Bi-allelic LETM1 variants perturb mitochondrial ion homeostasis leading to a clinical spectrum with predominant nervous system involvement

Graphical abstract

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Kaiyrzhanov et al. describe 18 affected individuals with bi-allelic variants in the leucine zipper-EF-hand containing transmembrane protein 1 gene presenting with clinical features suggestive of a mitochondrial disease. Functional studies showed defective mitochondrial K⁺ efflux, swollen mitochondrial matrix structures, and loss of mitochondrial oxidative phosphorylation protein components.
Bi-allelic \textit{LETM1} variants perturb mitochondrial ion homeostasis leading to a clinical spectrum with predominant nervous system involvement

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Summary

Leucine zipper-EF-hand containing transmembrane protein 1 (\textit{LETM1}) encodes an inner mitochondrial membrane protein with an osmoregulatory function controlling mitochondrial volume and ion homeostasis. The putative association of \textit{LETM1} with a human disease was initially suggested in Wolf-Hirschhorn syndrome, a disorder that results from \textit{de novo} monoallelic deletion of chromosome 4p16.3, a region encompassing \textit{LETM1}. Utilizing exome sequencing and international gene-matching efforts, we have identified 18 affected individuals from 11 unrelated families harboring ultra-rare bi-allelic missense and loss-of-function \textit{LETM1} variants and clinical presentations highly suggestive of mitochondrial disease. These manifested as a spectrum of predominantly infantile-onset (14/18, 78%) and variably progressive neurological, metabolic, and dysmorphic symptoms, plus multiple organ dysfunction associated with neurodegeneration. The common features included respiratory chain complex deficiencies (100%), global developmental delay (94%), optic atrophy (83%), sensorineural hearing loss (78%), and cerebellar ataxia (78%) followed by epilepsy (67%), spasticity (53%), and myopathy (50%). Other features included bilateral cataracts (42%), cardiomyopathy (36%), and diabetes (27%). To better understand the pathogenic mechanism of the identified \textit{LETM1} variants, we performed biochemical and morphological studies on mitochondrial K$^{+}$/H$^{+}$ exchange activity, proteins, and shape in proband-derived fibroblasts and muscles and in \textit{Saccharomyces cerevisiae}, which is an important model organism for mitochondrial osmotic regulation. Our results demonstrate that bi-allelic \textit{LETM1} variants are associated with defective mitochondrial K$^{+}$ efflux, swollen mitochondrial matrix structures, and loss of important mitochondrial oxidative phosphorylation protein components, thus highlighting the implication of perturbed mitochondrial osmoregulation caused by \textit{LETM1} variants in neurological and mitochondrial pathologies.

Introduction

Leucine zipper-EF-hand containing transmembrane protein 1 (\textit{LETM1}) (MIM: 604407) is a ubiquitously expressed and phylogenetically highly conserved nuclear gene. \textit{LETM1}, also named SLC55A1, is part of the mitochondrial transporter protein SLC55 family that belongs to the SLC su-
membrane protein with an osmoregulatory function that controls cation homeostasis, preventing their equilibration with the H⁺ electrochemical gradient. While first identified to function as an electroneutral mitochondrial K⁺-H⁺ exchanger (KHE), LETM1 has also been connected to the regulation of the uptake or extrusion of Ca²⁺.

The pathological hallmark of LETM1 depletion is mitochondrial matrix swelling, fragmentation, and loss of cristae structure, consistently found in all studied organisms, whereas LETM1 overexpression causes mitochondrial elongation, cristae swelling, and matrix condensation due to imbalance in osmotic homeostasis. Silencing LETM1 homologs in yeast, Fusarium graminearum, and Toxoplasma gondii results in lethality or loss of virulence. Drosophila melanogaster with tissue-specific depleted LETM1 displays compromised tissue growth and locomotor behavior, as well as impaired evoked synaptic release of neurotransmitters. The homozygous deletion of LETM1 leads to developmental and embryonic lethality in flies, worms, and mice.

Consistent with the vital role of mitochondrial osmeregulation, matrix swelling and cation imbalance due to LETM1 inactivation have wide-reaching and pleiotropic effects on mitochondrial biogenesis and bioenergetics, perturbing glucose and pyruvate utilization, tryptophan and mtDNA metabolism, and outer mitochondrial membrane integrity and causing necrotic cell death.

The importance of LETM1 in neuronal function and pathology was initially suggested in Wolf-Hirschhorn syndrome (WHS [MIM: 194190]). This genetic syndrome results from de novo monoallelic deletion of several genes on the short arm of chromosome 4. Depending on the length of the deletion, WHS might present with a combination of congenital malformations, specific facial dysmorphism, growth and cognitive impairment, microcephaly, hypotonia, and epilepsy. LETM1 is localized in WHS critical region 2 (WHSCR2), less than 80 kb from WHS critical region 1 (WHSCR1), and is deleted in almost all individuals with the full WHS phenotype. LETM1 is proposed to be associated with epilepsy and neuromuscular features of WHS. Analysis of WHS fibroblasts linked LETM1 haploinsufficiency with mitochondrial defects. One study reports intracellular Ca²⁺, decreased Ca²⁺ sensitivity of the mitochondrial permeability transition pore (PTP), and increased superoxide and hyperpolarization of the inner membrane; another study reports mtDNA aggregation, pyruvate dehydrogenase (PDH) deficiency, and a preferential shift from pyruvate oxidation to ketone body utilization. How the cation transport properties of LETM1 and the broad effects of its dysfunction on other mitochondrial and cellular functions mechanistically contribute to the WHS disease phenotypes is not well understood and is complicated by the multigenic cause for WHS. Other implications of LETM1 impairment in genetic diseases include temporal lobe epilepsy, diabetes, and obesity.

Here, we describe 18 affected individuals from 11 unrelated families presenting with clinical features suggestive of a mitochondrial disease largely involving the CNS in which exome sequencing (ES) identified novel and ultra-rare bi-allelic segregating LETM1 variants.

To functionally characterize the bi-allelic LETM1 variants, we explored cellular growth and mitochondrial respiratory chain, morphology, osmotic regulation, and KHE activity in proband-derived fibroblasts, muscle samples, and yeast carrying the variants of interest.

Subjects and methods

Study subjects
Using the GeneMatcher platform and data sharing with collaborators around the world, 11 families with bi-allelic LETM1 variants...
| F ID | Center                                      | Method                      | gDNA Change (chr4 hg 19) | Variant type       | nt change | aa change          | gnomAD V3.1.2 and V2.1.1 | Other databases | CADD | GERP | SIFT | PolyPhen | AF      |
|------|--------------------------------------------|-----------------------------|--------------------------|--------------------|-----------|-------------------|--------------------------|-----------------|------|------|------|----------|--------|
| 1    | Queen Square Genomics                      | proband only ES25–27        | g.1834673A>T             | missense, splice   | c.878T>A   | p.Ile293Asn       | 0                        | 0               | 28.8 | 4.61 | D    | PD       |         |
| 1    | Queen Square Genomics                      | proband only ES25–27        | g.1816277T>T             | frameshift         | c.2094del  | p.Asp699Metfs*13  | 0                        | 1 het allele (UKBB) | –    | –    | –    | –        |         |
| 2    | Copenhagen University Hospital             | proband only ES26           | g.1816151C>G             | stop_loss          | c.2220G>C  | p.*740Tyext26     | 0                        | 0               | –    | –    | –    | –        |         |
| 7    | Queen Square Genomics                      | proband only ES26           | g.1816277T>T             | frameshift         | c.881G>A   | p.Arg294Gln       | 4 het alleles (V2.1.1); 2 het alleles (V3.1.2.) | 2 het alleles (UKBB); 2 het alleles (GeneDx); 2 het alleles (TOPMed) | 26.3 | 4.61 | D    | PD       |         |
| 3    | Welcome Center for Mitochondrial Research  | proband only ES27           | g.1836692CTT>             | inframe deletion   | c.754,756del | p.Lys252del       | 0                        | 0               | –    | –    | –    | –        |         |
| 4    | Welcome Center for Mitochondrial Research  | proband only ES27           | g.1834670C>T             | missense           | c.881G>A   | p.Arg294Gln       | 4 het alleles (V2.1.1); 2 het alleles (V3.1.2.) | 2 het alleles (UKBB); 2 het alleles (GeneDx); 2 het alleles (TOPMed) | 26.3 | 4.61 | D    | PD       |         |
| 8    | Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan | proband only ES27 | g.1834479C>T | missense | c.1072G>A | p.As358Asn | 0 | 0 | 23.5 | 4.61 | D | B |
| 5    | Institute of Medical Genetics and Applied Genomics, University of Tuebingen, Germany | proband only ES27 | g.1834479C>T | missense | c.1072G>A | p.As358Asn | 0 | 0 | 23.5 | 4.61 | D | B |
| 6    | GeneDX                                    | trio ES31–33                | g.1827313-C>T           | missense           | c.1178G>A  | p.Arg393His       | 13 het alleles (V2.1.1); 2 het alleles (V3.1.2) | AF 0.0002 (1K GP); 1 het allele (UKBB); 7 het alleles (GeneDx); 7 het alleles (TOPMed) | 26.6 | 5.06 | D    | PD       |         |
| 9    | Exeter Genomics Laboratory                 | trio ES34                   | g.1827352C>G             | missense           | c.1139G>C  | p.Arg380Pro       | 0                        | 1 het allele (UKBB) | 27.4 | 5.06 | D    | PD       |         |
| 10   | Institute of Human Genetics, Technical University of Munich | proband only ES35 | g.1814582G>C | splice defect | c.2071–9C>G | p.Val691fs*4 | 0 | 0 | – | – | – | – |

(Continued on next page)
were identified. The affected individual from family 8 was recruited from the report by Catania et al. describing a person with a combined pituitary hormone deficiency, ocular involvement, myopathy, ataxia, and mitochondrial impairment carrying variants in several putative disease-causing genes, including rare bi-allelic variants in OTX2 (orthodenticle homeobox 2 [MIM: 600037]) and LETM1 as well as rare heterozygous variants in AFG3L2 (AFG3 like matrix AAA peptidase subunit 2 [MIM: 604581]) and POLG (DNA polymerase gamma, catalytic subunit [MIM: 174763]). Clinical details of the cohort were obtained by the follow-up of the living affected individuals and retrospective analysis of the available clinical records for deceased individuals. Parents and legal guardians of all affected individuals gave their consent for the publication of clinical and genetic information according to the Declaration of Helsinki, and the study was approved by The Research Ethics Committee Institute of Neurology University College London (IoN UCL) (07/Q0512/26) and the local Ethics Committees of each participating center. Consent has been obtained from families 1, 5, and 8 to publish medical photographs and video examinations. Brain magnetic resonance imaging (MRI) scans were obtained from 6 affected individuals and were reviewed by an experienced pediatric neurodiagnosticist (FA).

Exome sequencing and data analysis
Proband only or trio ES in 11 families was carried out in DNA extracted from blood-derived leukocytes in 9 different centers following slightly different protocols (see Table 1). ES data analysis and variant filtering and prioritization were performed using in-house implemented pipelines of the local genetic centers (Table 1). Sanger sequencing was performed to confirm co-segregation in all available family members.

