



# Homozygous mutation in *MFSD2A*, encoding a lysolipid transporter for docosahexanoic acid, is associated with microcephaly and hypomyelination

Tamar Harel<sup>1</sup> · Debra Q. Y. Quek<sup>2</sup> · Bernice H. Wong<sup>2</sup> · Amaury Cazenave-Gassiot<sup>3,4</sup> · Markus R. Wenk<sup>3,4</sup> · Hao Fan<sup>5,6,7</sup> · Itai Berger<sup>8</sup> · Dorit Shmueli<sup>9</sup> · Avraham Shaag<sup>1,10</sup> · David L. Silver<sup>2</sup> · Orly Elpeleg<sup>1,10</sup> · Shimon Edvardson<sup>8,10</sup>

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## Abstract

The major facilitator superfamily domain-containing protein 2A (MFSD2A) is a constituent of the blood-brain barrier and functions to transport lysophosphatidylcholines (LPCs) into the central nervous system. LPCs such as that derived from docosahexanoic acid (DHA) are indispensable to neurogenesis and maintenance of neurons, yet cannot be synthesized within the brain and are dependent on MFSD2A for brain uptake. Recent studies have implicated *MFSD2A* mutations in lethal and non-lethal microcephaly syndromes, with the severity correlating to the residual activity of the transporter. We describe two siblings with shared parental ancestry, in whom we identified a homozygous missense mutation (c.1205C > A; p.Pro402His) in *MFSD2A*. Both affected individuals had microcephaly, hypotonia, appendicular spasticity, dystonia, strabismus, and global developmental delay. Neuroimaging revealed paucity of white matter with enlarged lateral ventricles. Plasma lysophosphatidylcholine (LPC) levels were elevated, reflecting reduced brain transport. Cell-based studies of the p.Pro402His mutant protein indicated complete loss of activity of the transporter despite the non-lethal, attenuated phenotype. The aggregate data of MFSD2A-associated genotypes and phenotypes suggest that additional factors, such as nutritional supplementation or modifying genetic factors, may modulate the severity of disease and call for consideration of treatment options for affected individuals.

**Keywords** MFSD2A · Docosahexanoic acid · Blood-brain barrier · Microcephaly · Lysophosphatidylcholine · Lysolipid transporters

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✉ Tamar Harel  
tamarhe@hadassah.org.il

✉ David L. Silver  
david.silver@duke-nus.edu.sg

<sup>1</sup> Department of Genetic and Metabolic Diseases, Hadassah-Hebrew University Medical Center, POB 12000, 9112001 Jerusalem, Israel

<sup>2</sup> Signature Research Program in Cardiovascular and Metabolic Disorders, Duke-NUS Medical School, Singapore 169857, Singapore

<sup>3</sup> Department of Biochemistry, National University of Singapore, 8 Medical Drive, Block MD 7, Singapore 117597, Singapore

<sup>4</sup> Singapore Lipidomics Incubator (SLING), Life Sciences Institute, National University of Singapore, 28 Medical Drive, Singapore 117456, Singapore

<sup>5</sup> Bioinformatics Institute (BII), Agency for Science, Technology and Research (A\*STAR), 30 Biopolis St., Matrix No. 07-01, Singapore 138671, Singapore

<sup>6</sup> Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543, Singapore

<sup>7</sup> Centre for Computational Biology, DUKE-NUS Medical School, 8 College Road, Singapore 169857, Singapore

<sup>8</sup> Pediatric Neurology Unit, Hadassah-Hebrew University Medical Center, 9112001 Jerusalem, Israel

<sup>9</sup> Child Developmental Center, Clalit Health Services, Jerusalem, Israel

<sup>10</sup> Monique and Jacques Roboh Department of Genetic Research, Hadassah-Hebrew University Medical Center, 9112001 Jerusalem, Israel

