2 –2.81 SD), and family 7 (IV:2 –3.91 SD) presented with microcephaly and secondary microcephaly was observed in individual V:1 from family 6 (–2 SD). In the rest of the patients studied the head circumference was in normal range. No striking growth abnormalities or common characteristic dysmorphic features were present. Detailed clinical phenotypes of all patients are provided in the Supplemental data. The clinical phenotype of heterozygous individuals was normal.

TMEM222 expression studies

Transmembrane protein 222, encoded by *TMEM222*, is comprised of 208 amino acid residues, and has a domain of unknown function (DUF778); however, the function of this protein has not yet been elucidated. To gain more insight into the role of *TMEM222*, we measured the gene expression level compared with three housekeeping genes (*GUSB*, *PPIB*, and *CLK2*)²⁴ in several human tissues. Our results show that *TMEM222* is highest

expressed in brain with ubiquitous but lower expression levels in non-neuronal tissues (Fig. 2). Similarly, *TMEM222* expression levels retrieved from the Genotype-Tissue Expression Project²⁹ (GTEx, Supplemental Fig. S3) show a relatively high expression levels in cerebellum, cerebellar hemisphere, and tibial nerve, supporting a more specific role for *TMEM222* in neurodevelopment.

To characterize the cellular localization of the protein, we used a commercially available polyclonal antibody for human *TMEM222* (catalog number NBP2–49295, Novus Biologicals). Western blot analysis of HEK293T cells transfected with expression vector pcDNA3native/DEST-*TMEM222* revealed that the anti-*TMEM222* polyclonal antibody specifically identifies a 23.2-KDa protein that was less abundant/not detectable in mock-transfected cells (Supplemental Fig. S1). Next, immunocytochemistry was used to test the endogenous expression of *TMEM222* in several cell lines. The highest *TMEM222* levels were detected in the mature human iPSC-derived glutamatergic cortical neurons (at DIV28), briefly iNeurons, whereas moderate levels were detected in immature iNeurons (DIV10) and SK-N-SH neuroblastoma cells (Fig. 3a), and very low levels in HEK293T, fibroblasts, and hTERT RPE-1.

Due to the unknown function of the protein, we used PSORT II (<https://psort.hgc.jp/form2.html>) to predict the subcellular localization sites of the protein. *TMEM222* is predicted to localize in vesicles of the secretory system³⁰. Considering that *TMEM222* has a high abundance in the brain and mature human iNeurons, and appears to play a role in vesicles, we sought to investigate the localization of *TMEM222* in mature iNeurons. The *TMEM222* antibody showed expression of the protein concentrated within the dendrites in a punctate pattern (Fig. 3b). We hypothesized that this expression could be in synaptic vesicles within the neuron. To test this, we performed colocalization analysis with different markers: *TMEM222* colocalized with MAP2 and partially colocalized with postsynaptic marker (PSD95), early endosomal and ER exit markers (EEA1, RAB5, KIF16B, and SAR1A respectively; Fig. 3b). These data show that *TMEM222* is localized in the dendrites and might play a role in postsynaptic vesicles in neurons.

DISCUSSION

In this study, we identified four missense homozygous variants, two frameshift homozygous variants, three splice-site variants, and one nonsense variant in *TMEM222*, in nine unrelated families, with a total of 17 affected individuals presenting with autosomal recessive ID and variable associated phenotypes, such as aggressive behavior, shy character, body tremors, decreased muscle mass in the lower extremities, and mild hypotonia.

It is interesting to note that interrogation of the gnomAD database²⁸ shows that *TMEM222* is a missense variation tolerant gene (Z -score = 0.44, probability of loss of function intolerance [pLI] = 0). In our study we found recessive variants in *TMEM222*; in this case heterozygous variants could be tolerated but homozygous ones are not. Even though the tolerance scores for this gene are low, we noticed that only two coding missense variants and no loss-of-function variants that were present in the gnomAD database were found in a homozygous state. Additionally, no homozygous variants with low allele frequency

(<0.01) were found in Bravo (<https://bravo.sph.umich.edu/freeze5/hg38/>), a variant browser with variants observed in 62,784 individuals. Both the available data from gnomAD and the recessive *TMEM222* variants identified (across multiple affected families) in this study suggest this gene may tolerate heterozygous variants but is not tolerant to biallelic variants.