Skin biopsy and primary fibroblast culture and muscle biopsy
Individuals F1:S1, F1:S2 and parents (mother-F1:M, father-F1:F), F2:S1, F5:S1, F10:S1, F11:S1, and F11:S2 provided each one skin biopsy, and affected individuals F11:S1, F11:S2, and F5:S1 provided also each one muscle biopsy. Details on fibroblast cell lines establishment and muscle sample preparations are described in the supplemental material and methods.

Western blotting analysis
Immunoblotting analysis was performed using standard protocols as described previously, detailed descriptions of sample preparation, quantification, and western blotting are in the supplemental material and methods. A list of antibodies used for this study is given in supplemental data.

Cell imaging
Confocal microscopy was performed for fibroblasts from F1, F2, F5, F10, and F11 and respective control subjects following established protocols for life and immune staining described in Durigon et al., Wilfinger et al., and supplemental material and methods. Transmission electron microscopy is described in the supplemental material and methods.

mtDNA copy number
DNA was extracted from muscle or fibroblasts by proteinase K treatment. The mtDNA content was determined by quantitative
As Mg2+ the plasmids. Yeast growth media were described in Zotova et al.44 grown on selective media (SD-URA) to ensure the retention of the yeast LETM1 homolog)42 was transformed with the multi-site-directed mutagenesis is given in supplemental data. A list of variants studied in yeast and primers used for this study. A list of variants studied in yeast and primers used for site-directed mutagenesis is given in supplemental data and methods. All antibodies used in this experiment are listed in supplemental material and methods.

Plasmid and LETM1 single-nucleotide variants
Full-length human LETM1 cDNA fused to C-terminal Hemagglutinin (HA)-tag and subcloned into the multi-copy plasmid pVT-103U42 served as a template to introduce the LETM1 variants by site-directed mutagenesis. Amino acid replacements and deletions were performed with non-overlapping back-to-back annealing mutagenic primers, using the Q5 site-directed mutagenesis kit (NEB #E0552S) with NEB 5-alpha competent E. coli cells (NEB #C2987). All primers were from Microsynth and all the identified variants were confirmed by DNA sanger sequencing. To distinguish the phenotypes of disease-associated LETM1 variants and non-pathogenic variants, two non-disease-associated LETM1 (GenBank: NM_012131.8) missense variants (rare LETM1 variants but with homozygotes in gnomAD v3.1.1), c.913A>C (p.Ile305Leu) and c.1760A>G (p.Lys587Arg), were included in this study. A list of variants studied in yeast and primers used for site-directed mutagenesis is given in supplemental data.

Yeast transformation
W303 (ATCC 201239) Saccharomyces cerevisiae strain mdm38/letm1Δ (lacking the open reading frame YOL027c, which encodes the yeast LETM1 homolog)42 was transformed with the multi-copy vector pVT-103U, either empty or containing wild-type human LETM1 or LETM1 variants using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method43 and grown on selective media (SD-URA) to ensure the retention of the plasmids. Yeast growth media were described in Zotova et al.44

Mitochondrial isolation and KOAc-induced swelling assay
Mitochondria were isolated from yeast cells logarithmically grown in SD-URA by homogenization and differential centrifugation method as described in Nowikovsky et al.42 and immediately used for KOAc-induced swelling assays. The protocols of Nowikovsky et al.42 were adapted to smaller volumes. In brief, isolated yeast mitochondria suspended in breaking buffer (0.6 M sorbitol, 20 mM Tris-HCl [pH 7.4]) were de-energized with antimycin A (2.5 μM) for 10 min at room temperature (25°C), washed, and re-suspended in breaking buffer at a concentration of 200 μg/20 μL. As Mg2+ is a brake to the KHE,45 mitochondria were depleted from Mg2+ with A23187 (0.5 μM) and EDTA (10 mM) and transferred onto 96-well plates for measurement (200 μL/well). When indicated, quinine (200 μM) served as a control to inhibit KHE-mediated swelling. The 96-well plates were placed in the Thermo Scientific Varioskan LUX Multimode Microplate Reader. The swelling was initiated by injection of KOAc media (55 mM KOAc, 5 mM TES, 0.1 mM EDTA) to a final volume of 200 μL/well and the optical density changes at OD_{440} were immediately recorded at 25°C. Each measurement was performed in 3 independent replicates. Raw swelling data were fitted into a curve showing changes in absorbance versus time to quantify the swelling rate.

Results
Clinical findings
The summary of the core phenotypic features of 18 affected individuals from 11 independent families with bi-allelic LETM1 variants is provided in Table 2, Figure 1C, and Table S1. Detailed clinical history is provided in the supplemental note (case reports). Video recordings are available for affected individuals from family 1 (Videos S1, S2, S3, and S4). The cohort comprises 10 males and 8 females, 9 of whom are currently alive with a median age of 15 years (range 1–39) at the latest available follow-up (Figure 2A). Half of the persons (9/18) succumbed to their rapidly progressing disease at an early age, ranging between 2 months and 8 years old. The ethnic composition of the cohort is diverse, including families of Pakistani, Caucasian, Middle Eastern, European, and Mexican origin, with 67% of the individuals (12/18) being from consanguineous unions. Only limited clinical data were obtainable from 6 deceased persons belonging to families 3 and 10.

The cohort members had unremarkable prenatal histories with full-term birth in 14/15 persons (93%). Admission to the special care baby unit was necessary in 5/15 affected individuals (33%) due to respiratory, cardiac, and feeding issues during the neonatal period. Most of the persons (14/18, 78%) had an infantile-onset disease manifestation, and 4/18 (22%) presented first symptoms between the ages of 1.5 and 2 years. The common presenting symptoms were global developmental delay, cognitive and motor regression, failure to thrive, central hypotonia, respiratory distress, and feeding difficulties. The disease progressed rapidly in 9/18 (50%), moderately fast in 4/18 (22%), and slowly in 5/18 (28%) affected individuals. Developmental regression was later present in 9/13 (69%) affected individuals with loss of independent ambulation by a mean age of 5.4 ± 3.2 years (range 2–12).

On the most recent follow-up, the affected individuals displayed clinical features suggestive of a mitochondrial disorder. Impaired vision (10/10, 100%) with a mean onset age of 5.2 ± 3.1 years, which was confirmed to be due to optic atrophy in 5/6 (83%), and bilateral sensorineural hearing loss (11/14, 78%) diagnosed at a mean age of 2.6 ± 1.9 years (range from congenital up to 6 years) with hearing aids fitted in 7/10 (70%) persons were the common neurosensory abnormalities. While cognitive delay and intellectual disability (7/8, 87.5%) and impaired speech acquisition (6/9, 67%) were among the common neurodevelopmental symptoms, more than half of the individuals displayed neuromuscular features including spasticity (8/15, 53%), hypotonia (11/18, 61%), muscular wasting (7/10, 70%), and cerebellar ataxia (7/9, 78%). Other frequent neurological symptoms were nystagmus
| Family ID | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 |
|-----------|----|----|----|----|----|----|----|----|----|-----|-----|
| Subject ID | S1 | S2 | S1 | S2 | S1 | S1 | S2 | S1 | S1 | S1  | S2  |

**Epidemiology and medical history**

| | F1 | M | M | F | F | M | M | M | F | F | M | F | F | M | M | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Current age/death age | 35 y | 25 y | 24 y | D | 2.7 y | D | 1 y | 17 m | 15 y | 8 y | D | 1 y | D | 10 y | D | 2 y | D | 1 y | D | 4.5 m |
| Age at onset | 1 y | 1.5 y | 2.5 y | 4 m | 6 m | 7 m | birth | 1.5 y | 2 y | 10 m | birth | 4 m | 1 m birth | birth |
| Type of progression | S | S | R | R | R | MD | MD | S | MD | MD | S | R | R | R | R | R | R | R |
| GDD/ID | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Regression in development | + | + | N/D | - | N/D | - | - | + | + | + | + | N/D | N/D | N/D | + | + |
| Loss of ambulation (age) | +, 12 y | +, 6 y | N/D | N/D | N/D | +, 2.5 y | N/D | +, 5 y | 5 y | +, 2 y | N/D | N/D | N/D | N/D | N/D | N/D | N/D |

**Main clinical features**

| | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 |
|---|---|---|---|---|---|---|---|---|---|-----|-----|
| Age at last examination | 35 y | 25 y | 24 y | > | 1 y | > | 1 y | 11 y | 2 m | 15 y | 8 y | 37 |
| Small weight and height | + | + | + | N/D | N/D | N/D | N/D | + | - | + | + | N/D | N/D | N/D | - | - |
| Facial dysmorphism | + | + | - | N/D | N/D | N/D | N/D | - | - | + | - | - | N/D | N/D | N/D | N/D | N/D |
| Optic atrophy/ impaired vision | + | + | N/D | + | N/D | + | N/D | + | + | N/D | N/D | N/D | + | N/D |
| Cataract | - | - | + | N/D | N/D | N/D | N/D | + | - | - | + | - | N/D | N/D | N/D | + | + |
| Sensorineural deafness | + | + | N/D | N/D | N/D | N/D | N/D | + | - | - | - | + | + | N/D | N/D | + | + |
| Hypotonia | - | - | N/D | N/D | N/D | N/D | N/D | + | - | - | - | - | - | + | + | N/D |
| Spasticity/hypertonia | + | + | N/D | N/D | N/D | N/D | N/D | - | - | + | + | - | - | - | + | - | - |
| Cerebellar ataxia | + | + | N/D | N/D | N/D | N/D | N/D | - | - | N/A | + | + | + | N/A | N/D | N/D | + | N/D |
| Myopathy | - | - | N/D | + | N/D | + | N/D | - | - | + | - | N/D | N/D | N/D | + | + | + |
| Hyperkinetic movement disorders | + | + | N/D | N/D | N/D | N/D | N/D | - | - | - | - | - | - | + | N/D | N/D | - | - |
| Peripheral neuropathy | + | + | N/D | N/D | N/D | N/D | N/D | - | - | N/D | - | - | - | - | N/D | N/D | N/D | + | N/D |
| Impaired speech/ language abilities | + | + | N/D | N/D | N/D | N/D | N/D | - | - | - | + | + | + | N/A | N/D | N/D | N/D | N/D |
| Impaired/spastic/ataxic gait | + | + | N/A | N/A | - | - | N/A | + | + | + | N/A | N/D | N/D | N/D | N/D | N/D | N/D |
| Seizures | + | + | N/A | N/A | - | - | N/A | + | + | + | + | + | + | + | + | + | + |
| Cardiac involvement | - | - | N/D | N/D | N/D | N/D | N/D | - | - | - | + | + | + | + | + | + | + |
| Diabetes | + | + | N/D | N/D | N/D | N/D | N/D | - | - | - | - | - | - | - | N/D | N/D | N/D | - |
| Lactic acidosis | - | - | N/D | + | N/D | + | N/D | N/D | N/D | N/D | N/D | N/D | - | + | + | + | + |
| Raised urinary 3-MGA | - | - | N/D | - | N/D | - | N/D | - | - | N/D | N/D | + | + | + | N/D |

**Investigations**

| | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 |
|---|---|---|---|---|---|---|---|---|---|-----|-----|
| MRC deficiencies | CI, II, III, IV | CI, IV | CI, III, IV | N/D | CI, II, III, IV | N/D | CI, III, IV | N/D | CI, III, IV | N/D | CI, IV, V |
| Muscle histochemistry | + | + | N/D | N/D | N/D | N/D | N/D | + | + | N/D | N/D | + | - |
| Brain MRI findings | CA, PA | N/D | UR | VM | UR | N/D | BA | ONA, CHA | CVH | N/D | ONA, CHA | BA, CA | UR | N/D | N/D | N/D | CVH, BSH, VM, DM | N/D |

Abbreviations: F, female; M, male; y, year; m, months; D, deceased; +, yes; - , no; N/D, no data; S, slow; MD, moderate; R, rapid; N/A, not applicable; GDD, global developmental delay; ID, intellectual disability; MCR, mitochondrial respiratory complex; C, complex; UR, unremarkable; 3-MGA, 3-methylglutaconic aciduria; CA, cerebellar atrophy; PA, pontine atrophy; VM, ventriculomegaly; BA, brain atrophy, ONA, optic nerve atrophy; CHA, chiasmal atrophy; CVH, cerebellar vermis hypoplasia, BSH, brain stem hypoplasia; DM, delayed myelination.
(7/13, 54%), myopathy (6/12, 50%), hyperkinetic movement disorders (4/12, 33%), and spastic-ataxic gait (3/9, 33%) combined with brisk deep tendon reflexes (4/10, 40%), upgoing plantar response (4/9, 44%), and peripheral neuropathy (3/9, 33%).