## Introduction

The blood-brain barrier (BBB), localized at the level of tight junctions between adjacent brain endothelial cells, regulates delivery of metabolites and essential nutrients into the brain while limiting entry of toxic plasma and cell constituents. Substrate-specific transport proteins are responsible for the transport of specific compounds across the BBB. Examples include the *SLC2A1*-encoded glucose transporter 1 (GLUT1) that ensures sufficient supply of glucose as the main energy substrate of the brain. The gradient of amino acids across the BBB is strictly controlled by amino acid transporters, such as the large neutral transporter LAT1, encoded by *SLC7A5*, and additional amino acid-specific transporters. Lactate and other monocarboxylates are dependent upon transporters of the SLC16 family. ATP-binding cassette (ABC) transporters limit penetration of drugs into the brain [1–3]. Recently, the major facilitator superfamily domain-containing protein 2A (MFSD2A, MIM 614397) was identified as a sodium-dependent transporter of docosahexanoic acid (DHA), an omega-3 long chain polyunsaturated fatty acid (LC-PUFA) highly enriched in the brain, across the BBB. DHA and other fatty acids are transported by MFSD2A in the form of lysophosphatidylcholine (LPC) [4]. The abundance of LPCs in the blood and efficient conversion to phosphatidylcholines (PCs) in cellular membranes suggest a key role for LPCs in cellular homeostasis and growth, with proposed roles in neural progenitor proliferation and neurite outgrowth [5].

In vitro and in vivo experiments in embryonic stem (ES) cells and rodent models have implicated DHA in differentiation of neural stem cells into neurons [6, 7], as well as in proliferation of cells undergoing differentiation into neural cells [7]. Moreover, DHA regulates membrane fluidity and thus supports signal transduction and neurotransmission. Dietary deficiency of omega-3 fatty acid during development is associated with decreased DHA in brain membrane phospholipids, inhibition or delay of neurogenesis, reduced learning ability and memory consolidation, altered activity of membrane receptors and proteins, and altered dopaminergic and serotonergic signaling in the fetal brain [8, 9].

*MFSD2A* is highly expressed in endothelium constituting the BBB [10] and encodes a 12-pass transmembrane protein which transports LPCs via a rocker-switch model [11]. In addition to its role in promoting specific transport of LPCs, MFSD2A lipid transport activity has been implicated in maintenance of BBB integrity by inhibiting transport of most molecules by transcellular vesicular transport (transcytosis) [10, 12]. *Mfsd2a*-knockout mice show microcephaly, cognitive defects and severe anxiety, markedly reduced levels of DHA in the brain, and neuronal cell loss in the hippocampus and cerebellum, overall reminiscent of omega-3 fatty acid deficiency [4]. Recently, inactivating and partially inactivating *MFSD2A* mutations were identified in lethal and non-lethal

microcephaly syndromes, respectively [5, 13]. Plasma concentrations of LPCs containing mono- and polyunsaturated fatty acyl chains were significantly increased in affected individuals, suggesting reduced brain uptake of LPCs in the pathogenesis [5, 13]. Here, we report two siblings with a non-lethal microcephaly syndrome despite a homozygous inactivating mutation in *MFSD2A*, suggesting that additional factors may be involved in modulating the clinical expression. Thus, prediction of phenotypic severity cannot be based on the residual activity of the transporter alone.

