Biallelic Mutations in *TMTC3*, Encoding a Transmembrane and TPR-Containing Protein, Lead to Cobblestone Lissencephaly

Julie Jerber,[1,2] Maha S. Zaki,[3] Jumana Y. Al-Aama,[4,5] Rasim Ozgur Rosti,[1,2] Tawfeg Ben-Omran,[6,7] Esra Dikoglu,[1,2] Jennifer L. Silhavy,[1,2] Caner Caglar,[1] Damir Musaev,[1,2] Beate Albrecht,[8] Kevin P. Campbell,[9] Tobias Willer,[9] Mariam Almuriekhi,[6,7] Ahmet Okay Çağlayan,[10] Jiri Vajsar,[11] Kaya Bilgüvar,[12] Gonul Ogur,[13] Rami Abou Jamra,[14,15] Murat Günel,[12] and Joseph G. Gleeson[1,2,*]

Cobblestone lissencephaly (COB) is a severe brain malformation in which overmigration of neurons and glial cells into the arachnoid space results in the formation of cortical dysplasia. COB occurs in a wide range of genetic disorders known as dystroglycanopathies, which are congenital muscular dystrophies associated with brain and eye anomalies and range from Walker-Warburg syndrome to Fukuyama congenital muscular dystrophy. Each of these conditions has been associated with alpha-dystroglycan defects or with mutations in genes encoding basement membrane components, which are known to interact with alpha-dystroglycan. Our screening of a cohort of 25 families with recessive forms of COB identified six families affected by biallelic mutations in *TMTC3* (encoding transmembrane and tetratricopeptide repeat containing 3), a gene without obvious functional connections to alpha-dystroglycan. Most affected individuals showed brainstem and cerebellum hypoplasia, as well as ventriculomegaly. However, the minority of the affected individuals had eye defects or elevated muscle creatine phosphokinase, separating the *TMTC3* COB phenotype from typical congenital muscular dystrophies. Our data suggest that loss of *TMTC3* causes COB with minimal eye or muscle involvement.

Cobblestone lissencephaly (COB), also known as type II lissencephaly, is a severe brain malformation characterized by cortical dysplasia, irregular borders between white and gray matter, dysmyelination, cystic cerebellar dysplasia, and brainstem hypoplasia.[1] These cortical malformations are due to overmigration of neurons and glial cells beyond the external basement membrane (BM, also known as the glial limitans in the brain) into the subarachnoid space, resulting in the COB appearance.[2–4] The cause of this overmigration defect is an impaired interaction between the glial limitans and the extracellular matrix (ECM) of the BM.[5,6] The dystroglycan-glycoprotein complex (DGC) is a multi-protein complex localized to the glial limitans. In this complex, glycosylated alpha-dystroglycan serves as a binding receptor for ECM proteins of the BM, including laminin, perlecan, or agrin, providing a physical link between the cytoskeleton of the glial cells and the BM.[7] Alpha-dystroglycan is a central component of the complex, and loss of its glycosylation reduces DGC binding to the ECM, thus contributing to functional defects during development.[8]

Type II lissencephaly is associated with disorders ranging from COB without other birth defects to a broader spectrum of conditions referred to as muscular congenital alpha-dystroglycanopathy with brain and eye anomalies (MIM: 253800), including Walker-Warburg syndrome (WWS [MIM: 236670]), muscle-eye-brain disease (MIM: 253280), and Fukuyama congenital muscular dystrophy (MIM: 253800). Symptoms are present at birth and can vary within the spectrum. Affected individuals usually have severe structural brain findings (including lissencephaly and ventriculomegaly), various developmental abnormalities of the eyes (e.g., unilateral or bilateral microphthalmia and retinal dysplasia), hypotonia, progressive muscle weakness, and degeneration. Affected children also display varying degrees of delayed milestones, including delayed speech and motor functions as well as seizures.

