BRAIN REPORT

Variants in the zinc transporter *TMEM163* cause a hypomyelinating leukodystrophy

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Hypomyelinating leukodystrophies comprise a subclass of genetic disorders with deficient myelination of the CNS white matter. Here we report four unrelated families with a hypomyelinating leukodystrophy phenotype harbouring variants in *TMEM163* (NM_030923.5). The initial clinical presentation resembled Pelizaeus–Merzbacher disease with congenital nystagmus, hypotonia, delayed global development and neuroimaging findings suggestive of significant and diffuse hypomyelination. Genomic testing identified three distinct heterozygous missense variants in *TMEM163* with two unrelated individuals sharing the same *de novo* variant. *TMEM163* is highly expressed in the CNS particularly in newly myelinating oligodendrocytes and was recently revealed to function as a zinc efflux transporter. All the variants identified lie in highly conserved residues in the cytoplasmic domain of the protein, and functional *in vitro* analysis of the mutant protein demonstrated significant impairment in the ability to efflux zinc out of the cell. Expression of the mutant proteins in an oligodendroglial cell line resulted in substantially reduced mRNA expression of key myelin genes, reduced branching and increased cell death. Our findings indicate that variants in *TMEM163* cause a hypomyelinating leukodystrophy and uncover a novel role for zinc homeostasis in oligodendrocyte development and myelin formation.

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Introduction

Genetic disorders with predominant involvement of the CNS white matter are termed leukodystrophies (LDs).[1] These entities are significantly heterogeneous with respect to the underlying genetic and aetiopathogenetic mechanisms.[2] A radiological diagnostic algorithm is of high value in phenotyping and clinical diagnosis of leukodystrophies.[2,3]

Hypomyelinating leukodystrophies (HLDs) are diverse but have prominent commonalities such as developmental delay and/or neuroregression, nystagmus, central hypotonia progressing to spasticity and ataxia.[4] The prototype of HLDs is X-linked recessive HLD1/Pelizaeus–Merzbacher disease (PMD) caused by variants in proteolipid protein 1 (*PLP1*, MIM# 312080).[4] At presentation, several disorders manifest with a clinical phenotype resembling PMD. However, the clinical course, degree of cognitive involvement, additional neurological manifestations like extrapyramidal signs and/or epilepsy and differences in the MRI findings may help distinguish these entities from PMD. Nearly all of the HLDs described to date are eventually progressive in nature, often after a long stable phase.[4]

Herein, we report subjects from four unrelated families presenting with hypomyelination and variable clinical and/or radiological improvement with age, suggesting a novel subtype of HLD caused due to heterozygous variants in *TMEM163*. To date, variants in TMEM163 have not been demonstrated to cause human disease.

Materials and methods

Detailed methodology is available in the Supplementary material.

Molecular testing and *in silico* analysis

Family 1 was investigated at Kasturba Medical College (Manipal, India), while Families 2, 3 and 4 were identified at the Children's Mercy Hospital (Kansas City, MO, USA), Columbia University Irving Medical Center (New York, NY, USA) and Amsterdam Leukodystrophy Center (The Netherlands), respectively. Informed consents approved by institutional ethics committees were obtained from all families. Trio exome sequencing was performed for families 1, 2 and 4 while proband-only exome sequencing was carried out for Family 3. The functional impact of variants was analysed with various prediction tools. Multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). *In silico* modelling and analysis was carried out using the structure of TMEM163 available in UniProt (AF-Q8TC26-F1) and the structural effect of variants was predicted using the automatic mutant analysis server, HOPE (http://www.cmbi.ru.nl/hope/).

Construction of wild-type and mutated TMEM163-mCherry expression vectors

The TMEM163-mCherry plasmid has been previously described[5] and was a kind gift from Dr Math P. Cuajungco. Point mutations were introduced into the wild-type (WT) TMEM163-mCherry plasmid using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) per the manufacturer's instructions. Plasmids were sequenced to ensure the accuracy of site-directed mutagenesis. Mutation nomenclature is relative to the transcription start site of *TMEM163*.

Data availability

Data associated with this study will be made available upon a request to the corresponding author.