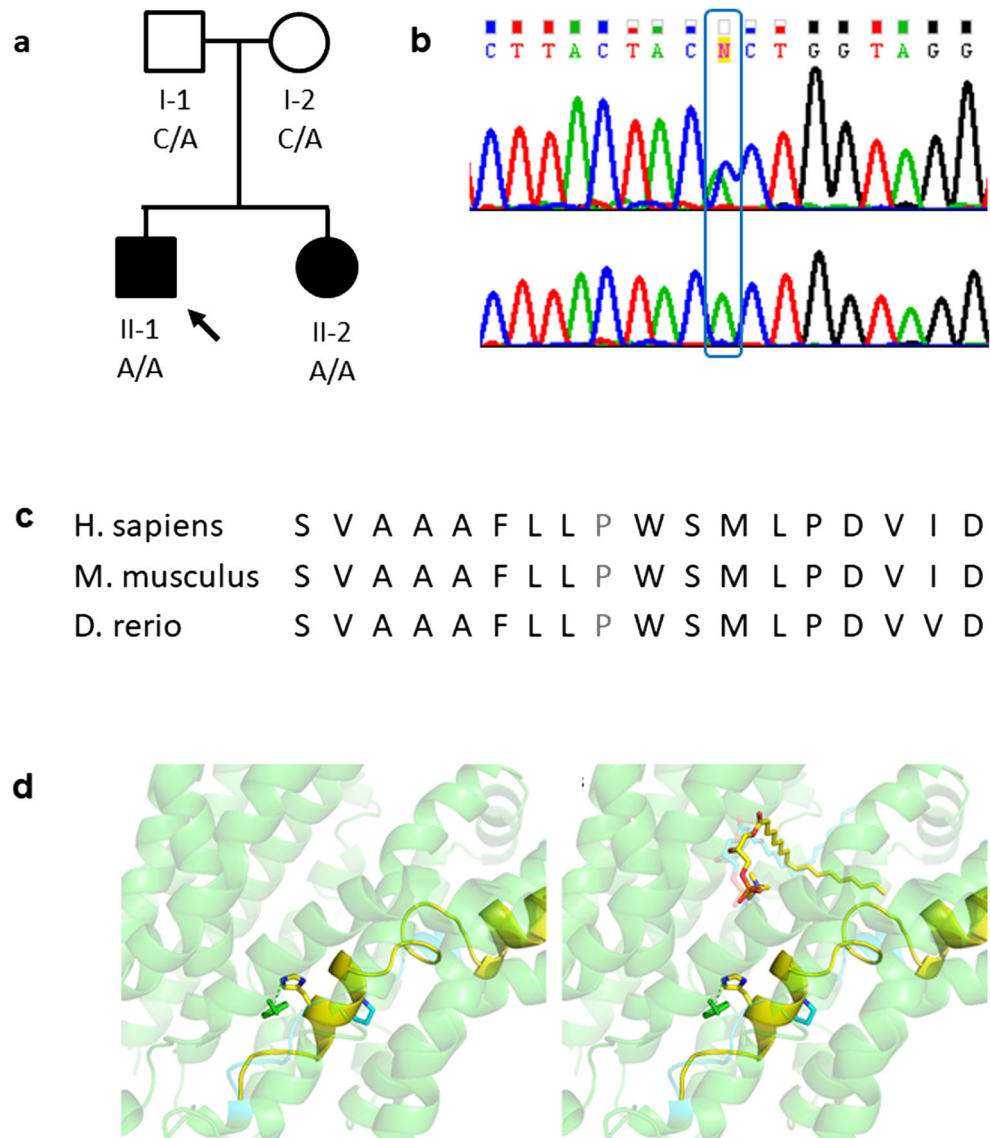
## Case reports

The subjects of this study were two siblings, a 5-year 8-month-old male (individual II-1 in Fig. 1a), and a 4-year 2-month old female (II-2 in Fig. 1a). These were the only children to non-consanguineous healthy parents of Jewish Moroccan ancestry. Individual II-1 was delivered after an uneventful pregnancy at 40 weeks of gestation with a birthweight of 2.8 kg (12th percentile) and a head circumference of 31 cm (−2.1 SD). He was brought to clinical attention at 2 months of age due to hypotonia, feeding difficulties, stridor, and esotropic strabismus. Within the next few months, obvious global developmental delay became apparent, without regression. At the time of his last exam, he spoke few words and indicated wants by gestures. He could understand simple commands such as pointing to colors or shapes and was weaned from diapers during daytime. Motor function was impaired; he was able to reach for objects with difficulty and ambulated with a walker and had stable sitting from 2 years of age.

On examination at 5.5 years, the boy was communicative and interested in his surroundings and had microcephaly (head circumference 46.5 cm, < 1st percentile, −3.5 SD) and a tented upper lip. Truncal hypotonia, appendicular spasticity with dystonia, and esotropic strabismus were prominent. Physical examination including fundoscopic examination was otherwise unremarkable. Routine laboratory investigations as well as metabolic work-up including plasma amino acids, acylcarnitines, lactate and ammonia and urine organic acids were all within the normal range. Brain magnetic resonance imaging (MRI) at 4 years of age disclosed paucity of white matter with attendant lateral ventricular widening (Fig. 2a).