All told, COB has been associated with mutations in 16 genes. 12 of them—protein O-mannosyltransferase 1 (*POMT1* [MIM: 607423]), protein O-mannosyltransferase 2 (*POMT2* [MIM: 607439]), protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1 (*POMGNT1*

[1]Laboratory for Pediatric Brain Disease, The Rockefeller University, New York, NY 10065, USA; [2]Howard Hughes Medical Institute, Rady Children's Institute for Genomic Medicine, University of California, San Diego, San Diego, CA 92093, USA; [3]Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Centre, Cairo 12311, Egypt; [4]Princess Al-Jawhara Al-Brahim Center of Excellence in Research of Hereditary Disorders, King Abdulaziz University, Jeddah 21453, Saudi Arabia; [5]Department of Genetic Medicine, Faculty of Medicine, King Abdulaziz University, Jeddah 21453, Saudi Arabia; [6]Clinical and Metabolic Genetics Section, Department of Pediatrics, Hamad Medical Corporation, PO Box 3050, Doha, Qatar; [7]Weill Cornell Medical College, Qatar, Education City, PO Box 24144, Doha, Qatar; [8]Institut für Humangenetik, Universitätsklinikum Essen, Universität Duisburg-Essen, 45122 Essen, Germany; [9]Howard Hughes Medical Institute, Departments of Neurology, Internal Medicine, and Molecular Physiology and Biophysics, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242-1101, USA; [10]Department of Medical Genetics, School of Medicine, Istanbul Bilim University, Istanbul 34394, Turkey; [11]Division of Neurology, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada; [12]Yale Program on Neurogenetics, Departments of Neurosurgery, Neurobiology, and Genetics, School of Medicine, Yale University, New Haven, CT 06510, USA; [13]Department of Genetics, School of Medicine, Ondokuz Mayis University, 55000 Samsun, Turkey; [14]Institute of Human Genetics, University of Leipzig Hospitals and Clinics, Philipp-Rosenthal-Str. 55, 04103 Leipzig, Germany; [15]Institute of Human Genetics, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91054 Erlangen, Germany
*Correspondence: jogleeson@mail.rockefeller.edu
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[MIM: 606822]), fukutin (*FKTN* [MIM: 607440]), fukutin related protein (*FKRP* [MIM: 606596]), acetyl glucosaminyl transferase like protein (*LARGE* [MIM: 603590]), isoprenoid synthase domain-containing protein (*ISPD* [MIM: 614631]), protein O-linked mannose N-acetylglucosaminyltransferase 2 (*GTDC2* [MIM: 614828]), transmembrane protein 5 (*TMEM5* [MIM: 605862]), protein O-mannose kinase (*POMK* [MIM: 615247]), beta-1,3-N-acetylglucosaminyltransferase 3 (*B4GAT1* [MIM: 605517]), and beta-1,3-N-acetylgalactosaminyltransferase 2 (*B3GALNT2* [MIM: 610194])—are required for the maturation of alpha-dystroglycan into a functional receptor[9–16] and are therefore identified as causing secondary dystroglycanopathies,[17] the most common form of congenital muscular dystrophies. Mutations in *DAG1* (MIM: 128239), the gene encoding alpha-dystroglycan, are rare and have been described in only a single subject with WWS.[18] Additionally, genes encoding BM constituents, including laminin subunit beta 1 (*LAMB1* [MIM: 150240]), laminin subunit gamma 3 (*LAMC3* [MIM: 614115]), and collagen type IV alpha 1 chain (*COL4A1* [MIM 120130]),[19–21] are mutated in COB. Despite intensive research, one-third of COB cases remain unexplained genetically.[4]

In this report, we show that biallelic mutations in *TMTC3* (transmembrane and tetratricopeptide repeat containing 3 [GenBank: NM_181783.3]) result in COB. Individuals harboring mutations in this gene exhibited intellectual disability, hypotonia, and delayed milestones. Most also presented with seizures and severe brain malformations including ventriculomegaly and brainstem and cerebellar hypoplasia, but ocular abnormalities or elevated creatine phosphokinase (CPK) were not a uniform feature.