All variants reported in this study are predicted to be deleterious (Table 1), but there are no known 3D structure or modeling templates available for *TMEM222* to predict the effects of amino acid changes on *TMEM222* protein structure. Considering that nonsense variants lead to degradation of mRNA via NMD^{31,32}, the loss-of-function variants found in families 4, 5, 7, 8, and 9 are predicted to undergo NMD, resulting in absence or an abnormally sized protein lacking the C-terminal part of the DUF778 domain and downstream amino acids. Additionally, based on protein sequence predictions and annotations publicly available, missense and in-frame variants are conserved across species (Fig. 1d) and the missense variants are predicted to affect protein function (Table 1, CADD score >29)³³. The in-frame deletion p.(Val179del) found in family 1 is predicted to be deleterious and disrupt the interactions of the transmembrane helix. The p.(Gly72Ser) variant found in family 2 likely disrupts protein localization due to the size of amino acid change. The p.(Gly176Arg) variant identified in family 3 is predicted to introduce a charged arginine in the transmembrane region, which would similarly interfere with its membranous localization. Finally, the p.(Val148Met) change found in family 6 might disturb the protein interactions, or the local core of the domain might be less stably folded, due to the larger size of the changed amino acid (<http://lbqp.unb.br/NetWheels/>).

The clinical symptoms in the patients, including severe developmental delay, hypotonia, significant speech delay, decreased muscle mass in lower extremity, neuropathy, secondary microcephaly, and other neurological symptoms such as tremor and lack of coordination and seizures, may suggest a neurodegenerative process, but a careful clinical reanalysis and longitudinal assessment of patients is required to establish this. In general, the patients presented here show significant clinical variability, which appears to be stronger between patients from different families than for affected individuals within the same family (Table 1). An explanation for this could be the differential functional consequences of the various allelic variants. In contrast to the clear loss-of-function frameshift, nonsense, and splice-site variants, some missense variants may be hypomorphic alleles capable of producing protein with residual activity. Another explanation for the clinical variability might be the presence of additional recessive hits. In three families, additional recessive variants were identified. In family 1, affected patients have a recessive missense variant passing our filtering criteria in *SHISA6* (c.1043C>T, p.[Pro348Leu]; Table 1), a gene previously reported to have a critical role in maintenance of high-frequency synaptic transmission at hippocampal synapses³⁴. This missense variant is found present 531 times in the gnomAD database, and four homozygotes. No clinical phenotype has been reported in individuals carrying pathogenic variants this gene. In family 3, affected individuals have recessive variants in three other genes (Table 1, Supplemental data): a splice-site variant c.6-1G>A in *RGMB*, and missense variants c.110C>T, p.(Thr37Met) in *BSDC1* and c.294C>A, p.(His98Gln) in *NDUFS5*. The splice-site variant in *RGMB* is indicated in gnomAD as a possible annotation and is homozygously present 27 times in the database. None of these three genes have previously reported variants leading to a clinical phenotype. A possible additive clinical effect cannot

be excluded for any of these homozygous variants, but the features of the patients from this family are not supportive of this possibility. In the patient of family 5, a variant of unknown significance paternally inherited was identified in the *NF1* gene, which may add to the severity of the phenotype in this case. Finally, the clinical variability seen for the patients in this study might also be due to (rare) variants in coding or noncoding regions affecting genes that influence the *TMEM222* phenotype. Further possible contributions to the phenotypes observed in this study might also be due to the effects of modifying genes that influence the *TMEM222* phenotype.