Ten of the fifteen affected individuals (67%) developed epileptic seizures by a median age of 5 years (range 0.5–14). The seizure type ranged from infantile spasms and myoclonic jerks to absences, focal, and generalized tonic-clonic seizures. Individuals with younger age of seizure onset had frequent episodes spanning from hourly clusters of spasms at peak to seizures once per day. Two affected siblings from family 1 with seizure onset after ages 9 and 14 years, respectively, had seizures recurring either in clusters 2–3 times every 2–3 months (F1:S2) or once in 2 years (F1:S1). Pharmacoresistance and epileptic encephalopathy were confirmed in one person from family 9. Electroencephalograms, available from 4 individuals, showed background slowing (F5:S1), excessive sharp transients (F6:S1), single 3–4 Hz potentials and short trains (F2:S1), and continuous spike-and-slow wave activity, with bursts of faster activity observed during sleep, consistent with epileptic encephalopathy (F9:S1).

Other features consistent with a mitochondrial phenotype included bilateral cataracts (5/11, 45%) cardiomyopathy (5/14, 36%) with pericardial effusion (3/11, 27%), and diabetes (3/11, 27%). Craniofacial abnormalities included occipitofrontal circumference below third percentile in 2/6 persons (33%) and facial dysmorphism (4/10, 40%) with
Figure 2. Pedigrees with the segregations of the LETM1 variants and LETM1 protein architecture with a partial sequence alignment of the variants

(A) Family trees of the individuals with bi-allelic LETM1 variants. Square, male; circle, female; black symbols, affected individuals; white symbols, unaffected individuals.

(B) Schematic representation of the human LETM1 organization in introns, shown as a line, and exons, shown as boxes, and of LETM1 domains as indicated by the residue numbers and the color code: coiled-coil motifs, light yellow; transmembrane helices, blue; (legend continued on next page)
a long thin face, prominent nose, low-set ears, micrognathia, high arched palate, and teeth abnormalities (Figure 1A).

While not every person had available electrophysiological investigations, biochemical, metabolic studies, and muscle histochemical analysis, the obtainable tests suggested the presence of mitochondrial dysfunction in the affected individuals. Hence, electromyography and nerve conduction studies available from 5 individuals showed neurogenic (3/5) and myopathic changes (2/4). Elevated serum lactate was confirmed in 8/12 (67%) affected individuals. Plasma amino acids were abnormal in 4/9 tested with mildly elevated alanine (501–597 µmol/L, normal range 232–494), glycine, and serine. CSF-alanine was tested and mildly increased in 2 probands. Urine amino acids were tested in 4 persons, and only one person showed abnormal results including increased levels of aspartic, serine, and glycine. Urine organic acids were analyzed in 11 affected individuals and were abnormal in 9 of them with 3-methylglutacetic acid excretion (5/11), moderately elevated beta-hydroxybutyrate and acetoacetate (1/11), and significant elevation of adipic acid (1/11). Muscle biopsy was available from 7 persons and of these, 5 had abnormal findings including scattered necrotic and regenerating COX-deficient fibers with an excess of internal nuclei, lipid depositions within fibers and prominent mitochondrial pattern in vacuolated fibers (F3:S2), COX-deficient multiple ragged-red fibers with increased fiber unisomity (F8:S1), type 1 fiber predominance with mild glycogen storage (F9:S1), and COX-deficient fibers (F11:S1). Respiratory chain enzyme (RCE) analysis was performed in 11 individuals showing isolated or combined mitochondrial respiratory chain deficiencies in all persons tested (Tables 2, S1, Figure 3C).

Brain MRI investigations were available for 6 persons, performed between 6 days and 32 years of age (Figure 1B). In some persons, only a few sections or low-quality images could be reviewed. In 4/6 affected individuals, optic nerve and chiasm atrophy were present and in two persons optic nerves were normal. Three individuals showed infratentorial abnormalities, with severe pontine hypoplasia and cerebellar atrophy in a proband from family 1 and mild vermian hypoplasia in 2 probands from family 3 and family 6. Other minor and non-specific findings were mild supratentorial atrophy and mild ventricular dilatation noted in 2 persons each.

The affected individual from family 8 was the oldest member of the cohort showing a phenotype consistent with the rest of the individuals that survived into adulthood.

### Molecular genetic findings

In all probands, ES at the local genetic centers did not identify causative variants in known disease-associated genes. Filtering for novel (i.e., not present in available databases) and rare protein-altering variants identified bi-allelic variants in LETM1 (GenBank: NM_012,318.3) in probands from all families (Table 1). Segregation by Sanger sequencing in families with proband-only ES and where available trio ES supported LETM1 as a candidate gene (Figure 2A). The proband from family 6 carried a homozygous c.1178G>A (p.Arg393His) variant in LETM1 resulting from maternal uniparental disomy. Known pathogenic variants in mtDNA and mtDNA rearrangements were excluded in all families.

The LETM1 variants (Table 1 for variant characterization and Figure 2B) comprised missense variants causing changes in amino acid charge, size, hydrophobic or “helix breaker” properties, and frameshift variants causing premature or delayed termination. All detected missense variants were located specifically within the conserved LETM domain, while the frameshift variants were localized to the C-terminal part of LETM1 (Figure 2B). Of all the amino acid changes, the only fully conserved amino acid across mammals, vertebrates, invertebrates, plants, and yeast is Asp358, and the semi-conserved ones are Lys252 and Ile293 (Figure 2B). Arg294, which is affected by the missense variant c.881G>A (p.Arg294Gln), is conserved in all sub-families excluding yeast, and it was found in two independent persons (F4:S1 and F8:S1) of Egyptian and Italian origin, respectively. Pro300, affected by the variant c.898C>T (p.Pro300Ser), is conserved in mammals and zebrafish. Four variants affect the C-terminal stretch of human LETM1 that is absent in the yeast LETM1 homolog (Letm1p/Mdm38p) as its protein sequence is shorter. The splice variant c.2071–9C>G (p.Val691fs4*) (Sashimi plot, Figure S2, supplemental material and methods) affects two residues conserved across mammals, zebrafish, worms, and plants and introduces a premature stop codon before the second EF loop. The variant c.2094del removes Asp699, a negatively charged residue, well-conserved in mammals, fish, worms, and plants that locates close to the second EF loop and prematurely terminates the protein sequence. The stop-loss variant c.2220G>C (p.*740Tyr>ext26) leads to an elongation of 26 amino acids. This variant was present in two independent families of Pakistani origin suggesting a possible founder effect. Five of the ten identified LETM1 variants were absent across a number of large genetic databases (~1 million alleles), whereas the remaining four variants appear to be ultra-rare (Table 1).
Figure 3. Effects of LETM1 variants on mitochondrial morphology and proliferation in fibroblasts

(A) LETM1 variants perturb the mitochondrial network. Confocal images of fibroblasts stained with Mitotracker Red. Shown is a representative overview of the cells (bars 5 μm, except F10 10 μm) and details magnified from the box (bars 5 μm). C1 and C2, healthy donors; F1:S2, c.878T>A (p.Ile293Asn) and c.2094del (p.Asp699Metts*13); F2, c.2220G>C (p.*740Tyret26); F5, c.1072G>A (p.Asp358Asn); F10, c.2071–9C>G (p.Val691fs4*). Arrow indicates representative fragmented mitochondria. For statistics, see Figure S1C.

(B) LETM1 variants cause swollen mitochondria and loss of cristae. The ultrastructure of control (C1) and affected individual (F5 and F10) fibroblasts was investigated by transmission electron microscopy and images show overviews (left panels, bars 2 μm) and details (right panels, bar 500 nm). Arrow indicates swollen mitochondria.

(C and D) Variants differently affect LETM1 stability and OXPHOS proteins in fibroblasts samples. Total lysates of fibroblasts were analyzed by immunoblotting using the indicated antibodies, GAPDH, or β-actin as loading control: C2 and C3, healthy donors; F1:S1 and F1:S2, c.878T>A [p.Ile293Asn]; F2, c.2220G>C [p.Asp699Metts*13]; F5, c.1072G>A [p.Asp358Asn]; F10, c.2071–9C>G [p.Val691fs4*]; F11, c.898C>T [p.Pro300Ser] (C). Quantitative graphs from independent experiments representing the protein bands, normalized to the housekeeping proteins, and calculated as a percentage of controls; data are expressed as mean ± SEM (n ≥ 3 independent experiments) (D).
Genotype-phenotype correlation

A remarkable interfamilial phenotypic variability was observed in the present cohort. Four persons from families 1, 2, and 8 have survived into adulthood albeit with a significant disability, while 10 individuals from families 3, 4, 9, 10, and 11 had a rapidly progressing disease course leading to early death in 9 of them. Affected individuals from family 5 (age 11 years), family 6 (age 17 months), and family 7 (ages 8 and 15 years) displayed less severe phenotypes. Affected individuals from family 4 and family 8 carrying the recurrent missense LETM1 c.881G>A (p.Arg294Gln) variant exhibited a similar range of symptoms, though for F4:S1 displayed more rapid disease progression with significant cardiac involvement and early mortality. Persons of Pakistani origin from family 2 and family 7 with loss-of-function (LoF) LETM1 c.2220G>C (p.*740Tyr>ext26) variant were reported with a similar phenotypic range, which was more severe in family 2, possibly due to older age and longer disease course. No significant intrafamilial phenotypic variability was observed in the cohort.

