Mitochondrial FAD shortage in SLC25A32 deficiency affects folate-mediated one-carbon metabolism

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Abstract

The SLC25A32 dysfunction is associated with neural tube defects (NTDs) and exercise intolerance, but very little is known about disease specific mechanisms due to a paucity of animal models. Here, we generated homozygous (*Slc25a32*$^{Y174C/Y174C}$ and *Slc25a32*$^{K235R/K235R}$) and compound heterozygous (*Slc25a32*$^{Y174C/K235R}$) knock-in mice by mimicking the missense mutations identified from our patient. A homozygous knock-out (*Slc25a32*$^{-/-}$) mouse was also generated. The *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mice presented with mild motor impairment and recapitulated the biochemical disturbances of the patient. While *Slc25a32*$^{-/-}$ mice die in utero with NTDs. All *Slc25a32* mutations did not hinder the mitochondrial uptake of folates but specifically blocked the uptake of flavin adenine dinucleotide (FAD). A positive correlation between SLC25A32 dysfunction and flavoenzyme deficiency was observed. Besides the flavoenzymes involved in fatty acid β-oxidation and amino acid metabolism impaired, *Slc25a32*$^{-/-}$ embryos still had a subunit of glycine cleavage system–dihydrolipoamide dehydrogenase damaged, resulting in glycine accumulation and glycine derived-formate reduction, which further disturbed folate-mediated one-carbon metabolism, leading to 5-methyltetrahydrofolate shortage and other folate intermediates accumulated. Maternal formate supplementation increased the 5-methyltetrahydrofolate levels and ameliorated the NTDs in *Slc25a32*$^{-/-}$ embryos. The *Slc25a32*$^{K235R/K235R}$ mice had no glycine oxidation defect but had another formate donor-choline metabolism interrupted and mitochondrial folates deficient. Formate supplementation increased mitochondrial folates amounts of mice, but this effect was not restricted to the *Slc25a32* mutant mice. In summary, we established novel animal models, which enabled us to better understand the function of SLC25A32 and to elucidate the role of SLC25A32 dysfunction in human disease development and progression.

Introduction

Riboflavin-responsive exercise intolerance (RREI, #616839) is an autosomal recessive disorder caused by mutations in the *SLC25A32* gene (#138480). It was discovered in 2016, and several cases have been reported to date. These cases all had disease onset at childhood and presented with neuromuscular phenotypes, such as exercise intolerance, ataxia, myoclonus, dysarthria, and dysphagia [1, 2], or with only hypoketotic and hypoglycemia but no neuromuscular complications [3]. Metabolites analysis revealed biochemical features of multiple acyl-coenzyme A dehydrogenation deficiency (MADD). Supplementation with riboflavin, the precursor of flavin adenine dinucleotide (FAD), improved the clinical symptoms of the patients.

The *SLC25A32* gene locates on chromosome 8q22.3 and contains seven exons. It encodes a 35 kDa protein SLC25A32 [4] and is ubiquitously expressed in all tissues [5]. SLC25A32 was proposed as a mitochondrial FAD transporter [6], and the pathogenesis of RREI was considered caused by the mitochondrial FAD transport defect, which reduced the activities of mitochondrial flavoproteins involved in fatty acid β-oxidation (FAO) and respiratory-chain [1]. However, earlier studies suggested that SLC25A32 was a mitochondrial folate transporter [4, 7]. Variation in *SLC25A32* SNP rs17803441 was found to be associated with lower intracellular 5-methyltetrahydrofolate (CH3-THF) concentrations in human red blood cells [8]. Recently, biallelic loss of function variants in *SLC25A32* gene had been identified in the human fetus with neural tube defects (NTDs), and *Slc25a32* gene trapped knock-out mice also presented with cranial NTDs in embryonic stage [9]. It is well known that the folate-mediated one-carbon metabolism (FOCM) abnormality is the most critical modifier of risk associated with neural tube closure [10, 11].

In clinical, we identified a patient who carried novel *SLC25A32* point mutations and presented with muscle weakness and intractable clubfeet. Neither riboflavin supplementation nor surgery blocked the development of clubfeet. Previous studies indicated that clubfoot deformity was a manifestation of NTDs [12], which prompted us to reconsider the pathophysiology and treatment strategy of RREI. In this study, we first reviewed the clinical history of the case and performed a morphology study and neuropsychological evaluation for the patient. By mimicking the mutations identified from our patient, we generated multiple mice models with variable degrees of SLC25A32 dysfunctions to study the pathophysiology of RREI. Our results advanced our understanding of the relationship between the *Slc25a32* mutant and NTDs, and how SLC25A32 dysfunction affected mitochondrial flavoenzymes. New findings suggested potential therapeutic intervention for patients suffering from mitochondrial FAD deficiency.

Materials And Methods

Study approval

This study was performed in line with the principles of the Declaration of Helsinki. The ethics committee of Guangzhou Women and Children's Medical Center authorized this study, and the Institutional Animal Care and Use Committee of Guangzhou Medical University approved the animal studies. Informed consent has been obtained from the patient and her guardian.

Generation of Slc25a32 mutant mice models

Human *SLC25A32 c.521A > G* (*p.Y174C*) and *c.704A > G* (*p.K235R*) are conserved to mouse *Slc25a32* (NM_172402) *c.521A > G* (*p.Y174C*) and *c.704A > G* (*p.K235R*), respectively. CRISPR/Cas9 technology was utilized to generate the mouse mutants with the corresponding missense mutations. The sgRNA used for generating the *p.Y174C* mouse mutants was TATAAATATGAAGGTGTGCGTGG to match the forward strand of *Slc25a32* (PAM sequence was underscored), and oligo donor was
GGTGTTGCTAGCCCTTCACAGAGACAGTATAAAGGAATGTTTGATGCACTTGTGAAAATATGTAAATATGAAGGTGTGAGAGGATTATACAAGGTAACAAATTATCATGAA
The *p.Y174C* (*c.521A > G*, TAT to TGT) mutation in donor oligo was introduced into exon 4 by homology-directed repair, and a silent mutation (*p.R180=*, CGT to AGA) was co-introduced to prevent the binding and re-cutting of the sequence by sgRNA after homology-directed repair. The sgRNA used for generating the *p.K235R* mouse mutants was ATACGGGTATGTTGCTGCTACGG to match the reverse strand of *Slc25a32* (PAM sequence was underscored), and oligo donor was
ATCCTGCTTGTGTTCTCATTTTAGAGTACAGCAGAATACATCTCTGTCGCAGCGCTATCCAGAATATTTGCGGTAGCAGCAACATACCCGTATCAGGTTGTGAGAGCCCGC

The *p.K235R* (*c.704A > G*, AAA to AGA) mutation sites was introduced into exon 6 by homology-directed repair, and a silent mutation (*c.714C > G*, GCC to GCG) was co-introduced to prevent the binding and re-cutting of the sequence by sgRNA after homology-directed repair.