The relatively high abundance of *TMEM222* in the brain, especially the parietal and occipital cortex, together with the high protein expression in mature human iNeurons and the probable localization to early endosomes and in synapses, support a more specific role for *TMEM222* in neurophysiological processes. The proteins of the *TMEM* family are predicted to be components of various cell membranes, such as mitochondrial, lysosomal, Golgi, and endoplasmic reticulum membranes. *TMEM222* is predicted to encode for a transmembrane protein, according to the Pfam database²², and is highly expressed in brain with ubiquitous but lower expression levels in non-neuronal tissues (Fig. 2). Moreover, anti-*TMEM222* antibody revealed the highest *TMEM222* levels detected in the mature iNeurons and colocalization with MAP2 and partial colocalization with postsynaptic marker (PSD95), and early endosomal and ER exit markers (EEA1, RAB5, KIF16B, and SAR1A respectively; Fig. 3b), suggesting a role in postsynaptic vesicles in neurons.

UniProtKB³³ lists several possible *TMEM222*-interacting proteins with a membranous localization. These interacting proteins have various cellular roles, including endosomal trafficking, synaptic vesicle shuttling, sterol metabolism, and mitochondrial calcium influx. For some of these proteins, variants have been identified in the corresponding gene that are associated with phenotypic features that overlap those associated with *TMEM222* variants reported here. Biallelic variants in *TMX2* cause a neurodevelopmental disorder (NEDMCMS; OMIM 618730) characterized by severe to profound global developmental delay, early-onset seizures, microcephaly, and polymicrogyria and/or cerebral atrophy. Most affected individuals are unable to walk or speak and have profound ID as well as axial hypotonia and peripheral spasticity. *TMX2* is an ER protein that functions in post-translational protein modification and folding, and mitochondrial calcium flux and cellular redox homeostasis³⁵. Hemizygous variants in *EBP* cause MEND syndrome (OMIM 300960) with neurologic defects is an X-linked recessive disorder representing a continuous phenotypic spectrum including ID, short stature, scoliosis, digital abnormalities, cataracts, and dermatologic abnormalities. The variable manifestations of MEND syndrome are associated with a defect in sterol biosynthesis. Interestingly, several other *TMEM222*-interacting proteins, such as *ERG28*, *ELOVL4*, and *COMT*, are involved in sterol metabolic pathways. *ELOVL4* is required for the synthesis of very long chain saturated fatty acids and very long chain polyunsaturated fatty acids uniquely present in retina, sperm, and brain³⁶. Causative *ELOVL4* variants have been identified in three different disorders, heterozygous frameshift and nonsense variants in Stargardt disease 3 (OMIM 600110), heterozygous missense variants in spinocerebellar ataxia 34 (OMIM 133190), and homozygous nonsense and frameshift variants in ichthyosis, spastic quadriplegia, and mental retardation (OMIM 614457). Phenotypic features associated with *TMEM222* variants have significant overlap

with the two latter conditions^{37,38}, suggesting related biological functions in *TMEM222* and *ELOVL4*.

In conclusion, we have identified 17 patients from nine unrelated families with deleterious autosomal recessive variants in *TMEM222* presenting with moderate–severe ID, speech delay, and nonspecific facial dysmorphisms. Additionally, some individuals presented with behavioral problems such as aggressiveness and shy character, body tremors, decreased muscle mass in lower extremities, seizures, and hypotonia. Further functional studies are necessary to understand the pathophysiological mechanism of this new autosomal recessive ID syndrome.