Effects of the LETM1 variants on patient-derived fibroblasts and muscle tissue

Loss of mitochondrial volume homeostasis is the most characteristic and universally accepted phenotype of LETM1 deficiency in human, animal models, plants, and yeast, which leads to mitochondrial fragmentation, matrix swelling, and disorganized cristae as reviewed in Austin et al. Therefore, we first evaluated the mitochondrial morphology in the available fibroblasts. Compared to fibroblasts from healthy donors (C1–C4), fibroblasts from F1:S1 and F1:S2 (compound heterozygous for c.[878T>A; 2094del], p.[Ile293Asn; Asp699Metf*13]), F10 (homozygous for c.2071–9C>G [p.Val691fs4*]), F2 (homozygous for c.2220G>C [p.*740Tyr>ext26]), F5 (homozygous for c.1072G>A [p.Asp358Asn]), and F11:S2 (homozygous for c.898C>T [p.Pro300Ser]) displayed mitochondrial alterations, with significantly increased fragmented shapes seen as donut segments and punctate and enlarged units often separated from the main network (Figures 3A and S1A–S1C). Elongated mitochondrial shapes were restored by ketone bodies and by nigericin, as both reverted the ratio of elongated tubules versus fragmented units to control levels (Figure S1C). The use of the membrane potential-dependent mitochondrial dye Mitotracker Red (MTR) revealed an irregular polarization pattern of the mitochondrial network of all affected individuals, with partly depolarized tubules and hyperpolarized patches, as well as a markedly reduced electric potential of mitochondria in F10 and trend-wise also in F5 (Figures 3A, S1A, and S1C). Impaired KHE activity in LETM1-deficient cells leads to uncompensated electrophoretic K⁺ uptake and consequent mitochondrial swelling. Treatment with the synthetic KHE nigericin to counteract the loss of K⁺ homeostasis reverted the decreased membrane potential to control levels in F10 and F5 (Figures S1A and S1C), while addition of ketone bodies had no beneficial effect. Consistent with the electrophoretic K⁺ influx rate exceeding the K⁺ release rate due to a lack of KHE activity, mitochondria in F11:S2 cells readily underwent swelling and depolarization (as assessed by in situ staining with the potentiometric probe TMRM) upon the addition of low concentrations of valinomycin, a selective K⁺ ionophore that allows electrophoretic K⁺ uptake unlike mitochondria of control fibroblasts. Treatment of F11:S2 fibroblasts with the ionophore nigericin restored the mitochondrial sensitivity to valinomycin, a strong indication that the response to valinomycin was indeed due to lack of KHE activity (Figure S1D). Based on the protective effect of ketone bodies as an energy source for LETM1-deficient cells, we tested next whether a tubular network could be better maintained as a result. Ketone bodies suppressed MTR fluorescence in fibroblasts from F1 and F2 and attenuated its intensity in F10:S1. However, increasing the laser intensity, an elongated tubular shape of the mitochondrial network also became apparent in the samples of F1:M and F5:S1 (Figure S1A). Thus, elongation of mitochondrial tubules was accompanied by a reduced inner membrane potential, a phenomenon previously described in the context of transient matrix contraction. Replacement of glucose with galactose, known to suppress glycolytic ATP production, in F1:S1 and F11:S2 for up to 5 days produced a more dramatic morphological phenotype, in some persons resembling LETM1 siRNA (Figures S1B) and Durigon et al. and it caused cell death after only 48–72 h in F11:S2. Transmission electron microscopy was performed for F5 and F10 fibroblasts as well as control fibroblasts and confirmed ultrastructural mitochondrial changes associated with LETM1 variants compared to the elongated tubular shapes of the healthy control mitochondria (Figure 3B). Different morphological stages of mitochondrial alterations were associated with LETM1 c.2071–9C>G (p.Val691fs4*) (F10), including short tubules containing enlarged sections with reduced cristae, swollen matrix devoid of cristae, and perinuclearly distributed spherical ghost shapes resembling a mixture of mitochondrial remnants and vacuoles. Similarly, fibroblasts with the variant LETM1 c.1072G>A (p.Asp358Asn) (F5) showed broad, short, and electron-lucent mitochondria, partly devoid of cristae and intermediate shapes between mitochondria and vacuoles.

Pathological variants frequently lead to altered expression or stability of the encoded proteins, and so we assessed LETM1 protein levels via immunoblotting. The steady-state levels of LETM1 in fibroblasts from F10 were comparable to those from control subjects. Instead, LETM1 was significantly decreased in bi-allelic LETM1 variant fibroblasts F1:S1 and F1:S2, and F11:S2, and more drastically in F2 (Figures 3C and 3D).

Because LETM1 dysfunction restricts mitochondrial respiratory capacity in yeast and mammals, and the clinical and metabolic findings in the affected individuals were consistent with a mitochondrial disorder, we next investigated the abundance of the oxidative phosphorylation (OXPHOS) subunits. Fibroblasts of affected individuals...
Figure 4. LETM1 variants affect the stability of LETM1 and OXPHOS components in muscle samples 
(A and B) Western blot analysis of LETM1 and components of the OXPHOS complexes I, II, III; and IV in muscle samples from F11 and quantitative graphs. Total lysates of muscle samples from healthy donors (C4, C5) and F11 c.898C>T (p.Pro300Ser) (S1, S2) were analyzed by immunoblotting using the indicated antibodies; VDAC served as a loading control (A). Quantitative graphs represent the protein levels relative to controls and normalized to VDAC. Data are expressed as mean ± SEM; n ≥ 3 independent experiments (B). 
(C) Immunohistochemical staining of OXPHOS subunits and VDAC of the muscle of F5 and control subjects. Muscle samples from healthy donors (C6, C7) and F5 c.1072G>A (p.Asp358Asn) were stained for each of the five OXPHOS subunits using the indicated antibodies; VDAC served as a control. Magnification 400X.

(legend continued on next page)
harboring bi-allelic LETM1 variants displayed reduced steady-state levels of selected respiratory chain proteins of complex I and IV, in opposite to increased levels in F10 (c.2071–9C>G [p.Val691fs4*]) (Figures 3C and 3D). OXPHOS proteins NDUFB8 and NDUFA9 were decreased in F1:S1 and F1:S2 fibroblasts and to a higher extent in F11:S2 (Figures 3C and 3D). Since mitochondrial defects can limit cellular growth, we assessed the proliferation rates of the fibroblast cell lines. While proliferation was comparable for fibroblasts with the single or compound heterozygous variants (F1), extension variant (F2), or wild-type LETM1 (LETM1 WT), it was significantly slowed down in LETM1 c.2071–9C>G (p.Val691fs4*) (F10) and absent in LETM1 c.1072G>A (p.Asp358Asn) (F5) fibroblasts (Figure S3).

Similar to fibroblasts, LETM1 was significantly reduced in the muscle of F11 (Figures 4A and 4B). NDUFA9 (complex I) was reduced in muscles samples from F11 while SDHB (complex II) displayed a strong tissue-specific upregulation (Figures 4A and 4B). The immunohistochemistry and western blotting analysis from F5 muscle tissue (Figures 4C–4E) revealed even greater reductions for components of complexes I, III, and IV, increased SDHA, accompanied by decreased enzyme activity of complex I, and upregulated activity of complex II and citrate synthase and increased mtDNA copy number (Table S2). Proteins of the ATP synthase remained not significantly changed in all tested cell lines and tissue samples.

Overall, altered LETM1 and OXPHOS protein levels in fibroblasts and muscle samples were observed in most of the individuals. Fibroblasts cell culture data indicated that bi-allelic LETM1 variants result in aberrant mitochondrial morphology, which was more pronounced under galactose challenge (Figure S1B) and was often lethal for F11-derived fibroblasts. Consistent with the frequently observed effect of mitochondrial defects on cellular functions and growth, cell proliferation was retarded in F10 and more drastically in F5 fibroblasts. The synthetic KHE nigercin restored mitochondrial morphological aberrations and membrane depolarization, coupling mitochondrial dysfunctions and impaired K+ homeostasis.

**Functional compensation analysis in yeast**

Considering that LETM1 controls mitochondrial volume by regulating KHE, we ectopically expressed LETM1 variants or wild-type in the S. cerevisiae letm1Δ strain to explore the functional impact of LETM1 variants on mitochondrial KHE activity. All LETM1 variants listed in the supplemental data were included in this analysis. The loss of KHE activity in yeast letm1 deletion mutants, the complementation by re-expression of the homologous human LETM1 WT, and the absence of a Ca2+ transport system in S. cerevisiae mitochondria make the system ideally suited for functional complementation analysis of LETM1 variants and determination of their pathogenicity with respect to KHE defects.

Light scattering recording of KOAc-induced swelling is a well-established method to measure the mitochondrial electroneutral exchange of K+ for H+. Exposure of de-energized mitochondria to hypotonic KOAc buffer elicits the rapid uptake of protonated acetic acid, acidification of mitochondrial matrix, and thereby activation of KHE, which results in mitochondrial K+ influx and water uptake and thus swelling. Isolated mitochondria from S. cerevisiae LETM1 wild-type cells and S. cerevisiae letm1Δ cells overexpressing human LETM1 WT or variants or the empty control vector were subjected to KOAc-induced swelling experiments. Recording KHE activity by measuring the decrease in optical density (OD) using light scattering techniques allows discrimination of its main determinants: initial OD, indicating the osmotic state of mitochondria before KOAc addition, and KHE exchange rate per second, indicated by the amplitude from initial to final OD as a function of the time required to achieve it. As shown in Figure 5A, KOAc-induced swelling was sensitive to the KHE inhibitor quinine, confirming the correlation of optical density with KHE activity. Knockout of S. cerevisiae LETM1 (S. cerevisiae letm1Δ) entirely abolished KHE activity, as illustrated by low initial OD and swelling amplitude, which were restored by expression of LETM1 WT. The non-pathogenic variants (p.Ile305Leu and p.Lys587Arg) performed as well as LETM1 WT for the initial OD, and almost as well for the kinetics values. LETM1 with the variant p.Val691fs4* (F10) almost restored the initial OD, and so did LETM1 p.Lys587Arg (F9) and p.Arg393His (F6) but their swelling amplitudes were very low. Expression of LETM1 variants c.754–756del (p.Lys252del) (F3), c.878T>A (p.Ile293Asn) (F1:M), or c.2220G>C (p.*740Tyrext26) (F2, F7) marginally compensated K+ fluxes with extremely slow swelling kinetics; swelling traces for S. cerevisiae letm1Δ transformed with LETM1 c.881G>A (p.Arg294Gln) (F4, F8) or LETM1 c.2094del (p.Asp699Metfs*13) (F1:F) suggested uncontrolled cation leakage (Figure 5A). Overexpression of LETM1 c.1072G>A (p.Asp358Asn) did not rescue KHE. Taken together, these results suggest that mitochondrial reduced K+ flux dynamics and swollen matrix are indicative of the functional impact of disease-associated LETM1 variants.