In a collaborative effort to identify additional mechanisms explaining the genetics of COB, we recruited 25 families with suspected recessive disease. This study was performed in accordance with the ethical standards set by our institutional review boards, and informed consent was obtained from each individual involved in the study. In-solution exome capture was performed with the SureSelect Human All Exome 50 Mb Kit (Agilent Technologies) with 125-bp paired-end read sequences generated on a HiSeq 2000 or 2500 (Illumina) and then analyzed by standard methods.

Approximately 32% of the evaluated families were found to harbor biallelic mutations in genes already associated with COB, including *POMGNT1* (n = 5), *POMT2* (n = 1), and *LAMB1* (n = 2) (Figure 1A). Of the remaining screened individuals, there were predicted rare deleterious mutations in genes previously not associated with COB in ten families (40%). Seven (28%) unrelated individuals remained without identified mutations. Six of the ten families harbored biallelic mutations in *TMTC3* (24%), which had a total of eight distinct mutations in nine affected individuals from six families (four homozygous and two compound heterozygous) (Figures 1B and 1C and Table S1).

The affected individual in family I carried a frameshift variant in exon 11 (c.1462delA [p.Arg488Glufs*6]) and a stop variant in exon 14 (c.2617C>T [p.Gln873*]), whereas the affected individual in family VI carried a missense variant in exon 8 (c.1151G>A [p.Gly384Glu]) and a frameshift variant in exon 14 (c.2521dupA [p.Ile841Asnfs*4]). Frameshift variants were found in exon 14 (c.1959_1960insTT [p.Arg654Leufs*6]) and exon 12 (c.1686_1701del [p.Phe562Leufs*8]) of families II and III, respectively. Affected individuals in family IV carried a missense variant in exon 3 (c.199C>G [p.His67Asp]), and affected individuals in family V had a variant affecting the initiation methionine (c.3G>A [p.Met1?]). The missense variants disrupt amino acids that are highly conserved across evolution (Figure 1D). The variants were unique in our dataset of >5,000 exomes from individuals with neurodevelopmental phenotypes; were not represented in dbSNP138, the Greater Middle Eastern Variome, 1000 Genomes, or the Exome Aggregation Consortium (ExAC) Browser; were confirmed by Sanger sequencing; and fully segregated with disease according to a recessive mode of inheritance for the four families with available samples (Figure S1). All known COB-associated genes were well covered, and no deleterious variants or deletions were found in any of these genes.

All nine affected children from the six families were born to consanguineous Arabic families except for family V (from Turkey) and family VI (from North America) (Figures 2 and S1 and Table 1). The study included five females and four males with ages ranging from 9 months to 15 years. All children were born full term without complications during pregnancy and delivery, except the child from family VI had occipital encephalocele, not infrequently seen in COB. Measurements at birth, including birth weight and height, were in the normal range for those reported. Head circumference at birth was available for three of nine subjects and ranged from −1 to +1 SD from the mean.

All affected individuals presented with moderate to severe psychomotor delay. They were able to achieve sitting but much later than unaffected peers. Most were able to ambulate between the ages of 4 and 5 years. All individuals had truncal hypotonia with variable appendicular spasticity and were able to achieve head control and visual tracking but lacked language skills typical for their age. Individuals from families III, IV, and VI presented with no verbal speech. None of the individuals could be placed at the severe end of the dystroglycanopathy spectrum, and unlike WWS individuals, they continued to achieve developmental milestones.

Follow-up measurements of head circumference showed microcephaly in children from families I and III. There were no individuals with macrocephaly or craniofacial dysmorphisms, and none showed progressive loss of milestones, which can be seen in severe forms of dystroglycanopathies.