Results

Subjects

Four individuals (S1, S2, S3 and S4) from four unrelated families (Fig. 1A) presented with PMD-like clinical findings, i.e. nystagmus, hypotonia and delayed global development (Table 1). S5 from Family 4, the father of S4, indicated a history of nystagmus in his sister and probably in himself. However, no medical records were available for the same. Other developmental milestones were self-reported to be normal and he currently works in regular employment. Of all subjects, S1, S3 and S4 showed clinical improvement with progressive attainment of motor as well as language milestones and resolution of nystagmus. The age of independent ambulation was quite variable in these individuals: 1 year and 6 months

Table 1 Clinical characteristics observed in the individuals

Identifier	S1 (Family 1)	S2 (Family 2)	S3 (Family 3)	S4 (Family 4)	S5 (Family 4)
Variant in *TMEM163* (NM_030923.5)	c.227T>C p.Leu76Pro	c.227T>C p.Leu76Pro	c.412C>T p.Arg138Cys	c.437A>G p.His146Arg	c.437A>G p.His146Arg
Zygosity (inheritance)	Heterozygous (*de novo*)	Heterozygous (*de novo*)	Heterozygous (*de novo*)	Heterozygous (paternally inherited)	Heterozygous (unknown)
Sex	Female	Female	Male	Female	Male
Age at last assessment	7 years	7 years 7 months	10 years	3 years	29 years
Anthropometry at last assessment	OFC: 48.2 cm (–3.5 SD) Height: 110 cm (–2 SD) Weight: 16 kg (–2.6 SD)	OFC: 55.5 cm (0 SD) Height: 131 cm (+1.4 SD) Weight: 23.2 kg (–0.4 SD)	NA	OFC: 48 cm (–2 SD) Height: 99 cm (+1.4 SD) Weight: 17.2 kg (–2.4 SD)	OFC: 57.2 cm (+0.13 SD) Height: 182 cm (+0.71 SD) Weight: 92 kg (+2.6 SD)
Global developmental delay	Yes	Yes	Yes	Yes, mild	No
Intellectual disability	Moderate	Severe	No	No	No
Seizures (age of onset)	Yes (4 months)	Yes (1 year and 2 months)	No	Febrile seizure (3 years)	No
Nystagmus					
Age at onset	Since birth	Since birth	6 weeks	2 weeks	No nystagmus
Age resolved	4 years	Still present	7 months	12–18 months	No nystagmus
Neurological findings					
Power	5/5	3/5	NA	Normal	Normal
Tone	Hypotonia in infancy progressed to hypertonia in lower limbs	Hypotonia	Hypotonia	Normal	Normal
Deep tendon reflexes	Normal	Normal after initial hyporeflexia	NA	Normal	Normal
Ataxia	Yes	Not ambulatory	NA	Yes	No
Other abnormal movements	No	Generalized dystonia, now resolved	Jitteriness	No	No
Investigations					
EEG findings	Normal EEG at 5 months Generalized epileptiform discharges noted in repeat EEG at 5 and 6 years	Normal EEG findings	NA	NA	NA
Fundus evaluation	Temporal pallor noted in both eyes	Foveal hypoplasia	NA	Normal	NA
MRI findings	Diffuse and significant hypomyelination, dysmorphic corpus callosum, slight progression of myelination at follow-up	Significant hypomyelination, thinning of the corpus callosum, myelin progression noted in serial MRIs	Diffuse hypomyelination, some myelination noted within bilateral corona radiata	Significant hypomyelination	Subtle white matter signal abnormalities in cerebral and cerebellar white matter

NA = not available; OFC = occipitofrontal circumference; S = Subject.

for S4 but 7 years for S1. The gait remained ataxic for both these subjects. S2, on the other hand, failed to achieve any clinical improvement and remained non-ambulatory at her last exam at 7 years of age. Nystagmus persisted in S2 while S3 showed predominantly uniocular nystagmus (right eye) at 7 months. S1 and S2 also manifested seizures but achieved medical seizure freedom by the age of 2 years. The MRI pattern was suggestive of significant hypomyelination in S1, S2, S3 and S4 since early infancy (Fig. 2). Concurrent with the improvement in neurological signs, MRI of two individuals (S1 and S2) showed minimal improvements in myelination. Given the young age of S4, follow-up at a later age will be needed to check on the expected improvement of myelination. MRI of S5 at age 29 years showed a focus of T_2 hyperintensity in the parietal deep white matter and mildly elevated T_2 signal of middle cerebellar peduncles and the peridentate cerebellar white matter (Fig. 2). Extended case reports are provided in the Supplementary material.