LETM1 protein levels associated with LETM1 variants were examined using total cell lysates and isolated mitochondria. In comparison to the mitochondrial loading control (Porin, Por1p), LETM1 total protein levels from ectopic LETM1 WT or variant expression were similar, except those from F1:S1-S2 (LETM1 c.878T>A;
LETM1 variants fail to restore KHE activity of yeast letm1Δ. Isolated and de-energized mitochondria were subjected to KOAc and changes of optical density at OD540, immediately measured. Left upper panel: representative traces of KOAc-induced swelling in S. cerevisiae LETM1 WT mitochondria (WT, blue) or S. cerevisiae letm1Δ mitochondria overexpressing the empty plasmid (e, yellow) or the plasmid carrying human LETM1 WT untreated (w, green) or treated (wq, gray) with quinine or the human LETM1 variants; color code as indicated in the inserted table: c.754–756del (p.Lys252del) (1, red), c.878T>A (p.Ile293Asn) (2, bottle green), c.881G>T (p.Arg294Gln) (3, aqua), c.898C>T (p.Pro300Ser) (4, dark green), c.913A>C (p.Ile305Leu) (5, lavender), c.1072G>A (p.Asp358Asn) (6, violet), c.1139G>C (p.Arg380Pro) (7, beige), c.1178G>A (p.Arg393His) (8, turquoise), c.1760A>G (p.Lys587Arg) (9, mauve), c.2071–9C>G (p.Val691fs4*) (10, purple), c.2094del (p.Asp699Metfs*13) (11, dark blue), compound (12, lilac), c.2220G>C (p.*740Tyrfs26) (13, olive). Right upper panel: quantified rates of KOAc-induced swelling amplitudes (t = 60 s) from 3 independent experiments. An overview of the swelling rate is given in Figure S5. One-way ANOVA with Dunnett’s multiple comparisons test performed against S. cerevisiae letm1Δ transformed with empty pVT-103U plasmid *p = 0.0426, **p = 0.0026, ***p = 0.0006, ****p < 0.0001. And for p.Ile305Leu and p.Lys587Arg relatively to S. cerevisiae letm1Δ transformed with WT, ns > 0.05, *p = 0.0169.

(B) Ectopic expression of LETM1 variants in S. cerevisiae letm1Δ. Isolated mitochondria (upper panel) and total protein lysates (lower panel) from the same strains as in (A). Subcellular fractions (T; total; SN; post-mitochondrial supernatant; M; mitochondria) (right lower panel) were immunoblotted using the indicated antibodies; Por1p and Act1p served as mitochondrial and total (and SN) loading control, respectively.

2094del], p.[Ile293Asn; Asp699Metfs13*]), F2 and F7 (both LETM1 c.2220G>C [p.*740Tyrfs26]), and F11 (LETM1 c.898C>T [p.Pro300Ser]), which showed reduced LETM1 levels in mitochondria (Figure 5B upper panel). The levels of LETM1 from the F5 LETM1 variant (c.1072G>A [p.Asp358Asn]) were also low, but not when normalized to Por1p, which was similarly decreased (Figure 5B). LETM1 levels from the variants identified in F3 (c.754–756del [p.Lys252del]) and in F4 and F8 (both c.881G>T [p.Arg294Gln]) were detectable in total lysates and mitochondria prepared from a large-scale intracellular fractionation (Figure 5B right lower panel) but were also

**Figure 5. Functional implication of LETM1 variants on yeast mitochondria**

(A) LETM1 variants fail to restore KHE activity of yeast letm1Δ. Isolated and de-energized mitochondria were subjected to KOAc and changes of optical density at OD540, immediately measured. Left upper panel: representative traces of KOAc-induced swelling in S. cerevisiae LETM1 WT mitochondria (WT, blue) or S. cerevisiae letm1Δ mitochondria overexpressing the empty plasmid (e, yellow) or the plasmid carrying human LETM1 WT untreated (w, green) or treated (wq, gray) with quinine or the human LETM1 variants; color code as indicated in the inserted table: c.754–756del (p.Lys252del) (1, red), c.878T>A (p.Ile293Asn) (2, bottle green), c.881G>T (p.Arg294Gln) (3, aqua), c.898C>T (p.Pro300Ser) (4, dark green), c.913A>C (p.Ile305Leu) (5, lavender), c.1072G>A (p.Asp358Asn) (6, violet), c.1139G>C (p.Arg380Pro) (7, beige), c.1178G>A (p.Arg393His) (8, turquoise), c.1760A>G (p.Lys587Arg) (9, mauve), c.2071–9C>G (p.Val691fs4*) (10, purple), c.2094del (p.Asp699Metfs13) (11, dark blue), compound (12, lilac), c.2220G>C (p.*740Tyrfs26) (13, olive). Right upper panel: quantified rates of KOAc-induced swelling amplitudes (t = 60 s) from 3 independent experiments. An overview of the swelling rate is given in Figure S5. One-way ANOVA with Dunnett’s multiple comparisons test performed against S. cerevisiae letm1Δ transformed with empty pVT-103U plasmid *p = 0.0426, **p = 0.0026, ***p = 0.0006, ****p < 0.0001. And for p.Ile305Leu and p.Lys587Arg relatively to S. cerevisiae letm1Δ transformed with WT, ns > 0.05, *p = 0.0169.

(B) Ectopic expression of LETM1 variants in S. cerevisiae letm1Δ. Isolated mitochondria (upper panel) and total protein lysates (lower panel) from the same strains as in (A). Subcellular fractions (T; total; SN; post-mitochondrial supernatant; M; mitochondria) (right lower panel) were immunoblotted using the indicated antibodies; Por1p and Act1p served as mitochondrial and total (and SN) loading control, respectively.
reduced. None of the ectopic expression of LETM1 variants, however, affected the mitochondrial subcellular localization. As previously noticed\textsuperscript{17,49,50} and shown here (Figure 5B), Cox2p (subunit of CIV) is reduced in \textit{S. cerevisiae} \textit{letm1Δ} strains. Cox2p levels were restored upon ectopic expression of human LETM1 WT or \textit{LETM1} c.878T>A (p.Ile293Asn) (F1:M), \textit{LETM1} c.2071–9C>G (p.Val691fs*4) (F10), or \textit{LETM1} c.[878T>A; 2094del], p.[Ile293Asn; Asp699Metfs13*] (F1:S1, F1:S2), but remained absent upon expression of \textit{LETM1} c.754–756del (p.Lys252del) (F3), \textit{LETM1} c.881G>A (p.Arg294Gln) (F4, F8), or \textit{LETM1} c.1072G>A (p.Asp358Asn) (F5) (Figure 5B).

\textit{S. cerevisiae letm1Δ} shows poor growth on non-fermentable (YPG) substrate.\textsuperscript{42} To determine the significance of the \textit{LETM1} variants in rescuing the growth defects of \textit{S. cerevisiae letm1Δ} compared to human \textit{LETM1} WT, serial dilutions of \textit{S. cerevisiae letm1Δ} strains overexpressing an empty plasmid or \textit{LETM1} variants or WT were spotted onto fermentable (YPD) and non-fermentable (YPG) plates and grown at 30°C or 37°C (Figure S4). We found a detrimental effect of the mutant phenotype by ectopic expression of \textit{LETM1} c.1072G>A (p.Asp358Asn) (F5) variant; this strain was able to grow on selective media but showed worsened growth defect on complete media. Growth was also slowed down at 37°C on YPD by \textit{LETM1} c.898C>T (p.Pro300Ser)) (F11). On YPG, a marginal rescue was obtained by ectopic expression of \textit{LETM1} c.881G>A (p.Arg294Gln) (F4, F8), \textit{LETM1} c.2071–9C>G (p.Val691fs*4) (F10), or \textit{LETM1} c.[878T>A; 2094del], p.[Ile293Asn; Asp699Metfs13*] (F1:S1, F1:S2) variants.

In summary, ectopic expression in \textit{S. cerevisiae letm1Δ} of human \textit{LETM1} variants associated with clinical presentations phenocopied \textit{S. cerevisiae letm1} loss of function, whereas expression of wild-type \textit{LETM1} restored the yeast deletion defects in non-fermentable growth and mitochondrial KHE exchange.

**Discussion**

\textit{LETM1} function is required for the maintenance of mitochondrial cationic and osmotic balance, and swelling of the matrix due to impaired \textit{LETM1} has far-reaching consequences. Matrix swelling is supported by the unfolding of inner membranes and loss of cristae invaginations and results in dilution of metabolic substrates. Here, we found that bi-allelic \textit{LETM1} variants identified in the affected individuals with severe clinical features differently affected the \textit{LETM1} levels and led to the typical aberrant mitochondrial morphology previously described for \textit{LETM1}-deficient cells. Several OXPHOS subunits were downregulated in fibroblasts or muscle tissue, enzymatic activities were reduced, and mtDNA copy number increased. The fact that nigericin, the synthetic KHE, restored morphological aberrations interconnects these phenotypes to impaired K\textsuperscript{+} homeostasis. Decreased membrane potential or increased sensitivity to valinomycin and normalization of this sensitivity by nigericin supports the presence of a defect in K\textsuperscript{+}/H\textsuperscript{+} exchange. Moreover, it is tempting to speculate that OXPHOS decreases proportionally to cristae loss. The finding that loss of KHE activity in \textit{LETM1}-defective yeast was restored by ectopic expression of wild-type \textit{LETM1} but not \textit{LETM1} variants strongly support the notion of deregulated mitochondrial K\textsuperscript{+} homeostasis caused by the \textit{LETM1} variants. Whether and how Ca\textsuperscript{2+} handling is also perturbed will need to be determined in future studies. Together with the fibroblasts, muscle biopsy, and yeast analyses, and with the prior knowledge that the mitochondrial phenotypes in cells match those caused by \textit{LETM1} haploinsufficiency, knockdown, or deletion in other eukaryotic species, the present findings amount to compelling evidence that the bi-allelic \textit{LETM1} variants are the cause of the disease in the pedigrees reported in this study.

**Diseases of mitochondrial morphology and dynamics**

Defects in non-OXPHOS genes responsible for mitochondrial homeostasis including mitochondrial fission and fusion have been suggested to cause primary MD (PMD).\textsuperscript{51} Primarily targeting the non-bioenergetic capabilities of the mitochondria, non-OXPHOS gene defects could indirectly affect the OXPHOS system,\textsuperscript{52} leading to a phenotype mimicking the inactivation of RCE.\textsuperscript{52} While there is a plethora of non-OXPHOS genes accounting for PMD,\textsuperscript{51,53,54} the examples relevant to the context of the present study are the genes regulating mitochondrial shape and interorganellar communication. They regulate mitochondrial dynamics through fusion and fission processes. Defects in these genes have been emerging as a cause of a novel class of inherited neurodegenerative disorders with variable onset ranging from infancy to adulthood.\textsuperscript{53,54} Residing in the outer and inner mitochondrial membranes or the cytosol, upon misregulation, they cause altered mitochondrial morphology including matrix swelling, fragmentation, elongation, and abnormal cristae structure, similar to what has been observed in abnormal \textit{LETM1} function.\textsuperscript{53–56} Reviews of the disease-causing genes responsible for mitochondrial dynamics are provided in Burté et al.\textsuperscript{53} and Navaratnarajah et al.\textsuperscript{15} To date, affected individuals diagnosed with diseases of mitochondrial dynamics present first and foremost with neurological symptoms.\textsuperscript{53,54} Being essential for the survival of all organisms tested so far and having important control over the mitochondrial osmotic balance, morphology, and dynamics, before now, bi-allelic variants in \textit{LETM1} have not been associated with any Mendelian disorder in humans.