The Cas9 mRNA, sgRNA, and oligo donor were co-injected into fertilized C57BL/6 eggs. Founders with the expected mutation were identified by sequencing the PCR amplicons of tail DNA. Primers for identifying *p.Y174C* mutation were: Forward 5'-GATCACGTGGGCCTTCTTTATGC-3' and Reverse 5'-TTTACACCAATGAAGACACAGCTTATGG-3'. Primers for identifying *p.K235R* mutation were: Forward 5'-AGAACCCCATATTAAATAGACCTCGTG-3' and Reverse 5'-AAATGAGGAAATCAGGCTTCAGAGAC-3'.

The mutant mice were crossed to wild-type C57BL/6 mice for at least three generations to eliminate potential off-targeting mutations. Potential off-targeting sites were predicted using CCTOP (https://cctop.cos.uni-heidelberg.de/), and the whole *Slc25a32* coding region and flanking sequences were sequenced for potential off-targeting mutations. Specifically, in the generation of the *p.Y174C* mouse mutant, we obtained a mutant mouse with an additional *c.552G > A* (AAG to AAA) mutation at the end of the 4th exon. This mutation interfered with the splicing and produced no SLC25A32 protein. This mouse mutant was kept and used as a “knock-out” allele in this study. Genotyping was performed by sequencing the PCR amplicons of tail or embryo amnion, and the primers were: Forward 5'-AATATGGATTGCATGAAACAGTACC-3' and Reverse 5'-TGTACTCTGTAGTCTTGGATGGGAA-3'.

Four types of mating with $Slc25a32^{Y174C/+}$×$Slc25a32^{Y174C/+}$, $Slc25a32^{K235R/+}$×$Slc25a32^{K235R/+}$, $Slc25a32^{Y174C/+}$×$Slc25a32^{K235R/+}$, and $Slc25a32^{+/-}$×$Slc25a32^{+/-}$, were set up to obtain homozygous ($Slc25a32^{Y174C/Y174C}$ and $Slc25a32^{K235R/K235}$) and compound heterozygous ($Slc25a32^{Y174C/K235R}$) knock-in and homozygous ($Slc25a32^{-/-}$) knock-out mice. Wild-type (WT, $Slc25a32^{+/+}$) mice or embryos were used as controls. All mice were housed in an SPF facility with a 12-h light/dark cycle and allowed access to water and mouse chow *ad libitum*. Tissues (skeletal muscle and brain) used for this study were isolated from mice aged at 2–3 months, and embryos used in this study were dissected from dams at E11.5.

Slc25a32 gene mRNA expression

Total RNA was isolated from skeletal muscle or embryo using the TRIzol® Reagent (ambion®, Life technologies, USA) according to the manufacturer’s protocol. cDNA was synthesized from total RNA using the PrimeScript™ RT Master Mix kit (TAKARA, Japan). For the mouse mutants with *Slc25a32* missense mutations, real-time quantitative PCR was performed to determine the abundance of *Slc25a32* mRNAs by using the primers 5'-ATGGGTGACGAAAACTCGCCTT-3' and 5'-CGCACCATGTGATGTTCCAAA-3' on a LightCycler® 480II system (Roche, Switzerland) with SYBR® Premix Ex Taq™ II (TAKARA, Japan) reagent. Each sample was analyzed in triplicate. For the $Slc25a32^{-/-}$ mice, primers locating on exon 2 and 7 of *Slc25a32* (5'-AATATGGATTGCATGAAACAGTACC-3' and 5'-TCCACCGATGCCTTCTTTCC-3') were used to amplify a 678-bp amplicon of the *Slc25a32* coding region. The amplicons were then separated by gel electrophoresis and purified for DNA sequence analysis.

Western Blot analysis.

Total proteins from pulverized tissues were extracted with RIPA lysis buffer (P0013C, Beyotime, China) according to the protocol from the manufacturer. The amounts of SLC25A32 and nine mitochondrial flavoenzymes in the samples were measured by a capillary-based automated Simple Western system (Wes, ProteinSimple, San Jose, CA, USA) according to the protocol from the manufacturer. Primary antibodies were purchased and diluted with antibody dilution solution from the Protein Simple kit (DM-001) before use: anti-SLC25A32 (SAB2103325 1:50 dilution; Sigma); anti-ACADS (ab156571, 1:100 dilution; Abcam, Cambridge, UK); anti-ACADM (ab92461, 1:50 dilution; Abcam); anti-ACADVL (ab155138, 1:50 dilution, Abcam); anti-ACAD8 (A68138, 1:50 dilution; Epigentek, NY, USA); anti-ACADSB (13122-1-AP, 1:50 dilution; Proteintech, Rosemont, USA); anti-IVD (10822-1-AP, 1:100 dilution; Proteintech); anti-ETFA (12262-1-AP, 1:50 dilution; Proteintech); anti-ETFB (#43776, 1:50 dilution; Signalway Antibody); anti-ETFDH (11109-1-AP, 1:100 dilution; Proteintech); anti-GCSH (NBP1-85842, 1:50 dilution; Novus Biologicals, CO, USA); anti-glycine decarboxylase (ab232989, 1:50 dilution; Abcam); anti-DLD(16431-1-AP, 1:200 dilution; Proteintech); and anti-AMT (orb374296, 1:100 dilution; Biorbyt ,Cambridge, United Kingdom). Anti-GAPDH (AF7021, Affinity, OH, USA), anti-α-tubulin (11224-1-AP, Proteintech), or anti-HSP60 (15282-1-AP, Proteintech) was chosen as a loading control according to the molecular weight of the target protein. The secondary antibody, goat anti-rabbit IgG, was from the ProteinSimple kit.