Molecular testing and *in silico* analysis

Genomic testing detected a heterozygous *de novo* missense variant in *TMEM163* (NM_030923.5) in S1 from Family 1. Family 2 was ascertained when the candidate gene was posted on GeneMatcher,[6] while Family 3 and Family 4 were ascertained by personal

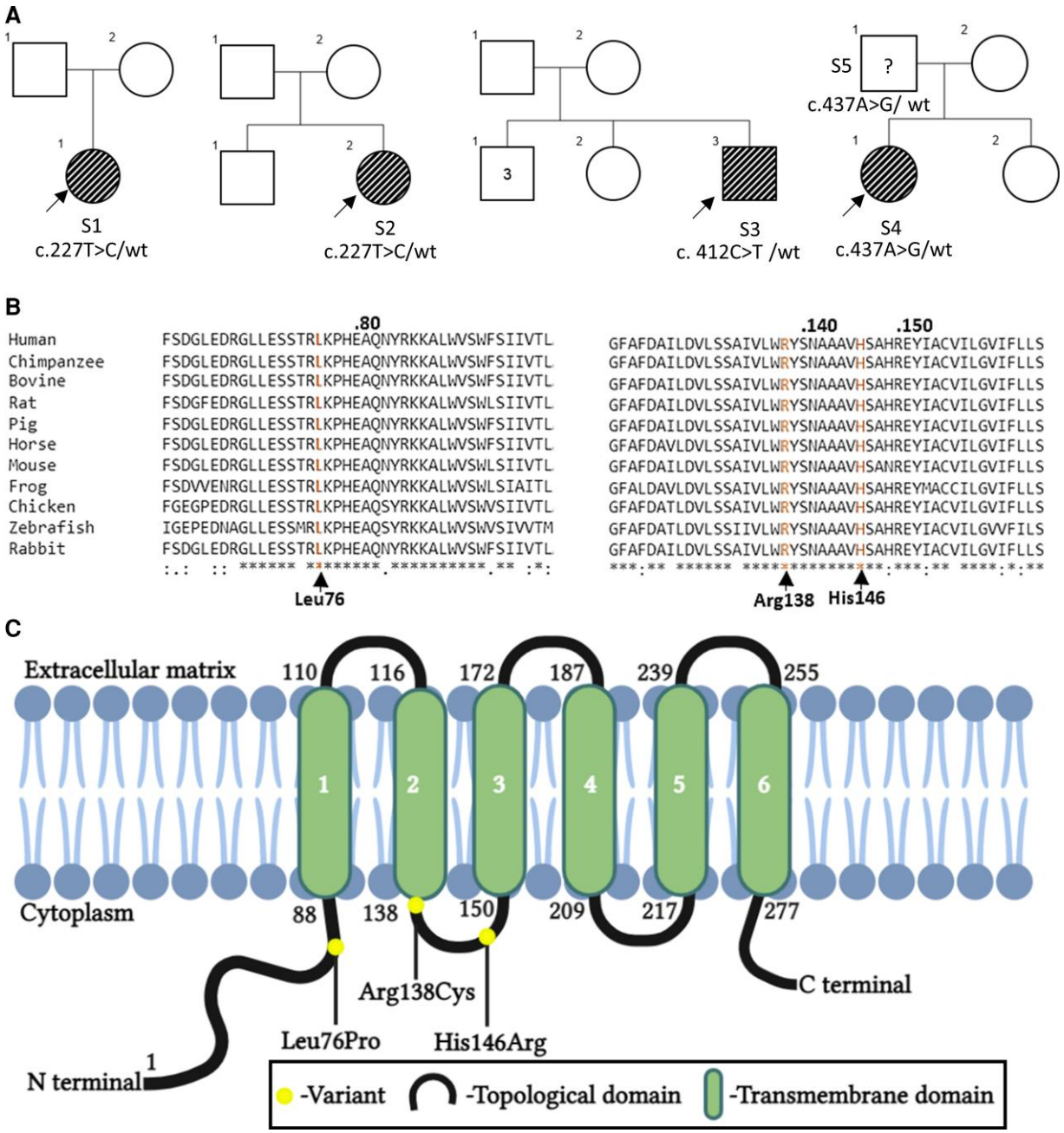

Figure 1 Pedigree, variant conservation and protein structure. (**A**) Pedigree of all four families. (**B**) Clustal alignment of TMEM163 protein from 11 vertebrate species. The residues altered in the presented individuals are marked in red. (**C**) Location of the three identified variants shown on a schematic representation of TMEM163 protein.