**Bi-allelic \textit{LETM1} variants present with a phenotypic spectrum of MD largely involving the CNS**

Here we report on the association of bi-allelic \textit{LETM1} variants with a spectrum of predominantly infantile-onset neurological, metabolic, dysmorphic, and multiple organ...
dysfunction syndromes in a cohort of 18 affected individuals from 11 unrelated families. Overall, the disease had a progressive course, though with variable rates of deterioration. Hence, the disease progression varied from rapid (as in families 3, 4, and 9–11) to a slow deterioration (as in the oldest persons from families 1, 2, and 8). Similar to the clinical presentation of the defective mitochondrial dynamics genes, bi-allelic LETM1 variants were associated with an infantile-onset neurodegenerative disorder with a complex phenotype as described for DNMT1/DRP1 (Dynamin 1 like [MIM: 603850]), OPA1 (OPA1 mitochondrial dynamin-like GTPase [MIM: 605290]), OPA3 (Outer mitochondrial membrane lipid metabolism regulator OPA3 [MIM: 606580]), MFF (Mitochondrial fission factor [MIM: 614785]), and MTO1 (Misato mitochondrial distribution and morphology regulator 1 [MIM: 617619]).

The shared phenotype mainly included global developmental delay, regression, and neurosensory impairment combined with neuromuscular symptoms, cerebellar ataxia, seizures, and early mortality. Akin to defects in OPA3, 3-methylglutaconic aciduria was a frequent finding in the subjects with bi-allelic LETM1 variants.57 Bilateral cataracts and facial dysmorphism observed in the present LETM1 cohort have also been reported in individuals with defective OPA3 and MSTO1, respectively.55,56

All persons with RCE analysis results in the present study showed defects in the OXPHOS system suggesting that LETM1 defects can affect the mitochondrial ability to generate ATP. This in turn might have mimicked the clinical presentation of OXPHOS MD. Therefore, distinguishing the LETM1 phenotype from OXPHOS MD or the aforementioned diseases of mitochondrial dynamics can be challenging without the help of genetic testing, particularly in affected individuals with a rapidly progressive disease course.

The phenotype of defective LETM1 and WHS

Monoallelic LETM1 deletion has been suggested to be responsible for epilepsy and neuromuscular features in WHS.5,19,21,58 Indeed, the current LETM1 cohort presented with hypotonia and epilepsy. Additionally, though, persons with bi-allelic LETM1 variants showed a milder spectrum of WHS signs that has not been previously ascribed to the LETM1 deletion. These included thin habitus, low set ears, microcephaly, micrognathia, and low body weight.59,60 It has been previously speculated that the most probable cause of growth deficiency, microcephaly, and the characteristic facial features in WHS is due to haploinsufficiency of WHSC1, a region located far from LETM1.61 The expression of mild non-neurological symptoms of WHS in our cohort could be due to either putative interaction between LETM1 and WHSC1 or other undiscovered mechanisms, including those intrinsically caused by LETM1 deficiencies.

We have observed some degree of clinical overlap between the presentation of defective LETM1 and small interstitial deletions in WHS presenting with a milder phenotype. The latter presents with a variable degree of growth and neurodevelopmental delay, microcephaly, thin faces with dysmorphic features, intellectual disability, language impairment, and seizures.62–64 Interestingly, persons with small 4p16.3 deletions encompassing LETM1 suggested that LETM1 might not be responsible for seizures in WHS as some individuals with LETM1 deletion did not have seizures by the age of 4 and 9 years, whereas persons with preserved WHSC2-2 including LETM1 developed seizures.64 Previous retrospective analysis suggests that several other genes in the terminal 4p region might potentially be involved in seizures in WHS.5

Clinical features including lactic acidosis, diabetes, cataract, neuropathy, and proximal myopathy combined with cerebellar ataxia, progressive spastic-ataxic gait, hypertensive movement disorders, and pontine/cerebellar atrophy were among the signs of the defective LETM1 phenotype that are not typical of WHS; instead, they are more typical of archetypal mitochondrial disorders.

Although there have been a handful of reports on microdeletions in WHS describing genotype-phenotype correlations, the association between the specific symptoms of WHS and LETM1 remains to be fully determined. To understand the full contribution of LETM1 in WHS cases, further studies would be needed to investigate which phenotypes of WHS can be restored by the re-expression of LETM1. Apart from this, the identification of phenotypes that were consistent with both LETM1 haploinsufficiency in WHS and LETM1 bi-allelic variants will advance our understanding of the contribution of LETM1 in WHS.

Genotype-phenotype correlation of bi-allelic LETM1 variants

The general distribution of the missense and frameshift variants to the highly conserved LETM domain and the C-terminal coiled coils, together with their comparable deleterious effects on mitochondrial morphology and KHE function, support the correlation of mitochondrial morphologic defects and imbalanced cation homeostasis. A previous variant analysis of the LETM domain found that Asp359 or the triple combination of Arg382, Gly383, and Met384 is necessary for the organization of cristae structure and growth complementation of S. cerevisiae letm1Δ strains.65 The missense variant c.1072G>A (p.Asp358Asn) identified here in family 5, which impaired mitochondrial morphology and KHE activity, is adjacent to Asp359. Based on cell-free data showing that the reconstituted LETM domain was sufficient to induce cristae invagination, Nakamura et al.65 concluded that cristae disorganization due to the single or triple variant occurred independently of ion homeostasis. Our findings are not in contradiction but propose that a regulatory contribution to cristae architecture by the LETM domain may depend on the swelling state of mitochondria in the cellular context.

Given the growing consensus that the hallmark of LETM1 deficiency is mitochondrial cation imbalance, we
used yeast as a model organism to analyze mitochondrial KHE activity of LETM1 variants from affected individuals and LETM1 variants not associated with the disease. Based on the results, we propose that light-scattering experiments that capture mitochondrial volume status and kinetics of K\(^+\)/H\(^+\) exchange are useful to predict the pathogenic potential of LETM1 variants (Figure S5).

Linking clinical features with in vitro data, we found that fibroblasts expressing LETM1 variants c.[878T>A; 2094del], p.[lle293Asn; Asp699Metfs13*], which were identified in the individuals F1:S1 and F1:S2 affected with epilepsy, neurosensory deficiencies, and diabetes, displayed mitochondria with disturbed morphology and membrane potential, reduced LETM1 levels, and a severe decrease in respiratory proteins of CI and CIV. Ectopic expression of the variants in yeast marginally rescued decrease in respiratory proteins of CI and CIV. Ectopic expression of the variants in yeast marginally rescued mitochondrial KHE activity. Persons harboring the variant expression of the variants in yeast marginally rescued decrease in respiratory proteins of CI and CIV. Ectopic expression of this variant failed to rescue wild-type lack of protein was associated with the severe phenotypes. Ectopic expression of this variant escaped the LETM1 deficiency, the OXPHOS components (although some compensatory pathway (Figure 4C). Further examination will be required to clarify molecular compensatory mechanisms.

Other LETM1 variants were analyzed in yeast, as fibroblasts from affected individuals were not available. Yeast data revealed poor complementation of S. cerevisiae letm1Δ by human LETM1 c.754–756del (p.Lys252del), a variant identified in affected individuals with a neurological, neuromuscular, and craniofacial presentation, rapid progression, and eventually death (F3). Ectopic expression of LETM1 c.881G>A (p.Arg294Gln), identified in persons with variable disease progression (F4, F8) but similar neuromuscular deficiencies, was not able to restore the activity of the mitochondrial KHE, since the swelling traces revealed continuous but very slow kinetics indicating minimal KHE activity per time unit, thus suggesting leaky mitochondrial membranes. Yeast growth was also impaired by overexpression of this variant. Phenotypic data were rather consistent with severe clinical presentation and early demise in F4.

The affected individual from family 9 was homozygous for the LETM1 variant c.1139G>C (p.Arg380Pro) and presented respiratory insufficiency, epileptic encephalopathy, neuromuscular disorder, and rapid disease progression. The missense variant is located in the middle of the LETM domain, in proximity to the three highly conserved amino acid residues (Arg382, Gly383, Met384) described in Nakamura et al. 45 (Figure 2B), supporting an essential functional role of the LETM stretch between residues 380 and 384.

**LETM1 role in cation homeostasis and neurodegenerative phenotype of the cohort**

Among the mitochondrial EF-hand-containing proteins, LETM1 has been identified as essential across several cell lines in genome-wide essentiality screens. Functionally, LETM1 is required for maintaining mitochondrial homeostasis of K\(^+\) and was considered an essential component of the KHE. After LETM1 was identified in a genomic Drosophila RNAi screen for mitochondrial Ca\(^{2+}\)/H\(^+\) exchanger (CHX), it has been suggested to catalyze the exchange of Ca\(^{2+}\) against H\(^+\) in both directions in a ruthenium red-sensitive pattern, which is difficult to reconcile with the CHX, and has been implicated in the pathogenesis of Parkinson’s disease through interaction with PINK1 (PTEN-induced kinase 1 [MIM: 608309]). The mitochondrial CHX is part of the mitochondrial Ca\(^{2+}\) release system, which compensates for electrophoretic mitochondrial Ca\(^{2+}\) uptake mainly through H\(^+\) - or Na\(^+\)-dependent Ca\(^{2+}\) extrusion. While
the role of LETM1 as a mitochondrial KHE or CHX has remained controversial, deregulation of the mitochondrial KHE has been shown to affect mitochondrial Ca$^{2+}$ buffering by impacting the Na$^+$-dependent Ca$^{2+}$ release pathway. Proper maintenance of mitochondrial Ca$^{2+}$ levels is critical to neurons, synaptic function, and neurodevelopment with mishandled mitochondrial Ca$^{2+}$ levels posing a risk of synaptopathies. In turn, synaptopathies may be a harbinger of neurodegenerative disorders. The neurodegenerative phenotype observed in the present LETM1 cohort could partially be explained by impaired mitochondrial Ca$^{2+}$ buffering and ensuing glutamate excitotoxicity, generation of reactive oxygen species, and apoptosis. Consistent with previous studies, exposure to nigericin or ketone bodies improved the mitochondrial morphological phenotypes of fibroblasts from affected individuals, supporting the link between LETM1 variant and impaired cation homeostasis. While nigericin enables K$^+$-H$^+$ exchange and prevents accumulation of matrix K$^+$, ketone bodies may bypass the deficient Ca$^{2+}$-dependent catalytic function of the pyruvate dehydrogenase.

Unlike in yeast, LETM1 orthologs of more complex organisms possess EF-hands, which may implicate LETM1 in Ca$^{2+}$ sensing or regulation. Focusing on K$^+$ analysis using yeast, we did not investigate the impact of the reported bi-allelic LETM1 variants on the mitochondrial Ca$^{2+}$ homeostasis. This would need to be investigated in further studies as it might have future therapeutic implications.

Collectively, our results demonstrate that bi-allelic pathogenic LETM1 variants are associated with defective mitochondrial K$^+$ efflux, swollen mitochondrial matrix structures, and a reduction in proteins levels and activity of the electron transfer chain. The former highlights the implication of perturbed mitochondrial osmoregulation caused by bi-allelic LETM1 variants in neurological and mitochondrial pathologies. Data showing that mitochondrial KHE activity is maintained above a functional threshold in non-pathogenic variants suggest that such functional yeast assays could be implemented to routinely determine the pathogenicity of a variant. While the beneficial effect of nigericin strengthened the link to KHE defects, that of ketone bodies, consistent with Durigon et al., supports the promising therapeutic role of ketogenic-based diets.