DATA AVAILABILITY

The *TMEM222* variant data were submitted to LOVD database (<https://www.lovd.nl/TMEM222>) hosted at Leiden University Medical Center, the Netherlands. Accession numbers are available in Table 1. Additional data and materials, such as primer sequences and Sanger sequencing electropherograms, are available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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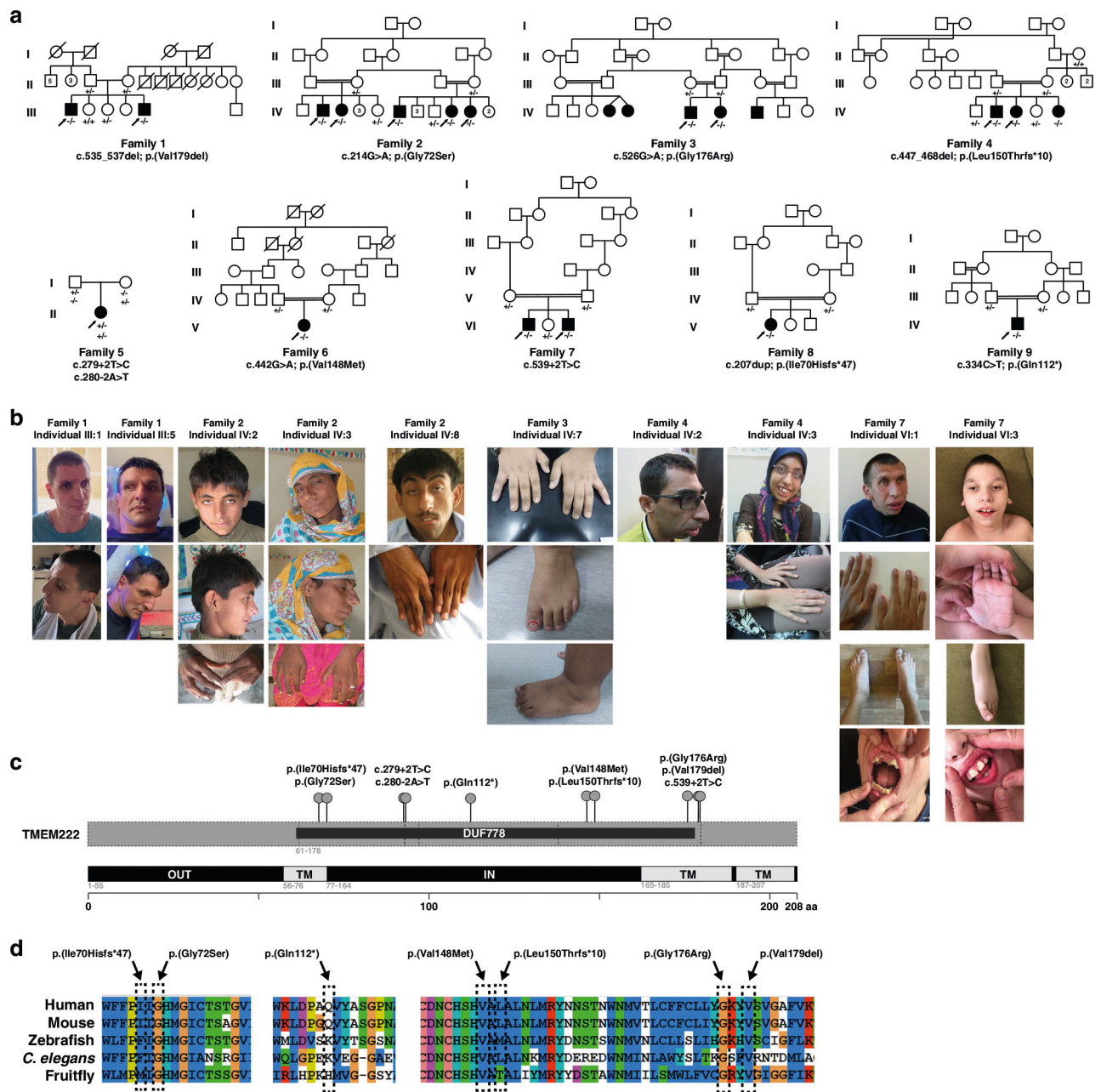


Fig. 1. Homozygous variants in *TMEM222* lead to intellectual disability. (a) Pedigrees of families and clinical appearance of affected individuals. Arrows show patients described on Table 1. (b) Phenotypic features of affected individuals. (c) Schematic representation of human *TMEM222* including the positions and the predicted effect on protein level of the eight identified variants in this study. Dark gray box represents the domain of unknown function DUF778 (positions 61–178). Dashed boxes represent exons. Black and light gray boxes represent the position of the protein in the membrane; IN inside (positions 77–164 and 208), OUT outside (positions 1–55 and 186), TM transmembrane (positions 56–76, 165–185, and 187–207). (d) Clustal W³⁹ amino acid sequence alignment

of TMEM222 from human, mouse, zebrafish, *C. elegans*, and fruit fly. Square dashed boxes indicate the position of the mutated amino acid conserved.