Analysis of mitochondrial uptake of folates and flavins

Mitochondria were isolated from skeletal muscle or embryos by differential centrifugation according to the published protocol [13]. About 200 μg of mitochondria were resuspended in MiRO5 respiration medium (110 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KH_2PO4, 20 mmol/L taurine, 60 mmol/L K-lactobionate, 3 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, and 1 g/L fatty acid-free BSA adjust pH to 7.2 with KOH). The reactions were initiated by adding stable isotope-labeled [$^{13}C_5$]-FAD, [^{15}N]-folate (FA), [$^{13}C_4$,$^{15}N_2$]-riboflavin (Rf), or 5-formyl-tetrahydrofolate to a final concentration of 10μmol/L. Samples were incubated at 37°C. Mitochondria were pelleted by centrifugation (4°C, 14,000g for 2 min) after the reaction. And the pellet was washed twice with ice-cold mitochondrial isolation buffer. The mitochondria absorbed [$^{13}C_5$]-FAD, [^{15}N]-folate, [$^{13}C_4$,$^{15}N_2$]-riboflavin, and CHO-THF were extracted in ice-cold 4% perchloric acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Metabolites analysis

Quantification of folates intermediates and flavins (FAD, flavin mononucleotide (FMN) and riboflavin (Rf)) in mitochondria or embryos homogenates were obtained by an LC-MS/MS system (Shimadzu Ultrahigh Pressure Nexera chromatograph system (Kyoto, Japan) interfaced to a Sciex QTRAP 5500 + mass spectrometer (AB Sciex LLC, Framingham, MA, USA)) according to the published protocols [14, 15]. Analysis of acylcarnitine, amino acids, organic acids, DMG, and formate in tissues was carried out according to the published protocols by using the LC-MS/MS system (Shimadzu Ultrahigh Pressure Nexera

chromatograph system interfaced to an AB Sciex 3200 Q TRAP mass spectrometer (Foster City, CA, USA)) [16–19], or a gas chromatography-mass spectrometry system (GC-MS, Agilent 5975GC-4890MS, USA) [20].

Flavoenzyme activity assays

Nitrogen-chilled skeletal muscle tissues were first pulverized by CryoGrinder™ System and then homogenized in PBS by sonication. The enzymatic activities of short/branched-chain acyl CoA- (SBCAD), isovaleryl CoA- (IVD), isobutyryl CoA- (IBD), glutaryl CoA- (GCDH), short-chain- (SCAD), medium-chain- (MCAD), and very-long-chain acyl-CoA dehydrogenases (VLCAD), were determined based on the published protocols [21, 22]. The mitochondrial dihydrolipoamide dehydrogenase (DLDH) activity was measured by a spectrophotometer (BioTek Synergy HTX) according to the protocol described in reference [23]. Relative enzymatic activity of each enzyme in *Slc25a32* mutant mice was expressed as a ratio compared to the activity levels of the WT mice at the reaction system without exogenous FAD supplementation.

Measurement of mitochondrial glycine generated formate content.

Isolated mitochondria were resuspended in the incubation buffer (pH 7.4) consisted of 140 mmol/L KCl, 5.0 mmol/L HEPES, 4.0 mmol/L KPO_4, 2.5 mmol/L $MgCl_2$, 1.5 mmol/L EDTA, and 1.6 mmol/L ADP according to the reference [24]. The reaction was initiated by adding 1.0 μL of 0.5M [1,2-$^{13}C_2$]-glycine solution to 20μg of mitochondria. Samples were incubated at 37°C for 24 h. The generated [^{13}C]-formate was derivatized along with its internal standard [^{13}C, D]-formate by *o*-benzylhydroxylamine and analyzed by the LC-MS/MS system.

Statistics

All results are presented as mean values ± standard error of the mean (SEM). Statistical analysis was performed using SPSS. The two-tailed unpaired student's t-test is used to compare two groups. $P < 0.05$ was considered significant.

Results

An RREI case with novel SLC25A32 mutations

We have a female patient who came to our department at 7-year old complaining of dyspnea, fatigue, and growth retardation (Height 108cm (-3SD); Weight 18kg (-2SD~-1SD)). Blood gas analysis demonstrated that she suffered from acidosis with the pH at 7.276 and lactic acid elevated to 10.7 mmoL/L. Plasma metabolites analysis revealed a MADD-like biochemical profile with dimethylglycine (DMG), sarcosine, and multiple acylcarnitines accumulated (Fig. 1A). The patient was diagnosed with MADD and immediately received a low protein diet and oral supplementation of riboflavin and L-carnitine. After a 2-week treatment, the patient's dyspnea disappeared, and the biochemical abnormality ameliorated. From then on, the patient received riboflavin (30mg P.O tid) and L-carnitine (1g P.O qid) supplementation with a normal diet. Now, the patient is 20-yr-old with nearly normal growth (Height 155cm (-1SD); Weight 58kg (mean 51kg)).

Before coming to our department, the patient was found to be unwilling to walk after suffering from influenza at 1.6-year old. Her lower extremity developed to equinovarus and talipes cavus progressively. The right foot was more severe than the left one initially. She received orthopedic surgery to correct the right leg at 5.5-year old, and the surgery was successful (Fig. 1B). Unexpectedly, the deformity of her left leg became serious gradually even under riboflavin treatment, and she received the second orthopedic surgery at 9-year old. However, this operation did not improve her symptom. In contrast, it proceeded.

A morphology study was performed to investigate the etiology of lower extremity weakness and clubfeet of the patients. The magnetic resonance imaging (MRI) revealed enlarged left lateral ventricle (Fig. 1C). Electromyography found no abnormality in the peroneal nerve, and the signal transmission between peroneal nerves to muscle was fluent. X-ray imaging of tibiofibula bones showed that the left fibulae of the patient were abnormally slim (Fig. 1D).

The patient then underwent a comprehensive neuropsychological evaluation at 15.8-year old to identify whether neurologic impairment was involved. The gross motor function measure (GMFM) was carried out at first, and results showed that the patient had scores in the D (standing) and E (walking, running, and jumping) domains at 90% and 60% of total domain scores, respectively, indicating an inferior motor function in these domains. The intelligence test (Wechsler Intelligence Scale for Children IV) demonstrated that the patient's intelligence was in the range of borderline with a full-scale intelligence quotient at 71. In addition, her auditory- and visual-working memory function had mild to moderate defects with scores at 9 and 6, respectively.

MADD is commonly caused by defects in electron transportation due to variants in the *ETFA*, *ETFB*, or *ETFDH* genes. However, Sanger sequencing found no mutation in these genes of the patient. Whole-exome sequencing was then performed, and compound heterozygous mutations in *SLC25A32* were identified: *c.521A > G* (*p.Y174C*) and *c.704A > G* (*p.K235R*) (Fig. 1E), which located at the fourth and sixth exon of *SLC25A32*, respectively. Testing the mutations in parental samples confirmed that the unaffected parents were heterozygous for the mutations. Neither variant was listed in the gnomAD database. Both affect residues were highly conserved across species (the *p.Y174* conserved up to *Drosophila melanogaster*, and the *p.K235* conserved up to *Saccharomyces cerevisiae*). Protein structure homology modeling analysis using the mitochondrial ADP/ATP carrier (PDB ID: 6GCI) as the template suggested that K235 was located at the substrate-binding pocket and Y174 at the second matrix loops facing the mitochondrial matrix (Fig. 1F). Both variants were likely to damage the SLC25A32 protein by 4 of 4 in silico tools -Protein Variation Effect Analyzer (PROVEAN), Sorting Intolerant from Tolerant (SIFT), PolyPhen, and MutationTaster.