His younger sister, individual II-2, followed a similar course with unremarkable perinatal course and congenital microcephaly (30.5 cm, −2.9 SD, at birth; 41 cm, −3.6 SD, at 14 months; and 45 cm, −3.0 SD, at 4 years of age), global developmental delay with a mean developmental quotient of 0.49, hypotonia with appendicular spasticity, esotropic strabismus, and dystonia. Physical exam at 4 years of age showed microcephaly, esotropia, wide nasal bridge with epicanthal folds, tented upper lip, and a dimpled chin. Brain MRI performed at age 21 months was similar to that of the older

**Fig. 1** Pedigree and molecular analysis. **a** Pedigree indicating two affected children (solid fill) and genotypes of family members. **b** Sanger sequencing of heterozygous parent (upper panel) and affected individual (lower panel) showing the c.1205C > A variant. **c** The Pro402 residue and neighboring amino acids are highly conserved throughout evolution. **d** Predicted consequences of P402H mutation on MFSD2A structure. The left panel depicts 3D structural models of MFSD2A WT and P402H mutant, highlighting specific structural changes. The common parts of both models are shown in green (partially transparent), the largely unstructured helix X in the WT model is shown in cyan (partially transparent), and the helix X in the P402H mutant is shown in yellow. The P402 sidechain in the WT models is highlighted in cyan. The H402 sidechain in the mutant model is highlighted in yellow, forming hydrogen bond with Y431 sidechain in helix XI. In the right panel, LPC-oleate is shown docked to both the MFSD2A WT (docking pose in cyan, partially transparent) and P402H mutant models. The main difference between the two binding orientations is in the hydrophobic hydrocarbon tail



brother, showing paucity of white matter with attendant lateral ventricular widening (Fig. 2b).

In view of the uneventful perinatal course, congenital microcephaly, parental common ancestry, and the similarity between the two siblings, further genetic molecular studies were pursued.

## Materials and methods

### Exome analysis

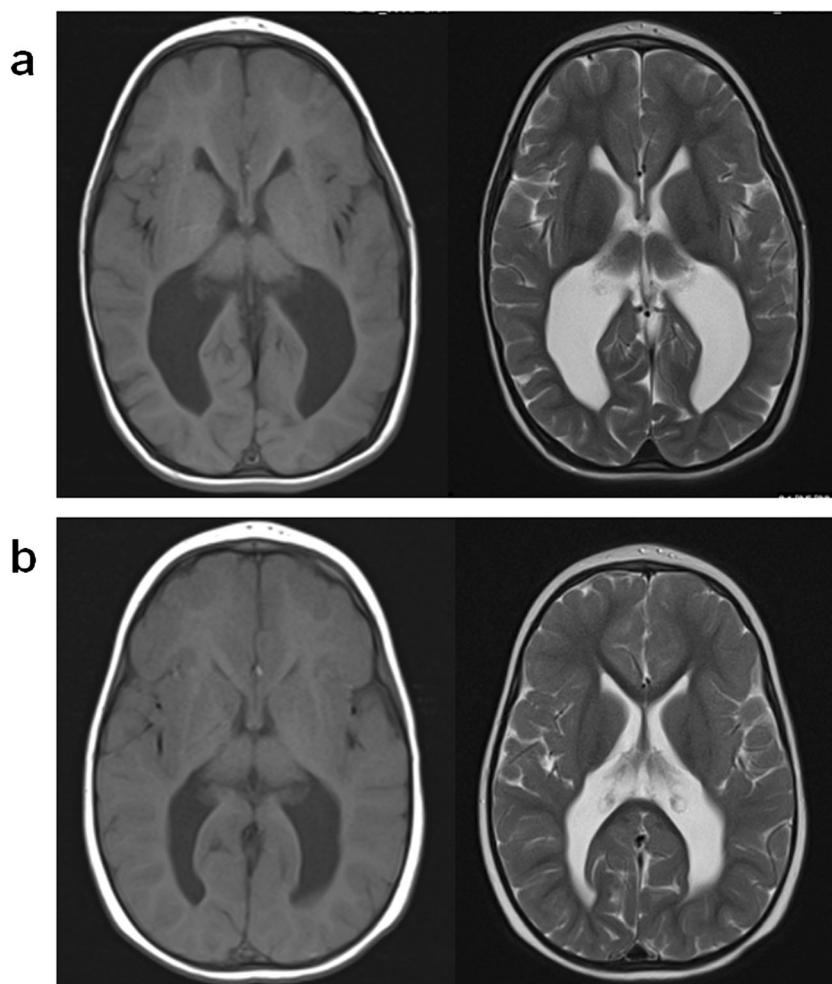
Exonic sequences were enriched in the DNA sample of individual II-2, using SureSelect Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA). Sequences were determined by HiSeq2500 (Illumina, San Diego, CA) as 100-bp paired-end runs. Data analysis including read alignment and