Data and code availability
The accession numbers for the genetic variants reported in this paper are ClinVar: SCV001981656, SCV001981657, SCV001981658, SCV001981659, SCV001981660, SCV001981661, SCV001981662, SCV001981663, SCV001981664, and SCV001981665.

Supplemental information
Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2022.07.007.

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Declaration of interests
The authors declare no competing interests.

Web resources
Brain RNA-seq, http://www.brainmaseq.org/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
ClinVar (summary of submissions), https://www.ncbi.nlm.nih.gov/clinvar/submitters/26957
Database of Genome Variants, http://dgv.tcag.ca/
Ensemble, https://www.ensembl.org/index.html
FlyBase, http://flybase.org/
GeneMatcher, https://genematcher.org/
gnomAD, https://gnomad.broadinstitute.org/
Iranome, http://www.iranome.ir/
OMIM, https://www.omim.org/
TOPMed, https://bravo.sph.umich.edu/freeze8/hg38/gene/snv/LETM1
UKBB, https://www.ukbiobank.ac.uk/
Uniprot, https://www.uniprot.org/
Varnomen, http://varnomen.hgvs.org/
Varsome, https://varsome.com/
References

1. Gyimesi, G., and Hediger, M.A. (2020). Sequence features of mitochondrial transporter protein families. Biomolecules 10, 1611.

2. Nowikovsky, K., Pozzan, T., Rizzuto, R., Scorrano, L., and Bernardi, P. (2012). Perspectives on: SGP symposium on mitochondrial physiology and medicine: the pathophysiology of LETM1. J. Gen. Physiol. 139, 445–454.

3. Hajnóczky, G., Booth, D., Csdorás, G., Debattisti, V., Golénár, T., Naghdi, S., Niknejad, N., Paillard, M., Seifert, E.L., and Weaver, D. (2014). Reliance of ER-mitochondrial calcium signaling on mitochondrial EF-hand Ca2+-binding proteins: Miro5, MICT1, LETM1 and solute carriers. Curr. Opin. Cell Biol. 29, 133–141.

4. Endele, S., Fuhry, M., Pak, S.J., Zabel, B.U., and Winterpacht, A. (1999). LETM1, a novel gene encoding a putative EF-hand Ca(2+)-binding protein, flanks the Wolf-Hirschhorn syndrome (WHS) critical region and is deleted in most WHS patients. Genomics 60, 218–225.

5. Austin, S., and Nowikovsky, K. (2019). LETM1: essential for mitochondrial biology and cation homeostasis? Trends Biochem. Sci. 44, 648–658.

6. Lin, Q.T., and Stathopulos, P.B. (2019). Molecular mechanisms of leucine zipper EF-hand containing transmembrane protein-1 function in health and disease. Int. J. Mol. Sci. 20, E286.

7. Waldeck-Weiermair, M., Jean-Quartier, C., Rost, R., Khan, M.J., Vishnu, N., Bondarenko, A.I., Imamura, H., Galli, R., and Graier, W.F. (2011). Leucine zipper EF-hand-containing transmembrane protein 1 (Letm1) and uncoupling proteins 2 and 3 (UCP2/3) contribute to two distinct mitochondrial Ca2+ uptake pathways. J. Biol. Chem. 286, 28444–28455.

8. Froschauer, E., Nowikovsky, K., and Schwyen, R.J. (2005). Electroneutral K+/H+ exchange in mitochondrial membrane vesicles involves Yol027/Letm1 proteins. Biochim. Biophys. Acta 1711, 41–48.

9. Jiang, D., Zhao, L., Clish, C.B., and Clapham, D.E. (2013). Letm1, the mitochondrial Ca2+/H+ antiporter, is essential for normal glucose metabolism and alters brain function in Wolf-Hirschhorn syndrome. Proc. Natl. Acad. Sci. USA 110, E2249–E2254.

10. Shao, J., Fu, Z., Ji, Y., Guan, X., Guo, S., Ding, Z., Yang, X., Cong, Y., and Shen, Y. (2016). Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) forms a Ca2+/H+ antiporter. Sci. Rep. 6, 34174.

11. Hasegawa, A., and van der Bliek, A.M. (2007). Inverse correlation between expression of the Wolfs Hirschhorn candidate gene Let1 and mitochondrial volume in C. elegans and in mamma-lian cells. Hum. Mol. Genet. 16, 2061–2071.

12. McGuibban, A.G., Joza, N., Megighian, A., Scorzeto, M., Zanini, D., Reipert, S., Richter, C., Schwyen, R.J., and Nowikovsky, K. (2010). A Drosophila mutant of LETM1, a candidate gene for seizures in Wolf-Hirschhorn syndrome. Hum. Mol. Genet. 19, 987–1000.

13. Dimmer, K.S., Navoni, F., Casarin, A., Trevisson, E., Endele, S., Winterpacht, A., Salviati, L., and Scorrano, L. (2008). LETM1 deleted in Wolf-Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability. Hum. Mol. Genet. 17, 201–214.

14. Durigon, R., Mitchell, A.L., Jones, A.W., Manole, A., Mennuni, M., Hirst, E.M., Houliden, H., Maragni, G., Lattante, S., Doronizio, P.N., et al. (2018). LETM1 couples mitochondrial DNA metabolism and nutrient preference. EMBO Mol. Med. 10, 1–20.

15. Piao, L., Li, Y., Kim, S.J., Byun, H.S., Huang, S.M., Hwang, S.K., Yang, K.J., Park, K.A., Won, M., Hong, J., et al. (2009). Association of LETM1 and mrpl36 contributes to the regulation of mitochondrial ATP production and necrotic cell death. Cancer Res. 69, 3397–3404.

16. Kuum, M., Veksler, V., Liiv, J., Ventura-Clapier, R., and Kaasik, A. (2012). Endoplasmic reticulum potassium-hydrogen exchanger and small conductance calcium-activated potassium channel activities are essential for ER calcium uptake in neurons and cardiomyocytes. J. Cell Sci. 125, 625–633.

17. Lupo, D., Vollmer, C., Deckers, M., Mick, D.U., Tews, I., Sinning, I., and Rehling, P. (2011). Mdm38 is a 14-3-3-like receptor and associates with the protein synthesis machinery at the inner mitochondrial membrane. Traffic 12, 1457–1466.

18. Li, Y., Tran, Q., Shrestha, R., Piao, L., Park, S., Park, J., and Park, J. (2019). LETM1 is required for mitochondrial homeostasis and cellular viability (Review). Mol. Med. Rep. 19, 3367–3375.

19. Schlickum, S., Moghekar, A., Simpson, J.C., Steglich, C., O’Brien, R.J., Winterpacht, A., and Endele, S.U. (2004). LETM1, a gene deleted in Wolf-Hirschhorn syndrome, encodes an evolutionarily conserved mitochondrial protein. Genomics 83, 254–261.

20. Hart, L., Rauch, A., Carr, A.M., Vermeesch, J.R., and O’Driscoll, M. (2014). LETM1 haploinsufficiency causes mitochondrial defects in cells from humans with Wolf-Hirschhorn syndrome: implications for dissecting the underlying pathomechanisms in this condition. Dis. Model. Mech. 7, 535–545.

21. Zhang, X., Chen, G., Lu, Y., Liu, J., Fang, M., Luo, J., Cao, Q., and Wang, X. (2014). Association of mitochondrial letm1 with epileptic seizures. Cereb. Cortex 24, 2533–2540.

22. Park, J., Li, Y., Kim, S.H., Yang, K.J., Song, K., Shrestha, R., Tran, Q., Park, K.A., Jeon, J., Hur, G.M., et al. (2014). New players in high fat diet-induced obesity: LETM1 and CTMP. Metabolism 63, 318–327.

23. Sobreira, N., Schiettecatte, F., Valle, D., and Hamosh, A. (2015). GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. 36, 928–930.

24. Catania, A., Legati, A., Peverelli, L., Marchet, S., Zanetti, N., Lamperti, C., and Ghezzi, D. (2019). Homozygous variant in OTX2 and possible genetic modifiers identified in a patient with combined pituitary hormone deficiency, ocular involvement, myopathy, ataxia, and mitochondrial impairment. Am. J. Med. Genet. 179, 827–831.

25. Horga, H.A. (2019). Clinical and Genetic Investigations of Inherited Neuropathies and Mitochondrial Disease. PhD thesis (University College London).

26. Makrythanasis, P., Marooian, R., Stray-Pedersen, A., Musaev, D., Zaki, M.S., Mahmoud, I.G., Selim, L., Elbadawy, A., Jhangiani, S.N., Coban Akdemir, Z.H., et al. (2018). .LETM 1 couples mitochondrial DNA metabolism and nutrient preference. EMBO Mol. Med. 10, 1–20.

27. Poole, O.V., Pizzamiglio, C., Murphy, D., Falabella, M., Macken, W.I., Bugiardini, E., Woodward, C.E., Labrum, R., Efthymiou, S., Salpietro, V., et al. (2021). Mitochondrial DNA analysis from exome sequencing data improves diagnostic yield in neurological diseases. Ann. Neurol. 89, 1240–1247.

28. Barington, M., Risom, L., Ek, J., Uldall, P., and Ostergaard, E. (2018). A recurrent de novo CUX2 missense variant associated with
with intellectual disability, seizures, and autism spectrum disorder. Eur. J. Hum. Genet. 26, 1388–1391.

29. Van Bergen, N.J., Guo, Y., Rankin, J., Paccia, N., Becker-Kettner, J., Kremer, L.S., Pyle, A., Conrotte, J.F., Ellaway, C., Procopis, P., et al. (2019). NAD(P)HX dehydrogenase (NAXD) deficiency: a novel neurodegenerative disorder exacerbated by febrile illnesses. Brain 142, 50–58.

30. Froukh, T., Nafie, O., Al Hait, S.A.S., Laugwitz, L., Sommerfeld, J., Sturm, M., Baraghiti, A., Issa, T., Al-Nazer, A., Koch, P.A., et al. (2020). Genetic basis of neurodevelopmental disorders in 103 Jordanian families. Clin. Genet. 97, 621–627.

31. Retterer, K., Juusola, J., Cho, M.T., Valtakka, P., Millan, F., Gibellini, F., Vertino-Bell, A., Smoaoui, N., Neidich, J., Monaghan, K.G., et al. (2016). Clinical application of whole-exome sequencing across clinical indications. Genet. Med. 18, 696–704.

32. Bai, R., Cui, H., Devaney, J.M., Allis, K.M., Balog, A.M., Liu, X., Schnur, R.E., Shapiro, F.L., Brautbar, A., Estrada-Veras, J.I., et al. (2021). Interference of nuclear mitochondrial DNA segments in mitochondrial DNA testing resembles biparental transmission of mitochondrial DNA in humans. Genet. Med. 23, 1514–1521.

33. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., et al. (2015). ACMG laboratory quality assurance committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. Genet. Med. 17, 405–424.

34. Williamson, S.L., Rasanayagam, C.N., Glover, K.J., Baptista, J., Naik, S., Satodia, P., and Gowda, H. (2021). Rapid exome sequencing: revolutions the management of acutely unwell neonates. Eur. J. Pediatr. 180, 3587–3591.