communication. S1 and S2 shared the same *de novo* missense variant, c.227T>C (p.Leu76Pro) in exon 2 and S3 from Family 3 had the *de novo* variant c.412C>T (p.Arg138Cys) in exon 4 of *TMEM163*. In Family 4, the variant, c.437A>G (p.His146Arg) in exon 4 in S4 was inherited from the father, S5. These variants are absent in the gnomAD database and are predicted to be damaging by multiple prediction tools (Supplementary Table 1). The three mutated residues are predicted to alter amino acid residues that are highly conserved across species as indicated by Clustal Omega multiple sequence alignment (Fig. 1B).

TMEM163 is a 31.5-kDa protein[7] with six transmembrane domains and seven topological domains (UniProt). All three variants are located within the topological–cytoplasmic domain of TMEM163. The Leu76Pro variant is located in the first cytoplasmic domain closer to the N terminal while the Arg138Cys and His146Arg are located in the second cytoplasmic loop between transmembrane domains two and three (Fig. 1C). *In silico* analysis and HOPE predicted that all three variants resulted in altered interactions with neighbouring residues (Supplementary Fig. 1).

In vitro studies

To study the cellular impact of the variants we identified in our subjects, we transfected WT and mutant TMEM163-mCherry plasmids into HEK293 cells. Transfection efficiencies were similar across the different constructs (Supplementary Fig. 2) and consistent with