In summary, the phenotypes of our patient include riboflavin responsive-dyspnea, -growth retardation, -muscle weakness, and -exercise intolerance, but riboflavin unresponsive-clubfeet and mild neuropsychological abnormality. We wondered whether mitochondrial folate transport deficiency was involved in RREI, and the intractable clubfoot was the phenotype of folate transport defect. To answer these questions, we performed this study.

Various phenotypes were observed in mice carrying different Slc25a32 mutations.

The homozygous (*Slc25a32*$^{Y174C/Y174C}$ and *Slc25a32*$^{K235R/K235}$) and compound heterozygous (*Slc25a32*$^{Y174C/K235R}$) knock-in mice were born with the expected Mendelian ratio. No genotype effect was observed on birth weight, neonatal growth, or adult weight. Clubfoot was not observed in mice models after examining the gait of 40 *Slc25a32*$^{Y174C/Y174C}$, 67 *Slc25a32*$^{K235R/K235R}$, and 39 *Slc25a32*$^{Y174C/K235R}$ mice. Quantitative real-time PCR revealed the *Slc25a32*$^{Y174C/Y174C}$ mice, but not the *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mice, had significantly lower *Slc25a32* mRNA expression in muscle when compared with the WT mice (Fig. 2A). The western blot analysis revealed that the muscular SLC25A32 protein amounts in the *Slc25a32* mutant mice were comparable to that in WT mice (Fig. 2B). To assess whether the mutant mice had motor impairment as our patient had, we performed hanging wire test, rotarod test, and forced swimming test (FST) following the published protocols [25–27] to test the muscle strength, exercise coordination, and anti-fatigue capacity of mice. Adult mice aged 2 to 3 months (weighed 18.6 ± 1.6 g) were used. The times hanging on the wire were shorter for the *Slc25a32*$^{Y174C/K235R}$ and *Slc25a32*$^{K235R/K235R}$ mice than for their WT littermates, though the difference did not reach significance (Fig. 2C). The *Slc25a32*$^{K235R/K235R}$ mice also performed worse in the rotarod test and FST than their WT littermates did (Fig. 2D). No difference was observed in these behavior tests between *Slc25a32*$^{Y174C/Y174C}$ and WT mice.

No *Slc25a32*$^{-/-}$ pup was born from the *Slc25a32*$^{+/-}$×*Slc25a32*$^{+/-}$ mating. Embryos collected at the embryonic day of 11.5 (E11.5, the post-neurulation-stage in normal mouse embryos) showed that 89.5% (n = 94) of *Slc25a32*$^{-/-}$ embryos had NTDs with exencephaly or craniorachischisis phenotypes, while the rest (n = 11) had normal neural tube closure but severe growth retardation (Fig. 2E). No *Slc25a32* mRNA expression was detected in *Slc25a32*$^{-/-}$ embryos (Fig. 2F). Reverse transcription PCR using primers located on exon 2 and 7 of the *Slc25a32* gene showed that a 678 bp cDNA fragment observed in WT embryo was absent in *Slc25a32*$^{-/-}$ embryos. Instead, fragments at 517 bp and 431 bp were presented (Fig. 2G). Sequence analysis revealed that the 517 bp and 431 bp fragments were truncated transcripts that lacked exon 4 and both exon 3 and 4, respectively. The SLC25A32 protein was almost undetectable in *Slc25a32*$^{-/-}$ embryos (Fig. 2H).

The SLC25A32 is not a mitochondrial folate transporter but plays an essential role in FOCM.

To investigate whether folate transport deficiency was involved in RREI, we first analyzed the folate profile of mitochondria isolated from the skeletal muscle of mice. The tetrahydrofolate (THF) and 5,10-methenyl-tetrahydrofolate (CH-THF) were the primary folate intermediates in mitochondria, accounting for > 90% of the total folate. The *Slc25a32* $^{K235R/K235R}$ mice, but not *Slc25a32*$^{Y174C/Y174CR}$ mice, had significantly lower levels of all folate intermediates than the WT mice had (Fig. 3A). We further tested the mitochondrial folates uptake rate. The stable isotope-labeled [^{15}N]-FA and CHO-THF were used as substrates. The results showed that neither the uptake of [^{15}N]-FA nor the uptake of CHO-THF was hindered by any of the *Slc25a32* mutants (Fig. 3B), which suggested that mitochondrial folates deficiency in *Slc25a32*$^{K235R/K235R}$ mice was not caused by folate transport defect.

To identify whether the *Slc25a32* mutation point or mice's developmental stage affected the function of SLC25A32, we isolated mitochondria from the embryos of *Slc25a32*$^{K235R/K235R}$, *Slc25a32*$^{-/-}$ and their WT siblings to tested their ability to uptake [^{15}N]-FA and CHO-THF. The experiment found no deficiency in the uptake of folates for mitochondria from *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{-/-}$ embryos (Fig. 3C). We further analyzed the folate profiles of *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{-/-}$ embryos. Unlike the mitochondrial folate profile, the whole embryo folate profile had the CH3-THF as the primary folate intermediate, while the level of CHO-THF was too low to be detected. Compared with controls, the *Slc25a32*$^{K235R/K235R}$ embryos had slightly disturbed folate profiles with only CH-THF levels decreased, while the *Slc25a32*$^{-/-}$ embryos had thoroughly disturbed folate profiles: the amounts of dihydrofolate (DHF), THF, CH-THF, and 5,10-methylene THF (CH2-THF) significantly increased, and the amounts of CH3-THF significantly decreased (Fig. 3D). As mentioned above, 10.5% of *Slc25a32*$^{-/-}$ embryos showed normal neural tube closure. We noticed that the *Slc25a32*$^{-/-}$ embryos without NTDs had the level of CH3-THF comparable to the WT embryos had (Fig. 3D), which were different from the *Slc25a32*$^{-/-}$ embryos with NTDs and suggested a close relationship between CH3-THF and NTDs.

The previous study had evidenced that maternal CH3-THF supplementation failed to rescue the NTDs of *Slc25a32* knock-out mice embryos, but formate could [9]. We repeated the same experiment but used 0.1mol/L sodium formate instead of calcium formate in the dam's drinking water. We collected 73 embryos from 9 litters, and genotyping revealed that there were 16 *Slc25a32*$^{+/+}$, 41 *Slc25a32*$^{+/-}$, and 16 *Slc25a32*$^{-/-}$ embryos. Of which 87.5% of *Slc25a32*$^{-/-}$ embryos completed neural tube closure (Fig. 3E). Analyzing the folate profile again showed that the levels of CH3-THF were significantly higher, and the levels of CH-THF were significantly lower in *Slc25a32*$^{-/-}$ embryos without NTDs than in their WT siblings (Fig. 3F). The levels of DHF, THF, and CH2-THF decreased in *Slc25a32*$^{-/-}$ embryos upon formate treatment, but they were still significantly higher when compared with the WT embryos. 12.5% of *Slc25a32*$^{-/-}$ embryos still had NTDs after formate supplementation, whose folate profiles were similar to the *Slc25a32*$^{-/-}$ embryos with NTDs before formate treatment. In summary, the data proved that the *Slc25a32* mutant did not interrupt mitochondrial folate transport, but folate metabolism disorder existed in *Slc25a32* mutant mice, which might derive from formate deficiency.