The majority of individuals (six of nine) had seizures: four had generalized tonic-clonic seizures, whereas III-IV-2 had myoclonic seizures and VI-IV-1 had infantile

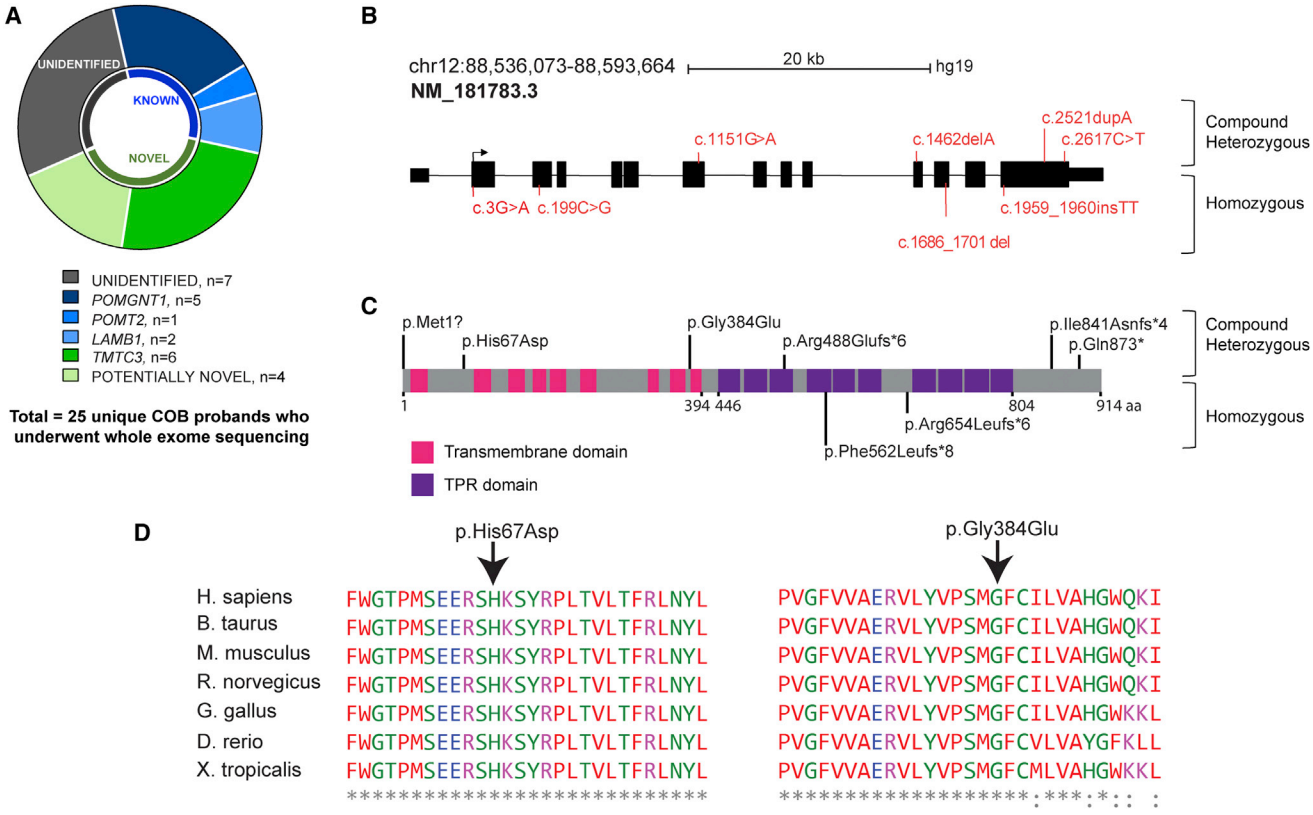

Figure 1. *TMTC3* Is Mutated in COB Brain Malformation
(A) Whole-exome sequencing results for 25 unique families. *POMT2* and *POMGNT1* mutations were found in one and five and families, respectively, and *LAMB1* mutations were found in two families. Six unique probands displayed mutations in *TMTC3* (dark green). In one-third of the affected individuals, no causative variant could be identified.
(B) *TMTC3*, located on chromosome 12, contains 14 exons (blocks), 13 of which are coding. Variants found in individuals are indicated in red (compound heterozygous above and homozygous below the gene). Scale bar represents 20 kb.
(C) TMTC3 (UniProt: Q6ZXV5) has 914 amino acids, 9 transmembrane domains (pink), and 10 TPRs (purple). Variants are indicated in black (compound heterozygous above and homozygous below the protein).
(D) Clustal alignment with multiple species shows conservation of the altered amino acids for the two missense variants. Asterisks indicate amino acids conserved in all noted species, and dots represent amino acid variants in at least one species.

spasms with secondarily generalized epilepsy. The onset of seizures was between 4 and 8 months, and they were intractable with medication. The frequency of epilepsy in dystroglycanopathies is 30%–62%, which is in accordance with our findings.[22,23]