variant calling was performed by DNAnexus software (Palo Alto, CA) using the default parameters with the human genome assembly hg19 (GRCh37) as reference. Parental consent was given for DNA studies. The study was performed with the approval of the ethical committees of Hadassah Medical Center and the Israeli Ministry of Health.

### Transport assays

Transport assays using HEK293 cells were performed as previously described [4]. Briefly, plasmids encoding wild-type, Asp97Ala, and Pro402His MFSD2A were transfected into HEK293 cells. Uptake assays were performed after 24 h of transfection with increasing concentrations of LPC-[<sup>14</sup>C]DHA. Experiments were repeated twice in triplicate in 12-well plates. Uptake activity is expressed in dpm per well. Radiolabeled LPC-[<sup>14</sup>C]DHA was purchased from

**Fig. 2** Neuroimaging studies of the affected individuals. **a** T1 and T2 weighted images (left and right, respectively) of individual II-1 at age 4 years showing paucity of white matter with lateral ventricular widening. **b** T1 and T2 weighted images (left and right, respectively) of individual II-2 at age 21 months showing paucity of white matter with lateral ventricular widening



ARC. Non-radiolabeled LPC-DHA was synthesized as described previously [14].

### Lipid extraction from plasma samples

Briefly, plasma phospholipids were extracted using a butanol/methanol-based protocol. Plasma samples (5  $\mu$ l) were resuspended in 50  $\mu$ l of butanol/methanol (1/1, v/v) containing a mixture of internal standards (Avanti Polar Lipids), vortexed for 30 s, and sonicated for 30 min on ice. Samples were centrifuged at 14,000 rpm for 10 min at 4  $^{\circ}$ C to pellet insoluble. The supernatants were transferred to MS vial and injected (2  $\mu$ l) as is into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument. Each sample was extracted in triplicate. Technical QC samples and blank extract were prepared together with the samples to ensure data quality.

### Lipidomics LC-MS/MS analysis

Samples were randomized for injection into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument (1290 Liquid Chromatography System, and

6460 QqQ, Agilent Technologies). Quality controls and blanks were injected after every six sample injections to monitor stability of the instrument response and carryover. The chromatographic column was a Kinetex HILIC (150  $\times$  2.1 mm, 2.6  $\mu$ M, 100  $\text{\AA}$ ; Phenomenex). Gradient elution was undertaken with solvents A (50% acetonitrile/50% 25 mM ammonium formate buffer, pH 4.6) and B (95% acetonitrile/5% 25 mM ammonium formate buffer, pH 4.6), with a gradient range from 75 to 25% solvent B for 6 min, 90 to 10% solvent B for 1 min, 0.1 to 99.9% solvent B for 0.1 min, followed by 0.1 to 99.9% solvent B for 3 min (total run time of 10.1 min). Phospholipids (see Supplemental Table 1) were quantified using multiple reaction monitoring with precursor to headgroup transitions. MS parameters were: gas temperature of 300  $^{\circ}$ C, gas flow of 5 l/min, sheath gas flow of 11 l/min, and capillary voltage of 3500 V. Quantification data were extracted using MassHunter Quantitative Analysis (QQQ) software, and data were manually curated to ensure correct peak integration. Areas under the curve for the extracted ion chromatogram peaks for each multiple reaction monitoring transition and lipid species were normalized to internal



standards. Isotope correction was then done on all relevant lipid species.

### Immunoblotting and immunofluorescence microscopy

Immunoblotting and immunofluorescence microscopy for MFSD2A were carried out as described previously using a rabbit polyclonal antibody against MFSD2A in HEK293 cells transfected overnight with constructs expressing WT and mutant human MFSD2A in a pcDNA3.1 vector [11].