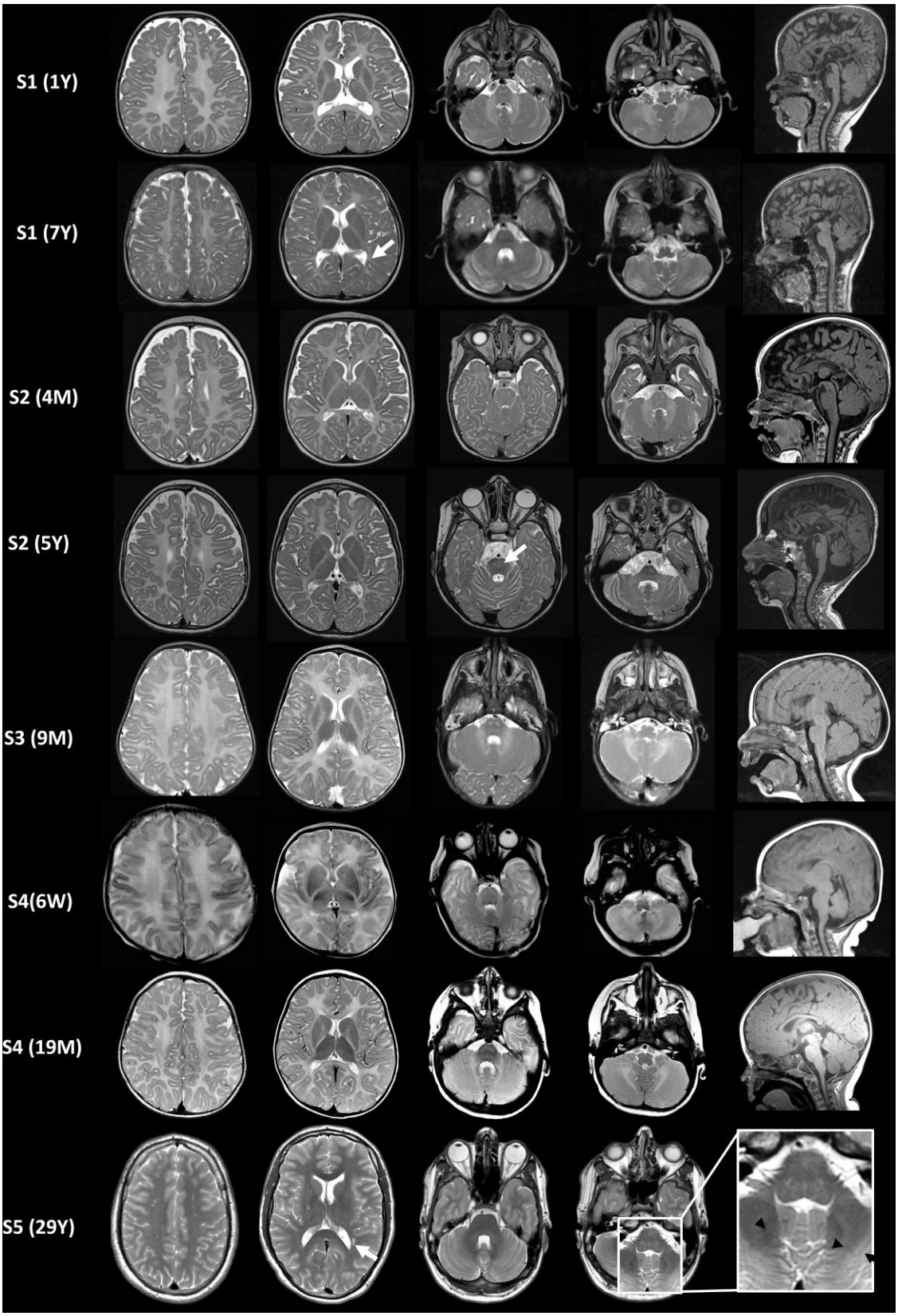

Figure 2 MRIs findings at different time intervals. Axial T_2-weighted and sagittal T_1-weighted images of S1–S5. S1–S4 show diffuse hyperintense signal in the supra- and infratentorial white matter suggesting hypomyelination. At the age of 7 years, S1 showed minimal progression of myelination in the middle cerebellar peduncles, the cerebellar white matter and the optic radiation (white arrow) and the corpus callosum was thin. MRI of S2 at age 5 years demonstrated improved myelination in the thalamus, cerebral peduncles, pons and medulla. MRI of S5 at age 29 years showed only a non-specific T_2-hyperintense signal abnormality in the parietal periventricular white matter (thick white arrow) and subtly elevated T_2 signal of the peridentate white matter including the hilus of the dentate nucleus (black arrows). Y = years; M = months; W = weeks.