35. Kremer, L.S., Bader, D.M., Mertes, C., Kopajtich, R., Pichler, G., Iuso, A., Haack, T.B., Graf, E., Schwarzmayer, T., Terriile, C., et al. (2017). Genetic diagnosis of Mendelian disorders via RNA sequencing. Nat. Commun. 8, 15824.

36. Calvo, S.E., Compton, A.G., Hershman, S.G., Lim, S.C., Lieber, D.S., Tucker, E.J., Laskowski, A., Garone, C., Liu, S., Jaffe, D.B., et al. (2012). Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. Sci. Transl. Med. 4, 118ra10.

37. Legati, A., Reyes, A., Nasca, A., Invernizzi, F., Lamantea, E., Piratini, V., Garavaglia, B., Lamperti, C., Ardissone, A., Moroni, I., et al. (2016). New genes and pathomechanisms in mitochondrial disease with targeted next-generation sequencing. Acta Physiol. Scand. 2016.1388–1391.

38. Saoura, M., Powell, C.A., Kopajtich, R., Alahmad, A., Balool, H.H., Albash, B., Alfadhel, M., Alston, C.L., Bertini, E., and Bonnen, P.E. (2019). Mutations in ELAC2 associated with hypertrophic cardiomyopathy impair mitochondrial tRNA 3′-end processing. Hum. Mutat. 40, 1731–1748.

39. Kušíková, K., Feichtinger, R.G., Csillag, B., Kaley, O.K., Weis, S., Duba, H.C., Mayr, J.A., and Weis, D. (2021). Case report and review of the literature: a new and a recurrent variant in the VAR2 gene are associated with isolated lethal hypertrophic cardiomyopathy, hyperlactatemia, and pulmonary hypertension in early infancy. Front. Pediatr. 9, 660076.

40. Wilfinger, N., Austin, S., Scheiber-Mojdehkar, B., Berger, W., Reipert, S., Praschberger, M., Paur, J., Trondl, R., Keppler, B.K., Zielinski, C.C., and Nowikovsky, K. (2016). Novel p53-dependent anticancer strategy by targeting iron signaling and BNP3L-induced mitophagy. Oncotarget 7, 1242–1261.

41. Acham-Roschitz, B., Plecko, B., Lindbichler, F., Bittner, R., Mache, C.J., Sperl, W., and Mayr, J.A. (2009). A novel mutation of the RRM2B gene in an infant with early fatal encephalomyopathy, central hypomyelination, and tubulopathy. Mol. Genet. Metab. 98, 300–304.

42. Nowikovsky, K., Froschauer, E.M., Zsurka, G., Samaj, J., Reipert, S., Kolisek, M., Wiesenberger, G., and Schweyen, R.J. (2004). The LETM1/YOL027 gene family encodes a factor of the mitochondrial Krebs cycle with a potential role in the Wolf-Hirschhorn syndrome. J. Biol. Chem. 279, 30307–30315.

43. Gietz, R.D., and Schiestl, R.H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2, 31–34.

44. Zotova, L., Aleschko, M., Sponder, G., Baumgartner, R., Reipert, S., Prinz, M., Schweyen, R.J., and Nowikovsky, K. (2010). Novel components of an active mitochondrial K(+)/H(+) exchange. J. Biol. Chem. 285, 14399–14414.

45. Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol. Rev. 79, 1127–1155.

46. Lee, H., and Yoon, Y. (2014). Transient contraction of mitochondria induces depolarization through the inner membrane dynamin OPA1 protein. J. Biol. Chem. 289, 11862–11872.

47. Baurerschmitt, H., Mick, D.U., Deckers, M., Vollmer, C., Funes, S., Kehrein, K., Ott, M., Rehling, P., and Herrmann, J.M. (2010). Ribosome-binding proteins Mdm38 and Mba1 display overlapping functions for regulation of mitochondrial translation. Mol. Biol. Cell 21, 1937–1944.

48. Mitchell, P., and Moyle, J. (1969). Translocation of some anions cations and acids in rat liver mitochondria. Eur. J. Biochem. 9, 149–155.

49. Frazier, A.E., Taylor, R.D., Mick, D.U., Warscheid, B., Stoezel, N., Meyer, H.E., Ryan, M.T., Guiard, B., and Rehling, P. (2006). Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery. J. Cell Biol. 172, 553–564.

50. Nowikovsky, K., Reipert, S., Devenish, R.J., and Schweyen, R.J. (2007). Mdm38 protein depletion causes loss of mitochondrial Krebs cycle activity, osmotic swelling and mitophagy. Cell Death Differ. 14, 1647–1656.

51. Wortmann, S.B., Mayr, J.A., Nuoﬀer, J.M., Prokisch, H., and Sperl, W. (2017). A guideline for the diagnosis of pediatric mitochondrial disease: the value of muscle and skin biopsies in the genetics era. Neuropediatrics 48, 309–314.

52. Niyazov, D.M., Kahler, S.G., and Frye, R.E. (2016). Primary mitochondrial disease and secondary mitochondrial dysfunction: importance of distinction for diagnosis and treatment. Mol. Syndromol., 122–137.

53. Burté, F., Carelli, V., Chinnery, P.F., and Yu-Wai-Man, P. (2015). Disturbed mitochondrial dynamics and neurodegenerative disorders. Nat. Rev. Neurol. 11, 11–24.

54. Navaratnarajah, T., Amand, R., Reichert, A.S., and Distelmaier, F. (2021). The relevance of mitochondrial morphology for human disease. Int. J. Biochem. Cell Biol. 134, 105951.

55. Nasca, A., Scotton, C., Zaharieva, I., Neri, M., Selvatici, R., Magnusson, O.T., Gal, A., Weaver, D., Rossi, R., Armaroli, A., et al. (2017). Recessive mutations in MTO1 cause...
mitochondrial dynamics impairment, leading to myopathy and ataxia. Hum. Mutat. 38, 970–977.

56. Ryu, S.W., Jeong, H.J., Choi, M., Karbowski, M., and Choi, C. (2010). Optic atrophy 3 as a protein of the mitochondrial outer membrane induces mitochondrial fragmentation. Cell. Mol. Life Sci. 67, 2839–2850.

57. Wortmann, S.B., Duran, M., Anikster, Y., Barth, P.G., Sperl, W., Zschocke, J., Morava, E., and Wevers, R.A. (2013). Inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature: proper classification and nomenclature. J. Inherit. Metab. Dis. 36, 923–928.

58. Zollino, M., Lecce, R., Fischetto, R., Murdolo, M., Faravelli, F., Selicorni, A., Butté, C., Memo, L., Capovilla, G., and Neri, G. (2003). Mapping the Wolf-Hirschhorn syndrome phenotype outside the currently accepted WHS critical region and defining a new critical region, WHSCR-2. Am. J. Hum. Genet. 72, 590–597.

59. Wilson, M.G., Towner, J.W., Coffin, G.S., Ebbin, A.J., Siris, E., and Brager, P. (1981). Genetic and clinical studies in 13 patients with the Wolf-Hirschhorn syndrome [del(4p)]. Hum. Genet. 59, 297–307.

60. Yamamoto-Shimojima, K., Kouwaki, M., Kawashima, Y., Ito, K., Momosaki, K., Ozasa, S., Okamoto, N., Yokochi, K., and Yamamoto, T. (2013). Mild Wolf-Hirschhorn syndrome derived from variable chromosomal abnormalities. Congenit. Anom. (Kyoto) 59, 169–173.

61. Zollino, M., and Doronzio, P.N. (2018). Dissecting the Wolf-Hirschhorn syndrome phenotype: WHSC1 is a neurodevelopmental gene contributing to growth delay, intellectual disability, and to the facial dysmorphism. J. Hum. Genet. 63, 849–857.

62. Okamoto, N., Ohmachi, K., Shimada, S., Shimojima, K., and Yamamoto, T. (2013). 109 kb deletion of chromosome 4p16.3 in a patient with mild phenotype of Wolf-Hirschhorn syndrome. Am. J. Med. Genet. 161A, 1465–1469.

63. Zollino, M., Orteschi, D., Ruiter, M., Pfundt, R., Steindl, K., Cañiero, C., Ricciardi, S., Contaldo, I., Chieffo, D., Ranalli, D., et al. (2014). Unusual 4p16.3 deletions suggest an additional chromosome region for the Wolf-Hirschhorn syndrome-Associated seizures disorder. Epilepsia 55, 849–857.

64. Nakamura, S., Matsui, A., Akabane, S., Tamura, Y., Hatano, A., Mityano, Y., Omote, H., Kajikawa, M., Maenaka, K., Moriyama, Y., et al. (2020). The mitochondrial inner membrane protein LETM1 modulates cristae organization through its LETM domain. Commun. Biol. 3, 99.

65. Maquat, L.E. (1995). When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. RNA 1, 453–465.

66. Filograna, R., Mennuni, M., Alsina, D., and Larsson, N.G. (2021). Mitochondrial DNA copy number in human disease: the more the better? FEBS Lett. 595, 976–1002.

67. Blomen, V.A., Maı́jek, P., Jae, L.T., Bigenzahn, J.W., Nieuwenhuys, J., Staring, J., Sacco, R., van Diemen, F.R., Olk, N., Stukalov, A., et al. (2015). Gene essentiality and synthetic lethality in haploid human cells. Science 350, 1092–1096.

68. Wang, T., Birsoy, K., Hughes, N.W., Krupczak, K.M., Post, Y., Wei, J.J., Lander, E.S., and Sabatini, D.M. (2015). Identification and characterization of essential genes in the human genome. Science 350, 1096–1101.

69. Jiang, D., Zhao, L., and Clapham, D.E. (2009). Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca2+/H+ antiporter. Science 326, 144–147.

70. Huang, E., Qu, D., Huang, T., Rizzi, N., Boonying, W., Krolak, D., Ciana, P., Woulfe, J., Klein, C., Slack, R.S., et al. (2017). PINK1-mediated phosphorylation of LETM1 regulates mitochondrial calcium transport and protects neurons against mitochondrial stress. Nat. Commun. 8, 1399.

71. Austin, S., Tavakoli, M., Pfeiffer, C., Seifert, J., Mattarei, A., De Stefani, D., Zoratti, M., and Nowikovsky, K. (2017). LETM1-mediated K+ and Na+ homeostasis regulates mitochondrial Ca2+ efflux. Front. Physiol. 8, 839.

72. Datta, S., and Jaiswal, M. (2021). Mitochondrial calcium at the synapse. Mitochondrion 59, 135–153.

73. Doonan, P.J., Chandramouorthy, H.C., Hoffman, N.E., Zhang, X., Cárdenas, C., Shanmugapriya, S., Rajan, S., Vallem, S., Chen, X., Foskett, J.K., et al. (2014). LETM1-dependent mitochondrial Ca2+ flux modulates cellular bioenergetics and proliferation. FASEB J. 28, 4936–4949.

74. Hashimi, H., McDonald, L., Stribrna, E., and Luke, J. (2013). TRYPA032 Letm1 protein is essential for mitochondrial potassium homeostasis. J. Biol. Chem. 288, 26914–26925.

75. Natarajan, G.K., Mishra, J., Camara, A.K.S., and Kwok, W.M. (2021). LETM1: a single entity with diverse impact on mitochondrial metabolism and cellular signaling. Front. Physiol. 12, 637852.