### 3D structural modeling of P402H

Starting from the published 3D model of MFSD2A WT in the outward occluded state [11], P402 in the WT model was mutated to histidine by sidechain prediction using SCWRL [15]. Then, the neighboring region of H402, from residue 379 to residue 407, was explored in conformational space by loop modeling method implemented in MODELER [16]. The resulted 2500 models were evaluated by the DOPE (discrete optimized protein energy) score [17] and the best ranked model was selected. Finally, known substrates of Mfsd2a including LPC-oleate and molecules not to be transported were docked into the selected 3D model of MFSD2A P402H with DOCK version 3.6 [18].

## Results

### Exome sequencing identifies a homozygous missense variant in *MFSD2A*

Exome analysis of the proband yielded 42.8 million mapped reads with a mean coverage of 75×. Following alignment to the reference genome [hg19] and variant calling, we performed a series of filtering steps. These included removing variants, which were called less than 8×, were off-target, on the X chromosome, synonymous, had MAF > 0.5% at ExAC (Exome Aggregation Consortium, Cambridge, MA, URL: <http://exac.broadinstitute.org>) or MAF > 1% at the Hadassah in-house database. A total of 358 variants remained, and only one of them was homozygous. This was chr1.hg19:g.40433585 C > A, NM\_032793.4: c.1205C > A; p.Pro402His in the major facilitator superfamily domain containing 2A (*MFSD2A*) gene. Using Sanger sequencing, we confirmed homozygosity in both affected individuals and heterozygosity in the parents (Fig. 1a, b). The variant was carried by only one of the ~125,000 individuals whose exome analyses were deposited at gnomAD (URL: <http://gnomad.broadinstitute.org/>, accessed Oct. 2016) and was not present in our in-house database (~2650 exome analyses).

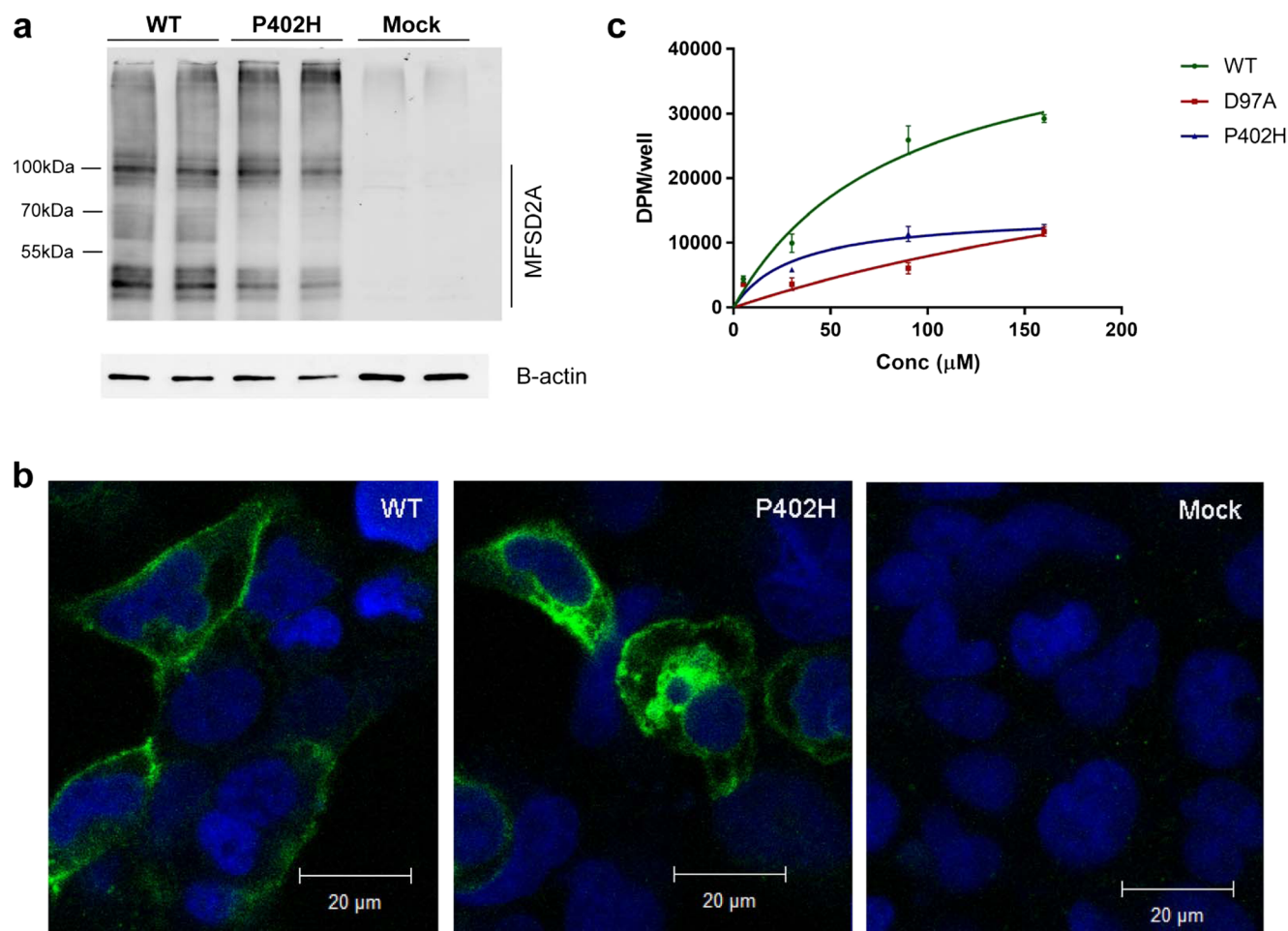
### The Pro402His variant is predicted to alter structure and transport function