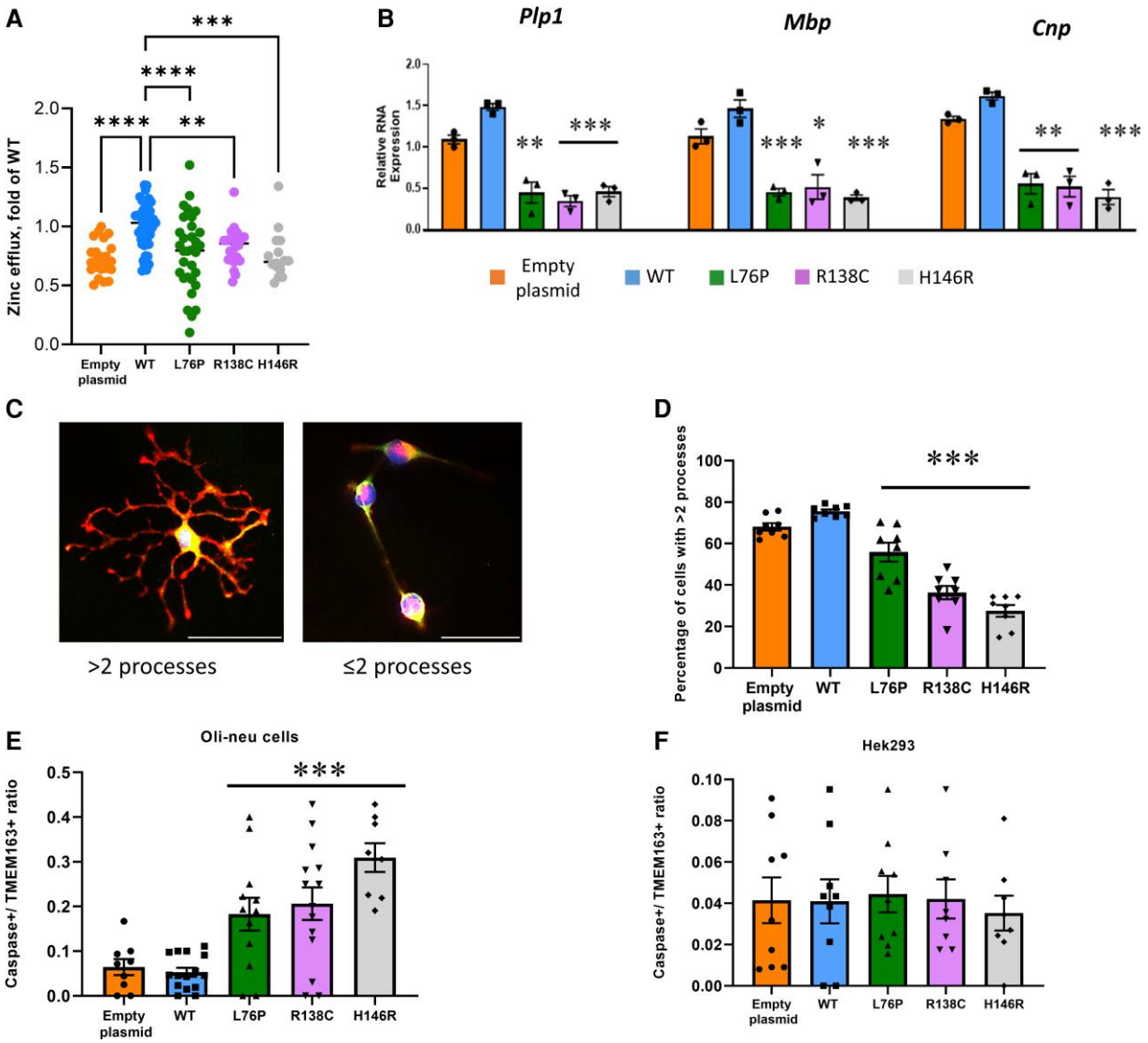

Figure 3 TMEM163 mutants exhibit functional defects in Zn efflux and oligodendrocyte function and survival. (**A**) Summary of the zinc efflux data obtained using HEK-293 cells electroporated with wild-type (WT) and mutant TMEM163-mCherry constructs. Each point is a biological replicate from 3–4 independent trials. During each trial zinc efflux values were normalized to average values of flux reported in WT samples in each set. Zn efflux is significantly reduced in HEK293 cells transfected with mutant constructs when compared to WT. $^{***}P < 0.001$, $^{****}P < 0.0001$ using ANOVA with Dunnett's test corrected for multiple testing. (**B**) Expression of myelin genes *Plp1*, *Mbp* and *Cnp* in oli-neu cells electroporated with WT and mutant TMEM163-mCherry constructs. Expression of individual genes was analysed by real-time PCR and normalized to *Actb* as an internal control and plotted as a ratio of WT expression. Expression was significantly reduced for all three genes in cells with the mutant plasmids when compared to WT. Error bars are standard error of the mean, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ analysed by Student's *t*-test. (**C**) Overlay epifluorescence images of the oli-neu cells transfected with TMEM163-mCherry (red channel) constructs and stained with β-tubulin (green channel). β-Tubulin marks the cytoplasmic processes in oli-neu cells (scale = 50 μM). Left panel is from the WT construct while right panel is from the L76P mutant construct. (**D**) Percentage of the cells with more than two processes. Error bars are standard error of the mean from more than 200 cells counted for each replicate, $^{***}P < 0.001$, calculated by Student's *t*-test compared to control. (**E**) Fraction of apoptotic oli-neu cells measured as the mean ratio of caspase positive cells to number of transfected cells from >200 cells. $^{***}P < 0.001$, calculated by Student's *t*-test compared to WT. (**F**) Similar experiment in HEK293 cells shows no significant difference in fraction of apoptotic cells between WT and mutants.

previous reports, the WT TMEM163-mCherry exhibited both cytoplasmic and plasma membrane localization.[5] However, we did not observe any difference in localization between the WT and mutant constructs (Supplementary Fig. 3).