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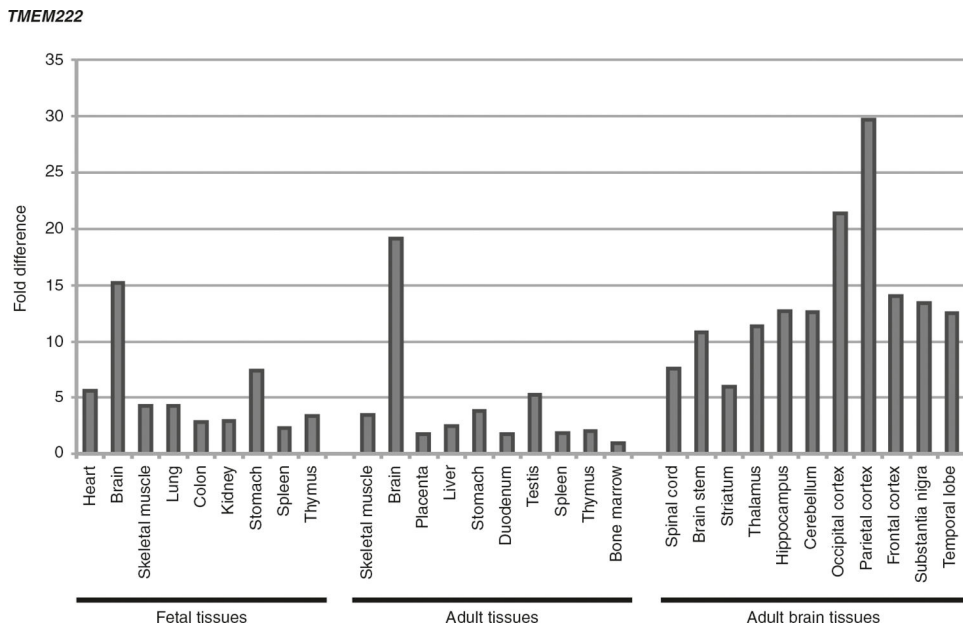


Fig. 2. Expression of *TMEM222* in selected human tissues. *TMEM222* expression levels as determined by messenger RNA (mRNA) analysis by quantitative polymerase chain reaction (qPCR) shows a higher expression in the brain.