Slc25a32 $^{-/-}$ mice embryos suffered from glycine cleavage defect due to deficiency in dihydrolipoamide dehydrogenase (DLDH).

We quantified the formate in the whole embryos and found that the formate amounts in *Slc25a32*$^{-/-}$ embryos were about 70% of controls (Fig. 4A). Formate is an intermediate of one-carbon metabolism and is produced through the mitochondrial metabolism of metabolic substrates, including serine, glycine,

sarcosine, and dimethylglycine (DMG) [11]. Analysis of these metabolic substrates in *Slc25a32*$^{K235R/K235R}$and *Slc25a32*$^{-/-}$ embryos showed that the *Slc25a32*$^{-/-}$ embryos had significantly increased glycine and serine contents, whereas *Slc25a32*$^{K235R/K235R}$ had significantly increased serine but significantly decreased glycine contents (Fig. 4B) when compared with WT embryos. No significant difference was detected between *Slc25a32* mutant and WT embryos in sarcosine and DMG levels.

The more than three-fold increase of glycine was specifically identified in the *Slc25a32*$^{-/-}$ embryos, which led us to hypothesize that formate deficiency found in *Slc25a32*$^{-/-}$ embryos resulted from glycine cleavage defect. To prove the hypothesis, we measured the mitochondria produced formate using a stable isotope-labeled [$^{13}C_2$]-glycine as the substrate. The amounts of [^{13}C]-formate produced by *Slc25a32*$^{-/-}$ embryo mitochondria was about 10.6% of controls (Fig. 4C), uncovering a causative role of glycine oxidation deficit in *Slc25a32*$^{-/-}$ embryos. We further analyzed the protein contents of the four subunits of glycine cleavage system (GCS), and results showed that the amounts of amino methyltransferase (AMT), glycine decarboxylase (GLDC), and hydrogen carrier protein (GCSH) in *Slc25a32*$^{-/-}$ embryos were comparable to that in WT embryos, but the amounts of DLDH were significantly lower in *Slc25a32*$^{-/-}$ embryos than in WT embryos (Fig. 4D). The enzymatic activity assay further revealed that the mitochondrial DLDH activity in *Slc25a32*$^{-/-}$ was about 23.9% of controls (Fig. 4E). DLDH is an oxidoreductase in the mitochondrial matrix, and its activity is dependent on the presence of both FAD and NAD [28].

The Slc25a32 mutations specifically blocked the mitochondrial uptake of FAD.

We then hypothesized that DLDH deficiency in *Slc25a32*$^{-/-}$ mitochondria resulted from the deprivation of FAD due to SLC25A32 dysfunction. SLC25A32 had been identified as a mitochondrial FAD transporter based on the experiment that transfection of the human *SLC25A32* gene functionally complemented the yeast strain with a mutant *FLX1* (gene encoding yeast FAD transporter) [6]. Here, we first measured the mitochondrial contents of FAD as well as its precursors, FMN and Rf, in mitochondria isolated form mice and embryos. Compared with WT controls, the *Slc25a32*$^{Y174C/K235R}$ and *Slc25a32*$^{K235R/K235R}$ mice, but not the *Slc25a32*$^{Y174C/Y174C}$ mice, had significantly lower FAD, FMN, and/or Rf contents (Fig. 5A). The *Slc25a32*$^{-/-}$ embryos also had mitochondrial FAD, FMN, and Rf shortages (Fig. 5B). By analyzing the mitochondrial uptake of stable isotope-labeled [$^{13}C_5$]-FAD and [$^{13}C_{4,}{}^{15}N_2$]-Rf, we found the *Slc25a32 K235R* mutation specifically blocked the mitochondria uptake of FAD. The [$^{13}C_5$]-FAD uptake rate was in the order of *Slc25a32*$^{+/+}$, *Slc25a32*$^{Y174C/Y174C}$, *Slc25a32*$^{Y174C/K235R}$, and *Slc25a32*$^{K235R/K235R}$, and significantly lower uptake rates were observed in *Slc25a32*$^{Y174C/K235R}$ and *Slc25a32*$^{K235R/K235R}$ mitochondria, when compared with controls (Fig. 5C). Instead, *Slc25a32* mutations did not hinder the uptake of [$^{13}C_{4,}{}^{15}N_2$]-Rf, and the *Y174C* mutation even promoted the uptake of Rf, which suggested that the mitochondrial Rf deficiency in *Slc25a32* mutant mice derived from FAD shortage instead of Rf transport deficiency. Knock-out of *Slc25a32* also disabled the mitochondria to transport [$^{13}C_5$]-FAD, as indicated by the low abundance of [$^{13}C_5$]-FAD in the mitochondria of *Slc25a32*$^{-/-}$ embryos and high abundance of [$^{13}C_5$]-FAD in the incubation buffer of *Slc25a32*$^{-/-}$ embryos (Fig. 5D).

Slc25a32 mutations caused various degrees of damage on mitochondrial flavoenzymes.

Our data proved that the *Slc25a32* mutations affected the uptake of FAD into the mitochondria. FAD was a cofactor of multiple mitochondrial dehydrogenases involved in FAO, amino acids, and choline metabolisms [29]. By quantifying acylcarnitine levels, we found *Slc25a32*$^{-/-}$ embryos had MADD-like acylcarnitine profiles with significantly elevated levels of butyryl-, isobutyryl-, 2-methylbutyryl-, isovaleryl-, glutaryl-, hexanoyl-, octanoyl-, and stearoyl-carnitine, and significantly decreased levels of free carnitine, acetyl-, propionyl-, 3-hydoxylbutyryl-, 3-hydroxyl-isovaleryl-, 3-hydroxylhexanoyl-, 3-hydroxyloctanoyl-, and 3-hydroxyldecanoyl-carnitine, when compared with WT embryos (Fig. 6A). For the adult *Slc25a32* mutant mice, the *Slc25a32*$^{K235R/K235R}$ mice had the severest metabolic disturbance, *Slc25a32*$^{Y174C/K235R}$ mice had a less severe metabolic disorder, and *Slc25a32*$^{Y174C/Y174C}$ mice had no disorder, in muscle (Fig. 6B) and brain tissues (Fig. 6C), which revealed a positive correlation between FAD transport deficiency and metabolic disturbance. In addition, organ-specific involvement was observed. The *Slc25a32*$^{K235R/K235R}$ mice had the skeletal muscle and brain most severely affected, but heart, kidney, and liver less affected (data not shown). The *Slc25a32*$^{Y174C/K235R}$ mice had almost normal muscle acylcarnitine profiles but abnormal brain acylcarnitine profiles (Fig. 6B and C). Further analysis of organic acids and choline metabolites found glutaric acid and DMG accumulated in skeletal muscle and/or brain tissues of *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mice (Fig. 6D and E), which was consistent with the plasma biochemical profile of the patient.