To measure zinc efflux, we incubated the transfected HEK293 cells with the cell-impermeable fluorescent dye, MI-Fluozin-3. This indicator is commonly used to evaluate alterations in relative zinc levels and only exhibits fluorescence when bound to zinc.[8] Transient expression of WT TMEM163-mCherry resulted in increased fluorescence compared to the empty plasmid (Fig. 3A), confirming previous results that it plays a role in transporting zinc out of the cell.[5] However, all three mutant plasmids tested demonstrated reduced Fluozin-3 fluorescence levels indicating reduced zinc efflux from the cell (Fig. 3A).

Although *TMEM163* is expressed in multiple tissues,[9] the phenotype observed in the current study is that of hypomyelination, indicating that oligodendrocytes (OLs), the glial cell type in the CNS that produces myelin, may be primarily impacted. We therefore sought to study the impact of these variants in an oligodendroglial-derived cell line, mouse oli-neu cells.[10] These cells can be easily transfected

and possess many characteristics of OL lineage cells and are considered to be OLs in early stages of maturity.[11] They can be induced to differentiate into cell types that express myelination-related genes at the RNA level and also exhibit branching patterns similar to mature oligodendrocytes.[10,11] Oli-neu cells electroporated with mutant TMEM163-mCherry plasmids exhibited significantly reduced mRNA expression of the major myelin genes *Mbp*, *Plp1* and *Cnp* when compared to cells transfected with the WT plasmid, suggesting that the variants impaired normal functioning in these cells (Fig. 3B). Endogenous *Tmem163* mRNA expression in oli-neu cells was not altered in either WT or mutant TMEM163-mCherry constructs when compared to the empty plasmid (Supplementary Fig. 4).

Oli-neu cells transfected with the WT construct exhibited complex branching patterns upon differentiation while cells transfected with the mutant plasmids demonstrated a significantly reduced number of branches (Fig. 3C and D). Further, higher rates of cell death, as measured by activated caspase-3 staining, in oli-neu cells transfected with the mutant plasmids were observed (Fig. 3E). Interestingly, no difference in cell death was observed in HEK293 cells transfected with either WT or mutant plasmids (Fig. 3E).

Discussion

TMEM163, previously referred to as synaptic vesicle 31,[12] was found to be localized in the plasma membrane and lysosomes and is expressed in many tissues, although to a higher extent in the brain (cortex and cerebellum), lung and testis.[13] Within the glial cell population, the highest expression of *TMEM163* is seen in foetal astrocytes and newly myelinating OLs (Brain RNA-seq database,[14,15] Supplementary Fig. 4). The rodent orthologue, *Tmem163*, was first discovered through proteomics in rat brain synaptosomes, where it was observed to bind to zinc in specific neuronal populations.[12] More recently, TMEM163 was demonstrated to serve as a zinc efflux transporter, transporting zinc out of living cells.[5,16]

All three pathogenic variants we have identified were located in the cytoplasmic domains of TMEM163 and impaired the zinc transport activity of the protein but did not alter protein localization (Figs 1C and 3A and Supplementary Fig. 3). Previously, a missense variant introduced at a highly conserved residue in the first cytoplasmic loop (Ser61Arg) also resulted in reduced zinc efflux without altered subcellular localization, comparable to what we have observed for the Leu76Pro variant.[5] The other conserved TMEM163 residues mutated in the previous study were in different transmembrane domains of the protein and reduced or completely abolished efflux activity. Only the variant in the fourth transmembrane domain (Ser193Pro) abolished localization to the plasma membrane. Similar to our results, none of the mutant constructs in this previous report resulted in reduced cell viability when transfected into HEK293 cells.

Oli-neu cells electroporated with mutant TMEM163-mCherry plasmids and induced to differentiate exhibited significantly reduced expression of the major myelin genes, reduced branching and higher rates of cell death (Fig. 3B–E). No difference in cell death was observed in HEK293 cells (Fig. 3E), suggesting that OL lineage cells are uniquely susceptible to putative pathogenic variants in *TMEM163*. These results suggest that the *TMEM163* variants may be detrimental to normal OL function and survival.