Truncal hypotonia with hyperreflexia and mild to severe appendicular spasticity but without muscular atrophy was present in all children. No retinal dysplasia, optic nerve hypoplasia, megalocornea, or buphthalmos was observed. One individual (V-IV-1) had microphthalmia and a cataract, and another (II-V-1) had a unilateral cataract. This is strikingly low in comparison to the reported high incidence of ocular abnormalities in the COB spectrum: 94% microphthalmia, 43% retinal dysplasia, 95% optic nerve hypoplasia, 50% glaucoma, and 58% pupil abnormalities.[24]

MRI of eight individuals showed features typical of COB (Figure 2), whereas individual IV-2 from family IV had subcortical and periventricular hypomyelination. Some individuals reported within the COB spectrum have shown infantile hypomyelination that later evolved to patchy dysmyelination, as well as overmigration of neuronal cells in the leptomeningeal space.[25] Keeping in mind that this affected individual was 10 months old at the time, this finding is suggestive of COB spectrum. Ventriculomegaly and hypoplastic corpus callosum were encountered in seven and five individuals (partially in VI-IV-1), respectively. Pontocerebellar and cerebellar hypoplasia were both seen in six of nine individuals. Two children, I-V-1 and VI-IV-1, had occipital encephalocele, and one child, III-IV-2, had a retrocerebellar cyst. Overall imaging findings were compatible with those of previous COB cases in the literature.[26] CPK, a muscle-damage marker that is usually elevated in muscular dystrophies, was elevated only in children V-IV-2 and VI-IV-1 (Table 1).

TMTC3 encodes a 914 aa protein composed of nine transmembrane domains and ten tetratricopeptide repeats (TPRs), a pattern conserved across evolution, at least to the fly. This protein was first identified in the context of renal transplant surgeries, where it was found to be upregulated in the blood of operationally tolerant subjects.[27] TMTC3 localizes to the endoplasmic reticulum (ER) in cultured