SBCAD, IVD, IBD, and GCDH are flavoenzymes involved in branched-chain amino acids and lysine metabolisms, and SCAD, MCAD, VLCAD are flavoenzymes participated in FAO. The enzymatic activity assays confirmed that all these flavoenzymes' activities were significantly lower in *Slc25a32*$^{K235R/K235R}$ mice than in WT controls (Fig. 7A-G). The activities of GCDH and SCAD were also found decreased in *Slc25a32*$^{Y174C/K235R}$ mice. FAD supplementation *in vitro* completely restored the function of SBCAD (Fig. 7A) and greatly increased the activities of IVD and GCDH (Fig. 7B and C), but only mildly improved the activities of SCAD, MCAD, VLCAD, and IBD (Fig. 7D-G), in *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mice. The western blot assays further revealed that the amounts of some flavoenzymes' apo-proteins were significantly lower in *Slc25a32*$^{K235R/K235R}$ mice than in WT controls (Fig. 7H), and the reduction degrees correlated with their responses to FAD supplementation. We still tested the amounts of flavoenzymes involved in electron transportation and found that the amounts of ETFα, but not ETFβ and ETFDH, were significantly lower in *Slc25a32*$^{K235R/K235R}$ mice than in controls (Fig. 7H).

Effect of riboflavin and formate supplementation on Slc25a32 mutant mice.

Since FAD could be synthesized from riboflavin in mitochondria [30], we tried riboflavin supplementation (1g/kg riboflavin in diet) for *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mice. After a one-month treatment, the mutant mice had no improvement in motor function. Further mitochondria flavins analysis found no increase of riboflavin and FAD levels, which suggested that the oral supplemented riboflavin had not been absorbed effectively by mice. Since the *Slc25a32*

mutant mice suffered from mitochondrial folate shortage, we added 0.1 M sodium formate in their drinking water to study whether formate could improve folate shortage. After a 3-week treatment, all mice had folates amounts elevated with the WT mice had total folates elevated to (13.03 ± 0.41) pmol/mg protein from (5.33 ± 0.58) pmol/mg protein, and *Slc25a32*$^{K235R/K235R}$ mice had total folates elevated to (6.08 ± 0.59) pmol/mg protein from (2.34 ± 0.70) pmol/mg protein (Fig. 8). This folates elevation effect was not restricted to the *Slc25a32* mutant mice, the disparity in folates amounts between *Slc25a32*$^{K235R/K235R}$ and WT mice still exist.

Discussion

It has long been appreciated that SLC25A32 was a mammalian mitochondrial folate transporter [4, 7]. The first aim of this study was to identify whether folate transport defect was involved in RREI. Our study uncovered a more complex relationship between SLC25A32 and folates. The SLC25A32 did not transfer folate into mitochondria, but SLC25A32 dysfunction disturbed FOCM. SLC25A32 was a mitochondrial FAD transporter as documented in this study. The *Slc25a32* mutations not only deprived the cofactor of mitochondrial flavoenzymes but also decreased their apo-protein amounts. A positive correlation between SLC25A32 dysfunction and mitochondrial flavoenzymes defect was found after studying the mice models with different *Slc25a32* mutations. Knock out of *Slc25a32* resulted in the most severe phenotype. It not only disrupted the function of multiple mitochondrial acyl-CoA dehydrogenases related to FAO and amino acid metabolism but also damaged the subunit of GCS–DLDH. A previous study indicated that the correct folding of the DLDH depended on both FAD and NAD [28]. NAD deficiency inhibited the overall activity of GCS by competitive conjugation of apo-DLDH with other subunits of GCS [31]. In this study, the severely impaired GCS activity (reduced 89.4%) and mildly decreased DLDH protein content (decreased 26.8%) in *Slc25a32*$^{-/-}$ embryos suggested that FAD deficiency should have the same effect as NAD deficiency to GCS. The glycine was a one-carbon donor in the neurulation stage of embryos, contributing to de novo purine biosynthesis [32]. The dysfunction of other subunits of GCS, such as GLDC and AMT, in humans and mice, had been evidenced with NTDs phenotype [33, 34]. Our study confirmed that GCS deficiency reduced formate generation, which further interrupted folate metabolism, leading to CH3-THF shortage and accumulation of other folate intermediates. The formate supplementation effectively increased the CH3-THF levels and ameliorated the NTDs in *Slc25a32*$^{-/-}$ embryos. However, formate supplementation was unable to extend normal fetal development to term. Growth retardation was still evidenced in *Slc25a32*$^{-/-}$ embryos as indicated by the shorter crown-rump length and undeveloped blood circulation system when compared with their WT siblings (Fig. 3E).

The *Slc25a32 K235R* mutation induced the second severe phenotype in mice. *Slc25a32*$^{K235R/K235R}$ mice recapitulated the biochemical disturbance observed in our patient, although the phenotype was mild and clubfoot was not observed in them. The mitochondrial folates deficiency found in the model provided insights into the cause of clubfeet, ventriculomegaly, and neuropsychological abnormality of the patient. Clubfeet is a feature of many neurological syndromes, and its etiology was related to genetic, maternal, and environmental factors. Improving maternal folate status through peri-conceptional folic acid supplementation or nutritional fortification with folic acid resulted in decreased prevalence of clubfoot-affected births [35, 36]. The babies with the less common variant of the *C677T* variant in the gene encoding methyltetrahydrofolate reductase were found less likely to develop clubfoot [37]. On the other hand, ventriculomegaly had been evidenced in rats with folate deficiency diet [38, 39]. A recent study uncovered that impaired folate one-carbon metabolism caused aqueduct stenosis, resulting in hydrocephalus and ventriculomegaly in mice with GLDC deficiency [40]. These studies revealed a strong link between folate and clubfoot and ventriculomegaly, which were consistent with our results. Unlike the *Slc25a32*$^{-/-}$ mice, *Slc25a32*$^{K235R/K235}$ mice had no glycine accumulation, suggesting glycine oxidation was undisturbed. However, they had another one-carbon source DMG accumulated. DMG is an intermediate of choline metabolism, which converts to sarcosine and donates one-carbon units to the folates cycle under the function of FAD-dependent dimethylglycine dehydrogenase (DMGDH) [41]. DMGDH deficiency alone had been identified in a patient with muscle fatigue and a fish-like odor [42]. The relationship between DMGDH deficiency and mitochondrial folate deficiency was unclear. In this study, elevated serine levels and decreased glycine levels in *Slc25a32*$^{K235R/K235}$ mice embryos were observed, and elevated serine levels were also noted in the adult *Slc25a32*$^{K235R/K235}$ mice brain tissues ((14.39 ± 0.65 vs. 16.64 ± 0.50) pmol/mg protein, WT vs. *Slc25a32*$^{K235R/K235}$), which probably due to a decreased utilization of serine for formate synthesis or an enhanced *de novo* synthesis of serine from the glycolytic intermediate 3-phosphoglycerate to support one-carbon requirement. No matter which mechanism contributed to the serine elevation, these data suggested one-carbon deficiency in *Slc25a32* mutant mice. It is reasonable to expect that the *Slc25a32* mutant interrupted the choline metabolism, leading to a reduction of mitochondrial one-carbon unit flux, which further affected mitochondrial folate and interrupted serine metabolism. Experiments to test this hypothesis are underway.