Maintenance of zinc homeostasis is of critical importance in the brain because uncontrolled release of zinc is reported to cause death of neurons and glial cells by apoptosis or necrosis.[17–20] During neuronal communication, zinc is usually released in the synapse along with glutamate and is present in the brain in sizeable amounts.[21] In OLs, release of zinc from intracellular stores in response to oxidative, nitrative, ischaemic and excitotoxic stimuli is a key step leading to cell death.[22] While a role for zinc in white matter injury has been described, much less in known about its role in normal myelination and OL development.

Transporters such as TMEM163 play an essential role in controlling the levels of intracellular zinc, while other zinc-binding proteins maintain the cytosolic zinc levels.[23] The reduced activity of mutant TMEM163 may impair the ability of oligodendrocytes to efflux zinc, rendering them more susceptible to zinc-mediated cell death. The variants we have identified may also act in a dominant negative manner because TMEM163 was demonstrated to function as a dimer.[7] TMEM163 is known to interact with other proteins such as the TRPML1 ion channel to regulate intracellular zinc.[13] Variants in *TRPML1* encoding Mucolipin cause mucolipidosis IV, a lysosomal storage disorder where hypomyelination is part of the symptom complex.[24] The variants we have identified may result in a dysfunctional dimeric version of TMEM163 and/or compromise TRPML1 activity to cause zinc dyshomeostasis leading to oligodendrocyte dysfunction and a hypomyelination phenotype.

Two recent reports have described the characterization of independently generated *Tmem163* knockout mice. One report found that these mice exhibited impaired biogenesis of platelet-dense granules[25] while the other revealed that *Tmem163* regulates the function of the neuronal ATP-gated ionotropic P2X receptors and pain-related ATP-evoked behaviour.[26] In both reports the myelination status in these mice was not analysed and it is therefore unclear how complete absence of TMEM163 impacts myelination. Genome-wide association studies have implicated common variants in this gene with susceptibility to diabetes mellitus and Parkinson's disease; however these findings were not always replicated in different populations.[16] Of note, we did not identify any clinical or biochemical findings of diabetes mellitus in our subjects.

The clinical improvement observed in S1, S3 and S4 and subtle radiological improvement seen in S1 and S2 suggests possibility of a non-progressive HLD in our cohort. S5 was ascertained only in adulthood and has minimal white matter abnormalities, which possibly represent the resolution of hypomyelination, paralleled by a normal neurodevelopmental outcome, although variable expressivity, non-penetrance and tissue-level mosaicism cannot be ruled out. Of all the previously described HLDs, two subtypes—i.e. HLD16 (*TMEM106B*, MIM# 617964) and HLD19 (*TMEM63A*, MIM# 618688), which also manifest with PMD-like findings initially—are known to have a favourable clinical outcome. Individuals with HLD16 acquire motor milestones and achieve ambulation.[27] However, MRI findings on serial scans documented even until the fourth decade of life did not show significant improvement in myelination. On the other hand, transient hypomyelination has been observed in HLD19.[28] Interestingly, a recent publication described a patient with a novel *TMEM63A* variant and persistent hypomyelination in combination with severe developmental delay, indicating that disease evolution in HLD19 is more heterogeneous than assumed. This is comparable to our cohort and suggests wide clinical variability even with variants in the same gene.[29] Overall, the clinical findings and natural history of individuals in the current study overlaps with both these HLDs. The non-progressive or transient nature of these disorders could be due to compensation of the defective protein by other proteins with similar functions. However, it is yet to be determined whether other zinc transporters can compensate for TMEM163 function. A limitation of this study is that the functional analysis was carried out *in vitro*. An *in vivo* validation in an animal model is thus essential to elucidate precise pathological

mechanisms. In addition, we are unable to accurately predict genotype–phenotype correlations and additional families will be required for such an analysis.

Our findings indicate that heterozygous variants in *TMEM163* result in a novel subtype of hypomyelinating leukodystrophy with a subset of affected individuals manifesting improvement in clinical and radiological findings. Long-term follow-up of these individuals will aid in understanding the comprehensive natural history of this disorder. While this manuscript was under revision, another report identified pathogenic variants in *TMEM163* in two individuals manifesting a similar HLD phenotype, further strengthening our findings.[30] These results also reveal a novel role for zinc homeostasis in oligodendrocyte development and myelin formation.

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Competing interests

Baylor College of Medicine receives revenue from clinical genetic testing completed at Baylor Genetics Laboratories. The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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