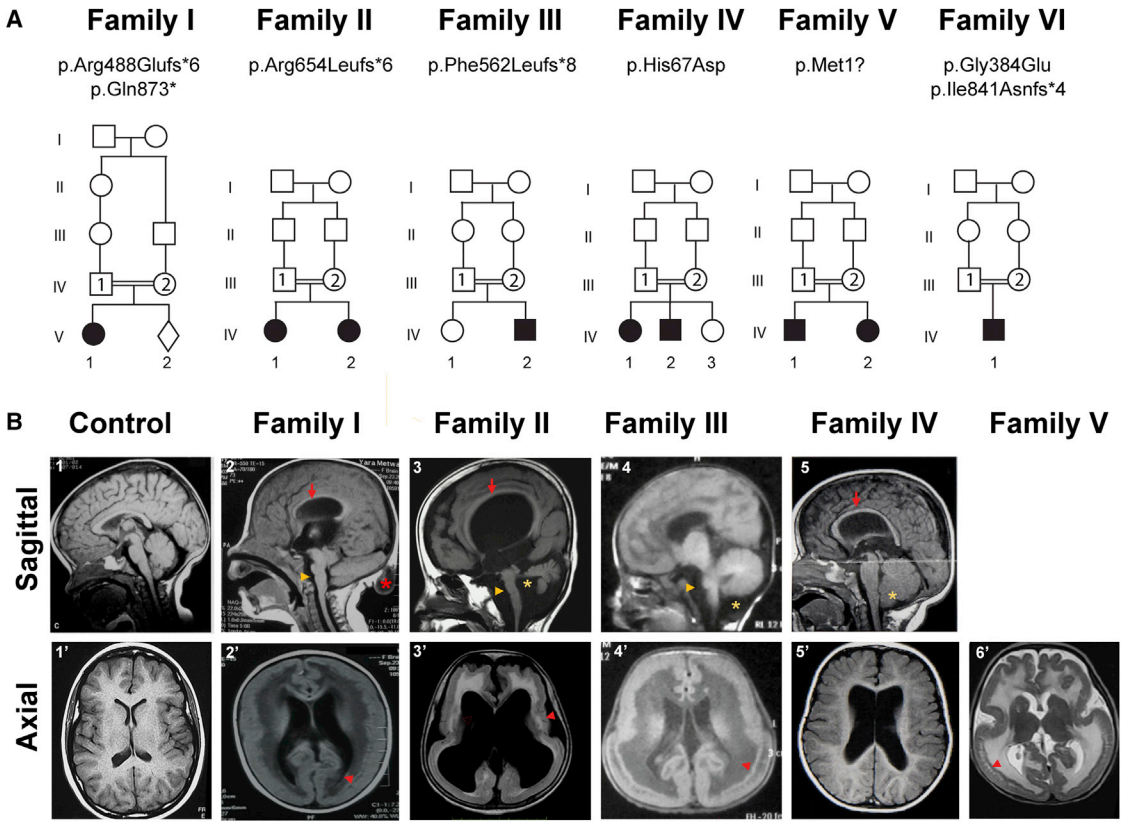

Figure 2. Family Pedigrees and Brain MRI of Individuals with *TMTC3* Variants
(A) Pedigrees harboring biallelic damaged variants in *TMTC3*. Double bars indicate consanguinity, and filled symbols represent affected individuals.
(B) Sagittal MRI (upper panel) showing hypoplasia of the corpus callosum (red arrow in B2, B3, and B5), hypoplastic brainstem (yellow arrowhead in B2–B4), cerebellum (yellow star in B3), or dysplastic cerebellum (yellow star in B4 and B5). Note the occipital meningocele (red asterisk in B2). Axial MRI (lower panel) reveals COB with heterotopic neurons (red arrow in B2′, B3′, B5′, and B6′). Ventriculomegaly was also observed in most individuals.

human odontoblasts and acts as a binding partner of the protein disulfide isomerase family A member 3 (PDIA3 or ERp57) in a yeast two-hybrid screen.[28] PDIA3, in turn, is an ER protein involved in the folding of glycoproteins by disulfide-bond formation in association with the chaperone calnexin, and it is overexpressed in ER stress conditions.[29] An in vitro study showed that, consistent with its interaction with PDIA3, silencing *TMTC3* in HeLa cells sensitizes the cells to ER stress by modulating the proteasome activity and *XBP-1* transcript expression, a key player in the unfolded protein response.[28] In addition, knockout of *mSmile*, the murine homolog of *TMTC3*, results in early postnatal death and altered Tgf-β signaling in embryonic fibroblasts.[30] However, the brain phenotype of knockout mice was not evaluated.