Mitochondrial FAD deficiency could be caused not only by SLC25A32 dysfunction but also by other inborn errors, such as electron transportation defects due to variants in *ETFα*, *ETFβ*, and *ETFDH* [43], riboflavin transport disorders due to variants in *SLC52A1*, *SLC52A2*, and *SLC52A3* [44], and FAD synthase deficiency due to variants in *FLAD1* [45]. This suggested the potential for a shared biochemical mechanism underlying these inborn errors. Mitochondrial folate deficiency might be a common biochemical character of these diseases and played a role in neurodevelopmental and cognitive dysfunctions of the patients. Riboflavin supplementation ameliorated some of the patients' clinical symptoms, but failed to improve others. In this study, oral riboflavin supplementation failed to rescue the NTDs of *Slc25a32*$^{-/-}$ embryos and motor impairment of *Slc25a32* mutant mice, further analysis identified the mitochondrial FAD contents were comparable before and after riboflavin supplementation. The limited uptake capacity of riboflavin transporters, or liver-specific riboflavin clearance function [46] might play roles in the low riboflavin bioavailability. Therefore, new drugs, such as esterified derivative of riboflavin (riboflavin-5'-lauric acid monoester) [47], should be considered for riboflavin-unresponsive patients. The effect of riboflavin-5'-lauric acid monoester on *Slc25a32* mutant mice will be evaluated in the near future. On the other hand, we found that formate supplementation raised the mitochondrial folates amounts, which suggested another potential therapeutic intervention for these diseases.

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. The ethics committee of Guangzhou Women and Children's Medical Center authorized this study, and the Institutional Animal Care and Use Committee of Guangzhou Medical University approved the animal studies. Informed consent has been obtained from the patient and her guardian.

Consent for publication

The authors affirm that human research participants provided informed consent for publication of the images in Fig 1b, 1c and 1d.

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Author contribution

Study concept and design were undertaken by M-Z P., Y-X S., and L L.. Data acquisition and analysis was undertaken by M-Z P., Y-X S., X-Z L., K-D Z., Y-N C., Y-T L., M-Y J., Z-C L., X-Y S., W Z., and X-L J. The manuscript was written by M-Z P. and revised by L L., Y-X S., and X-L J.

Conflict of interest

The authors have no conflicts of interest to declare.

Data Availability

The datasets generated during and/or analysed during the current study are all available in the manuscript.

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Figures

Figure 1

An RREI case with novel mutations in the *SLC25A32* gene

(A) Metabolic analysis of plasma amino acids and acylcarnitines suggested MADD. (B) X-ray imaging of the feet of patient after orthopedic surgery. The left foot developed clubfoot again after surgery. (C) Brain MRI revealed that the left lateral ventricle of the patient was slightly broadened. (D) X-ray imaging showed that the left fibula of the patient was abnormally slim. (E) A compound heterozygous mutation in SLC25A32 was identified from the patient, and testing for the mutations in parental samples confirmed that the unaffected parents were heterozygous for the mutations. (F) Homology modeling analysis using the mitochondrial ADP/ATP carrier (PDB ID: 6GCI) as the template predicted that K235 located at the substrate-binding pocket and the Y174 located at the second matrix loops facing mitochondrial matrix.

Figure 2

Characterization of mouse models with *Slc25a32* gene mutants

(A) *Slc25a32* mRNA expression in skeletal muscle of *Slc25a32* mutant mice was determined by quantitative real-time PCR (n=3). A significant difference was observed between $Slc25a32^{+/+}$ and $Slc25a32^{Y174C/Y174C}$ mice. (B) SLC25A32 protein in skeletal muscle of *Slc25a32* mutant mice was analyzed by the capillary-based automated western blot assay (n=2). No significant difference was detected between *Slc25a32* mutant and WT mice. (C) The hanging wire test showed that the times hanging on the wire were shorter for the $Slc25a32^{Y174C/K235R}$ and $Slc25a32^{K235R/K235R}$ mice than for $Slc25a32^{+/+}$ mice, but the difference did not reach significance (n=7-8). (D) The rotarod test and FST revealed that the $Slc25a32^{K235R/K235R}$ mice performed worse than their WT littermates did, but the difference did not reach significance (n=8). (E) Various NTDs were observed in E11.5 $Slc25a32^{-/-}$ embryos. a, no NTDs but growth retardation; b-d, exencephaly; e, craniorachischisis (scale bars represent 1mm). (F) Quantitative real-time PCR for *Slc25a32* mRNA expression in E11.5 $Slc25a32^{+/+}$ and $Slc25a32^{-/-}$ embryos (n=3). (G) RT-PCR for *Slc25a32* mRNA expression showed that a 678 bp cDNA fragment observed in $Slc25a32^{+/+}$ embryo was absent in S$lc25a32^{-/-}$ embryos. Instead, fragments at 517 bp and 431 bp were presented in $Slc25a32^{-/-}$ embryos. (H) Western blot assay for SLC25A32 protein in embryos (n=2). The SLC25A32 protein was almost undetectable in $Slc25a32^{-/-}$ embryos. HSP60 was used as a loading control. *n* represents biologically independent replicates. Data are Mean ± SEM. The *P* values were determined by unpaired, two-tailed Student's t-test. *** P<0.001 versus $Slc25a32^{+/+}$.

Figure 3

The *Slc25a32* mutations interrupted folate metabolism

(A) Mitochondrial folate profiles of *Slc25a32* mutant mice (n=5). The abundances of all folate intermediates were significantly lower in $Slc25a32^{K235R/K235R}$ than in controls. (B) Mitochondria uptake of [^{15}N]-FA and CHO-THF (n=3). Mitochondria were isolated from skeletal muscle of *Slc25a32* mutant and WT mice.