*MFSD2A* consists of 14 exons encoding the 543 amino acid MFSD2A protein, the sodium-dependent lysophosphatidylcholine (LPC) transporter which is the major transporter for uptake of omega-3 fatty acid docosahexaenoic acid (DHA) into the brain [4]. Since the Pro402 residue is highly conserved throughout evolution (Fig. 1c), we set to determine the effect of the mutation on the function of the MFSD2A protein. To examine the predicted consequences of the P402H mutation on the structure of MFSD2A, we generated 3D structural models of human MFSD2A P402H mutant based on our predicted 3D model of human MFSD2A WT [11]. Mutation of P402 to His resulted in a higher alpha helix content in the neighboring region (helix X) with respect to that in the WT model. The H402 sidechain forms a hydrogen bond with the Y431 sidechain in helix XI (Fig. 1d). These predicted changes in the mutant model may lead to multiple consequences in the protein structure and dynamics, including increased structural rigidity and reduced mobility due to enhanced helix packing.

To investigate how the mutant model may affect transport, we docked to this mutant model known LPC substrates of MFSD2A as well as molecules that are not transported. In comparison to the WT model, the mutant model could not distinguish the substrates from the non-substrates. The docking predicted that the binding geometry of the known substrate LPC-oleate in the mutant model mainly differed from that in the WT model with respect to the orientation of the LPC hydrophobic fatty acid tail (Fig. 1d). Taken together, the P402H mutant would be predicted to be inactivating.

### The Pro402His variant disturbs LPC transport and results in increased plasma LPCs

To test the hypothesis that P402H is an inactivating mutation, we expressed wild-type (WT), D97A (sodium binding mutant that is completely inactive as a negative control), and P402H (proband mutant) human MFSD2A in HEK293 cells; of note, these cells do not endogenously express MFSD2A and are thus essentially null. Expression of P402H in HEK293 cells resulted in plasma membrane localization and expression similar to WT MFSD2A (Fig. 3a, b). The cells were then treated with increasing concentrations of [<sup>14</sup>C]-LPC-DHA. The amount of the [<sup>14</sup>C]-LPC-DHA transported by the P402H mutant cells was significantly reduced compared to that transported by the WT cells. Moreover, the transport curve of the P402H mutant cells was comparable to that of the D97A mutant cells, indicating complete inactivity of the transporter (Fig. 3c).