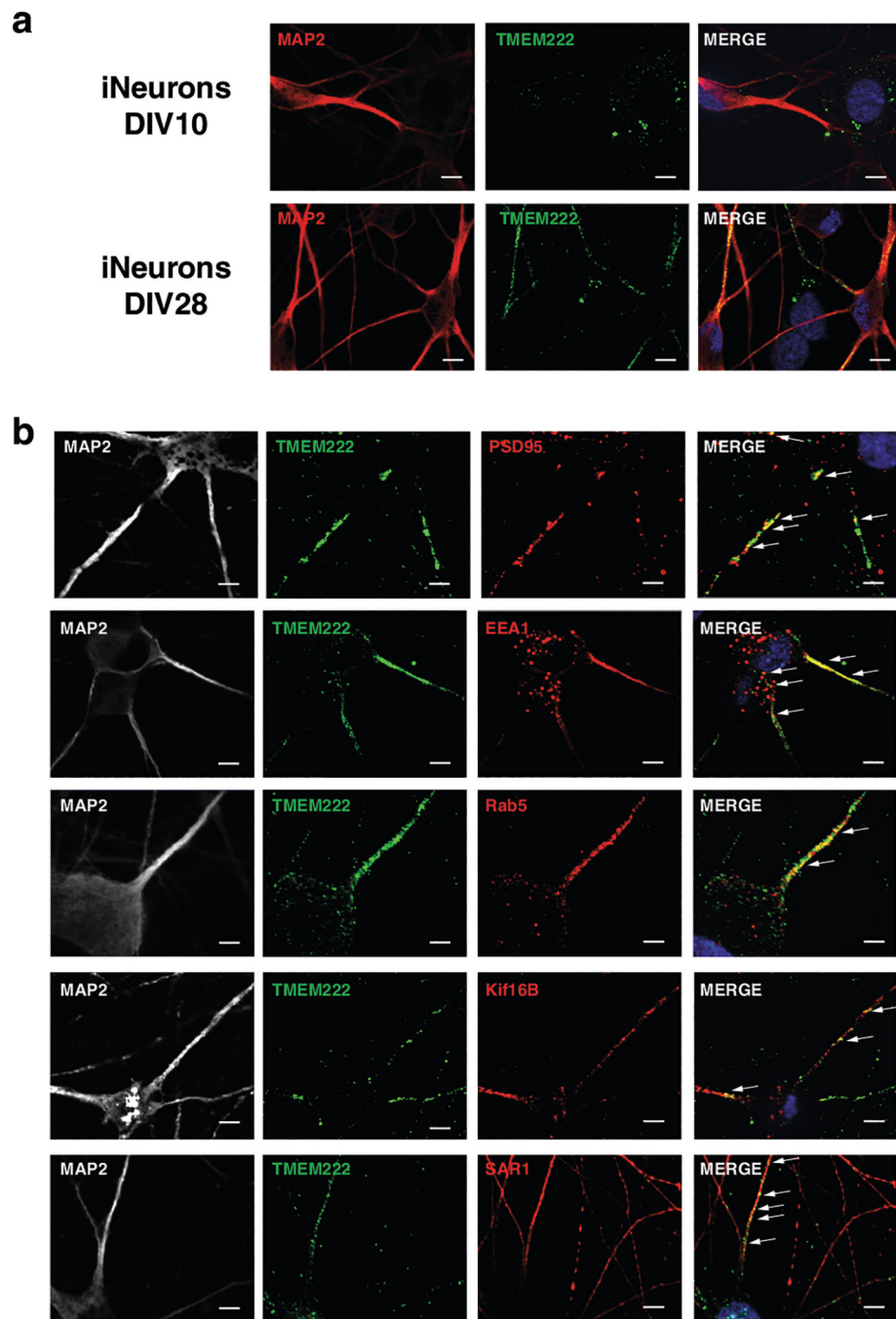


Fig. 3. Human TMEM222 is highly expressed in dendrites of mature iNeurons and partially colocalizes with postsynaptic and early endosomal markers.

(a) TMEM222 protein expression is specific to dendrites (MAP2 in white) in mature iNeurons at DIV28. (b) TMEM222 (in green) partially colocalizes with the postsynaptic marker PSD95 and early endosomal markers EEA1, Rab5, Kif16B, and SAR1 (in red). Scale bar 10 μ m.

Table 1.

Clinical and Genetic characteristics of families included in this study^a.