The folates uptake rates were comparable for *Slc25a32* mutant and WT mice. (C) [^{15}N]-FA and CHO-THF absorbed by mitochondria in 60 min (*n*=4-8). Mitochondria were isolated from *Slc25a32*$^{+/+}$, *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{-/-}$ embryos. The folates uptake amounts were comparable for them. (D) Folate profiles of whole embryos (*n*=5). Different folates profiles were observed in *Slc25a32*$^{K235R/K235R}$, *Slc25a32*$^{-/-}$ with NTDs, and *Slc25a32*$^{-/-}$ without NTDs, and *Slc25a32*$^{+/+}$ embryos. (E) Neural tube closure completed in E11.5 *Slc25a32*$^{-/-}$ embryo following maternal sodium formate supplementation (scale bars represent 1mm). (F) Folate profiles of E11.5 embryos following maternal sodium formate supplementation (*n*=6). Different folates profiles were observed in *Slc25a32*$^{-/-}$ with NTDs, and *Slc25a32*$^{-/-}$ without NTDs, and *Slc25a32*$^{+/+}$ embryos. *n* represents biologically independent replicates. Data are Mean ± SEM. The *P* values were determined by unpaired, two-tailed Student's t-test, * *P*<0.05, ** *P*<0.01, *** *P*<0.001 versus *Slc25a32*$^{+/+}$ control.

Figure 4

Glycine cleavage defect was identified in *Slc25a32*$^{-/-}$ embryos

(A) Formate amounts in whole embryos (*n*=5-6). The *Slc25a32*$^{-/-}$ embryos had significantly lower amounts of formate than *Slc25a32*$^{+/+}$ embryos had. (B) The contents of one-carbon donors in whole embryos (*n*=4-14). Significant differences in abundances between *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{+/+}$ embryos and between *Slc25a32*$^{-/-}$ and *Slc25a32*$^{+/+}$ embryos were noted for glycine and serine. (C) Isolated mitochondria produced [^{13}C]-formate amounts in 24 hours. A significant lower amount of [^{13}C]-formate was detected in *Slc25a32*$^{-/-}$ embryos than in *Slc25a32*$^{+/+}$ embryos. (D) Western blot analysis of four subunits of GCS in embryos (a-d, *n*=3). The relative abundance of each protein in *Slc25a32*$^{-/-}$ embryo was expressed as a ratio compared to the mean value of *Slc25a32*$^{+/+}$ embryos (e). HSP60, α-tubulin, or GAPDH was used as a loading control for each subunit. The *Slc25a32*$^{-/-}$ embryos had significantly lower amounts of DLDH. (E) DLDH activity assay (*n*=5-6). The activity of DLDH was significantly lower in *Slc25a32*$^{-/-}$ embryos than in *Slc25a32*$^{+/+}$ embryos. *n* represents biologically independent replicates. Data are Mean ± SEM. *P* values were determined by unpaired, two-tailed Student's t-test. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 versus *Slc25a32*$^{+/+}$ controls.

Figure 5

Slc25a32 mutations specifically affected the mitochondrial uptake of FAD

(A) The contents of flavins in mitochondria isolated from skeletal muscle of mice (*n*=6). Deficiencies of FAD, FMN, and Rf were observed in *Slc25a32*$^{Y174C/K235R}$ and *Slc25a32*$^{K235R/K235R}$ mice. (B) The contents of flavins in mitochondria isolated from embryos (*n*=5-6). Deficiencies of FAD, FMN, and Rf were observed in *Slc25a32*$^{-/-}$ embryos. (C) Mitochondria uptake of [$^{13}C_5$]-FAD and [$^{13}C_4$,$^{15}N_2$]-Rf (*n*=3). Mitochondria were isolated from the skeletal muscle of mice. Significantly lower [$^{13}C_5$]-FAD uptake rates were detected in *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mitochondria than in *Slc25a32*$^{+/+}$ mitochondria. (D) Mitochondria uptake of [$^{13}C_5$]-FAD. Mitochondria were isolated from embryos and incubated with 10 μmol/L [$^{13}C_5$]-FAD for 60-min. The LC-MS/MS TIC spectra revealed a low abundance of [$^{13}C_5$]-FAD in *Slc25a32*$^{-/-}$ mitochondria and a high abundance of [$^{13}C_5$]-FAD in *Slc25a32*$^{-/-}$ mitochondria incubation buffer. *n* represents biologically independent replicates. Data are Mean ± SEM. *P* values were determined by unpaired, two-tailed Student's t-test. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 versus *Slc25a32*$^{+/+}$ controls.

Figure 6

Slc25a32 mutant mice recapitulated the MADD-like biochemical features of the RREI patient

(A-C) Acylcarnitine profiles of embryos, skeletal muscle and, brain tissues of mice (*n*=5). Accumulation of multiple acylcarnitines and deficiency of free carnitine were found in *Slc25a32*$^{-/-}$ embryos, *Slc25a32*$^{K235R/K235R}$ and *Slc25a32*$^{Y174C/K235R}$ mice. (D-E) Glutaric acid and DMG amounts in skeletal muscle, and brain tissues of *Slc25a32* mutant mice (*n*=5). Glutaric acid and DMG were accumulated in S*lc25a32*$^{Y174C/K235R}$ and *Slc25a32*$^{K235R/K235R}$ mice tissues. *n* represents biologically independent replicates. Data are Mean ± SEM. *P* values were determined by unpaired, two-tailed Student's t-test. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 versus *Slc25a32*$^{+/+}$ control.

Figure 7

Slc25a32 mutations affect multiple mitochondrial flavoenzymes

(A-G) Enzymatic activities of muscular SBCAD, IVD, SCAD, MCAD, VLCAD, GCDH, and IBD, in the reaction system with or without FAD supplementation (*n*=5). Differences in activities between genotypes were noted for these enzymes. (I) Western blot analysis of nine mitochondrial flavoproteins in skeletal muscle tissues of *Slc25a32*$^{K235R/K235R}$ mice compared to *Slc25a32*$^{+/+}$ mice (*n*=3). HSP60 or GAPDH was used as a loading control for each protein. Significant differences in protein contents between genotypes were noted for IBD, SCAD, MCAD, VLCAD, and ETFα. *n* represents biologically independent replicates. Data are Mean ± SEM. *P* values were determined by unpaired, two-tailed Student's t-test. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 versus *Slc25a32*$^{+/+}$ control.

Figure 8

Effect of formate supplementation on mitochondrial folate profile of *Slc25a32*$^{K235R/K235R}$ mice

The abundances of all folate intermediates were significantly lower in *Slc25a32*$^{K235R/K235R}$ mice than in *Slc25a32*$^{+/+}$ mice (*n*=5-6). *n* represents biologically independent replicates. Data are Mean ± SEM. *P* values were determined by unpaired, two-tailed Student's t-test. *** *P*<0.001 versus *Slc25a32*$^{+/+}$ control.