TMTC3 is one of four human paralogs (TMTC1–TMTC4) known or predicted to be transmembrane proteins with TPRs.[28,31] TPRs, which are found in many proteins, mediate protein-protein interactions in various cellular processes, such as synaptic vesicle fusion, protein folding, and protein translocation.[32] TMTC1 and TMTC2 are ER membrane proteins that interact with the ER calcium uptake pump SERCA2B (both) and calnexin (TMTC2 only).[31] Although no clear function has been associated with any of these TMTC family members, their ER localization and association with known ER essential proteins suggest that they actively participate in ER function and homeostasis. Except for the transmembrane and TPR domains, TMTC3 does not have any recognized domains. Because the glycosylation of alpha-dystroglycan begins in the ER, it is tempting to speculate that TMTC3 regulates the glycosylation of alpha-dystroglycan, potentially explaining the functional defects observed during the development in the absence of a functional TMTC3 protein.

In addition to *TMTC3*, other genes encoding TPR-containing proteins have been associated with human diseases. In *AIPL1* (MIM: 604393), nonsense mutations affecting the TPR of the encoded protein, aryl-hydrocarbon-interacting protein-like 1, cause Leber congenital amaurosis (MIM: 20400),[33] and in neutrophil cytosolic factor 2 (*NCF2* [MIM: 608515]), a missense mutation affecting the encoded TPR is linked to chronic granulomatous disease (MIM: 233710).[34]

In summary, we implicate *TMTC3* in COB with moderate to severe intellectual disability and intractable,

Table 1. Clinical Features of Individuals with *TMTC3* Variants

	I-V-1	II-V-1	II-V-2	III-IV-2	IV-IV-1	IV-IV-2	V-IV-1	V-IV-2	VI-IV-1
cDNA mutations	c.1462delA, c.2617C>T	c.1959_1960insTT	c.1959_1960insTT	c.1686_1701del	c.199C>G	c.199C>G	c.3G>A	c.3G>A	c.1151G>A, c.2521dupA
Proteins variants	p.Arg488Glufs*6, p.Gln873*	p.Arg654Leufs*6	p.Arg654Leufs*6	p.Phe562Leufs*8	p.His67Asp	p.His67Asp	p.Met1?	p.Met1?	p.Gly384Glu, p.Ile841Asnfs*4
Gender	female	female	female	male	female	male	male	female	male
Country of origin	Egypt	Yemen	Yemen	Egypt	Lebanon	Lebanon	Turkey	Turkey	US
Consanguinity	+	+	+	+	+	+	+	+	–
Weight at birth (kg)	NA	NA	NA	2	3.55	3.63	3.45	2.6	2.7
Length at birth (cm)	NA	NA	NA	47	55	53	NA	NA	NA
HC at birth (SD)	NA	NA	NA	−1	−1	+1	NA	NA	NA
Psychomotor Development									
Gross motor skills[a]	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed
Fine motor skills[a]	delayed	delayed	delayed	absent	delayed	delayed	delayed	delayed	delayed
Language[a]	delayed	delayed	delayed	absent	absent	absent	delayed	delayed	absent
Social[a]	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed
Neurological Examination									
HC at last examination (cm)	41 (−7.5 SDs)	NA	NA	39.5 (−4 SDs)	46 (−1.34 SDs)	46.5 (−0.22 SD)	49.5 (−1 SD)	45 (−2 SDs)	53 (+1.5 SDs)
Age at diagnosis	NA	NA	NA	6 months	18 months	6 months	1 month	5 months	prenatal: encephalocele on ultrasound
Hypotonia	+	+	+	+	+	+	+	+	+
Intellectual disability	+	+	+	+	+	+	+	+	+
Autistic-like behavior	NA	+	+	–	NA	NA	–	–	NA
Seizures									
Onset	4 months	6 months	8 months	7 months	–	–	–	5 months	6 months
Type	GTC	GTC	GTC	myoclonic	–	–	–	GTC	infantile spasms
Frequency	weekly	weekly	under control	infrequent	–	–	–	weekly	daily