	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6	Family 7	Family 8	Family 9
Patient ID	III:1-III:5	IV:2;IV:3;IV:8;IV:13;IV:14	IV:6	IV:2	II:1	V:1	VI:1	V:1	IV:1
cDNA variant	c.534_536delICGT	c.214G>A	c.526G>A	c.445_466del	c.279+2T>C; c.280-2A>T	c.442G>A	c.539+2T>C	c.207dupC	c.334C>T
Amino acid variant	p.(Val179del)	p.(Gly72Ser)	p.(Gly176Arg)	p.(Leu150Thrfs*10)	Intronic	p.(Val148Met)	Intronic	p.(Ile70Hisfs*47)	p.(Gln112*)
gnomAD allele frequency	0	3.98E-06	3.98E-06	0	0; 7.07e-6	3.99E-06	0	0	0
Gene	NM_032125.2	NM_032125.2	NM_032125.2	NM_032125.2	NM_032125.2	NM_032125.2	NM_032125.2	NM_032125.2	NM_032125.2
CADD Score	-	32	32	-	-	29	-	-	-
Ethnicity	Turkish	Pakistani	Jordanian	Iranian	US	Polish	Russian	Arab	Iranian
Gender	M;M	M;F;M;F;F	M;F	M;F	F	F	M;M	F	M
Diagnostic or research	Research	Research	Research	Research	Diagnostic	Diagnostic	Research	Research	Research
Age of last examination (years)	36; 23	14;22;16;26;28	16;14	30;18	3.25	4.5	31;11	0.8	5.5
Growth Features									
Height (cm)	175;163	140;152;147;160;160	162;155	151;137	91	100	167;135	72 (+0.18 SD)	NA
Weight (kg)	66;59	35;45;40;62;63	85.5;68.5	45;58	12.1	15	63;30.5	10.7 (+1.57 SD)	NA
Head circumference (cm)	NA	52.1;50.8;53;50.8;50.8	57(+1.32)	53;53	49.5	47	57.5;48	46 (+0.73 SD)	NA
Neurologic Features									
ID	Severe	Moderate	Yes	Severe	Severe	Severe	Moderate-Severe	Severe	Severe
Behavior abnormalities	Stereotypic movements	Shy and usually afraid in some situations	Hyperactive	Shy Character	Shy character, usually happy around family	No	Stereotypic movements, autistic features	Stagnation of development, short visual contact, autistic features	ADHD, Obsessive behaviors

	Family 1	Family 2	Family 3	Family 4	Family 5	Family 6	Family 7	Family 8	Family 9
Aggressive behaviour	No	Yes	No	No; Yes	No	No	No	No	No
Motor delay	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Standing	NA	>24 months	NA	18;24 months	No	3 years	3 years	No	2 years
Speech delay	No Speech	Yes	Yes	No speech	No speech	No speech	Yes/No speech	No speech	Yes
Seizure	Yes (III.1); No	No	Yes	No	Yes	No	Yes	Yes	Yes
Hypotonia	NA	Yes (Mild)	No	No	Yes	Severe in early childhood	Severe in early childhood	Yes	No
Gait	Broad	Normal	Broad	Normal	NA	Broad	NA	NA	Normal
Brain MRI	NA; Normal	NA	Hypersignal intensity in periventricular region	Diffusely simplified gyral pattern	Mild diffuse brain volume loss	Normal	Parietal white matter reduction, atrophic ventriculomegaly, hydrocephaly	Supratentorial bifrontotemporal brain volume reduction	Normal
Dysmorphic Features									
Facial dysmorphism	Yes (mild)	No	Yes	Yes	Yes	Yes	Yes	No	No
Others									
Other genetic findings	<i>SHISA6</i> c.1043C>T p.Pro348Leu		<i>RGMB</i> c.6-1G>A <i>BSDC1</i> c.110C>T p.(Thr37Met) <i>NDUFE5</i> c.294C>A p.(His98Gln)		<i>NFI</i> c.5243G>C (p.R1748P) chr17:29654554 NM_000267.3				
Others ^a	Hypoplastic nails, decreased muscle mass, scoliosis, pes planovalgus, strabismus, visual impairment, otitis media, inguinal hernia	Tremors, weak limbs, walking difficulties	Broad first toe, mild contracture of elbow	Tremors. Decreased muscle mass in lower extremities, mild strabismus, mild pectus excavatum	Optic nerve atrophy, trivial pulmonary valve stenosis.	Microcephaly, hypotrophy of calves, reduced muscle tone, diminished reflexes, axonal sensory-motor polyneuropathy	Stereotypic movements, lack of coordination, disorder of motor neurons, microcephaly, spastic tetraparesis, convergent strabismus, foot deformities	Plagiocephalus, Impaired postural reflexes	-

NA Not assessed, M male, F female.

^aSee Supplementary data for details.