(*Continued on next page*)

Table 1. ***Continued***

	I-V-1	II-V-1	II-V-2	III-IV-2	IV-IV-1	IV-IV-2	V-IV-1	V-IV-2	VI-IV-1
Ophthalmologic Findings									
Cataract	–	+ (L)	NA	–	–	–	+ (R)	–	–
Micropthalmia	–	–	NA	–	–	–	+ (L)	–	–
Buphthalmos	–	–	NA	–	–	–	–	–	–
Megalocornea	–	–	NA	–	–	–	–	–	–
Optic atrophy	–	+	NA	–	–	–	–	–	–
Retinal dysplasia	–	–	NA	–	–	–	–	–	–
Other Systemic Findings									
Cardiovascular	NA	–	NA	–	–	–	NA	–	–
Respiratory	NA	–	NA	RD at birth	–	–	NA	–	–
Musculoskeletal	NA	–	NA	club feet	club feet	–	NA	contractures of elbow	G-tube feeding due to hypotonia
Genitourinary	NA	unilateral pubic adhesion	–	–	–	–	NA	–	–
Other	–	–	occipital lipoma	squint	–	–	facial asymmetry	hypertelorismus, strabismus, sacral hemangioma	squint
MRI									
Agyria	+	+	+	+	–	–	–	–	– (PMG)
Cobblestone lissencephaly	+	+	+	+	–	–	+	+	+
Ventriculomegaly	+	+	+	–	+	+	+	–	+
Encephalocele	+ (occipital)	–	–	–	–	–	–	–	+ (occipital)
Corpus callosum	hypoplasia	–	–	hypoplasia	hypoplasia	hypoplasia	–	–	partial hypoplasia
Brainstem	dysplasia, hypoplasia	hypoplasia	hypoplasia	hypoplasia	hypoplasia	hypoplasia	–	–	–
Cerebellum	dysplasia	hypoplasia	hypoplasia	hypoplasia	dysplasia	dysplasia	–	–	–
Retrocerebellar cysts	–	–	–	+	–	–	–	–	–

(*Continued on next page*)

Table 1. *Continued*

	I-V-1	II-V-1	II-V-2	III-IV-2	IV-IV-1	IV-IV-2	V-IV-1	V-IV-2	VI-IV-1
Investigations									
CPK level (units/liter)	normal	NA	NA	normal	normal	normal	normal	elevated (650)	elevated (1,025)
Other metabolic findings	NA	normal	increased GGT and ALP	normal	normal	normal	normal	normal	normal
VEP/ERG	NA	NA	NA	normal	NA	NA	NA	NA	normal
EMG	normal	NA	NA	normal	NA	NA	NA	NA	NA
Muscle biopsy	NA	NA	NA	NA	NA	NA	NA	NA	mild myopathic change, increased interstitial collagen, fiber-size variation

Abbreviations: ALP, alkaline phosphatase; CPK, creatine phosphokinase; EMG, electromyography; GGT, gamma glutamate transferase; GTC, generalized tonic clonic; HC, head circumference; L, left; NA, not available; PMG, polymicrogyria; R, right; RD, respiratory distress; and VEP/ERG, visual evoked potential and electroretinogram.
[a]Normal, delayed, or absent.

infantile-onset epilepsy without eye or muscle involvement. We previously reported mutations in *LAMB1* in families with individuals similarly presenting with a pure form of COB lacking eye and muscle defects.[20] Similarly, recessive mutations in *LAMC3* showed isolated brain involvement.[21] These shared phenotypes suggest common functions for *TMTC3* and the laminin genes.

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.09.007.

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Web Resources

1000 Genomes, http://www.1000genomes.org/
Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
ExAC Browser, http://exac.broadinstitute.org/
GME, http://igm.ucsd.edu/gme/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
OMIM, http://www.omim.org/
UniProt, http://www.uniprot.org/uniprot/

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