**Fig. 3** Functional analysis of the MFSD2A variant. **a** Western blot analysis of WT and P402H expressed in HEK293 cells relative to non-transfected control cells (Mock). Duplicate transfected wells of cells are shown for each construct; beta-actin served as a loading control. MFSD2A shows a typical complex banding pattern due to glycosylation. **b** Immunofluorescence localization of WT and P402H

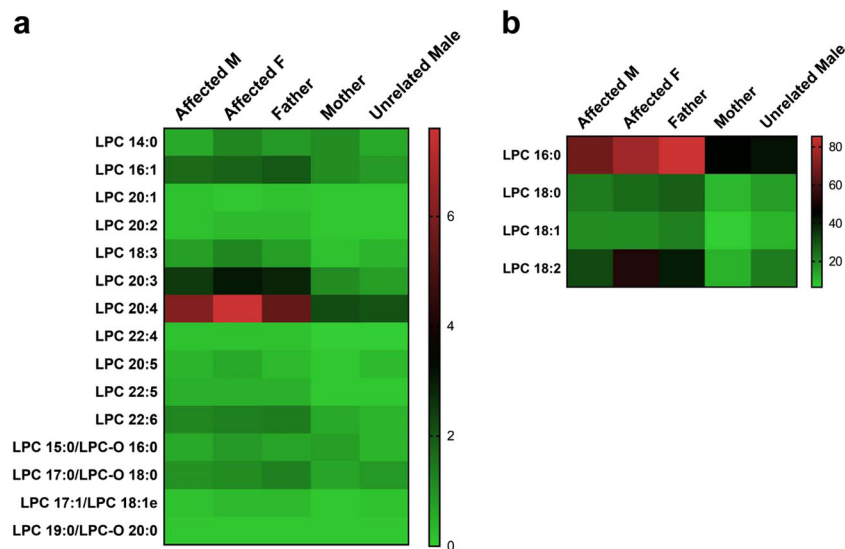
expressed in HEK293 cells indicated that P402H is expressed on the plasma membrane similar to WT MFSD2A. **c** Transport assay of LPC-[<sup>14</sup>C]DHA in WT, P402H, and D97A mutants in HEK293 cells. Transport of LPC-[<sup>14</sup>C]DHA in cells expressing P402H is similar to D97A mutant, indicating complete inactivity

We also performed a targeted plasma lipidomic analysis including quantification of LPCs in plasma in the affected children, parents, and a control, hypothesizing that inactivation of MFSD2A would result not only in LPC depletion within brain tissue but also in a reciprocal increase of plasma LPC levels as previously observed in humans and mice with inactivating *MFSD2A* mutations [5, 13]. Consistent with this hypothesis, we found that the levels of plasma LPCs were increased in the affected individuals as compared to the control and the mother (Fig. 4; Supplemental Table 2); the significance of the increase of LPC level correlated with the chain length of the LPC and with its degree of unsaturation, consistent with higher affinity of unsaturated fatty acyl chains for MFSD2A compared to the saturated acyl chains [4]. Surprisingly, the father exhibited similarly elevated plasma LPCs as the affected children; repeat plasma sampling confirmed initial

findings. This unexpected result may be due to MFSD2A haploinsufficiency coupled with other unknown factors that influence plasma LPC metabolism.

## Discussion

Recently, two consortia reported *MFSD2A* mutations in association with clinical findings that may be classified as a genetic leukoencephalopathy [19]. Alakbarzade et al. [2015] reported multiple affected siblings from a Pakistani family who suffered from microcephaly, spastic quadriplegia, and intellectual disability with absent speech. Neuroimaging disclosed paucity of white matter volume, particularly posteriorly around the lateral ventricles [13]. Guemez-Gamboa et al. [2015] studied two affected families, from Libya and Egypt, with microcephaly,



**Fig. 4** Targeted lipidomic analysis of plasma lysophosphatidylcholine (LPC). Heatmaps show levels of saturated, mono- or polyunsaturated fatty acid species in LPCs in blood plasma. **a** Low abundant LPC species and **b** high abundant LPC species in plasma. Fatty acid identity

is designated as number of carbons/number of double bonds, e.g., 20:4 denotes a lysophospholipid with 20 carbons and 4 double bonds. Scale bar represents normalized, isotope-corrected LPC levels

developmental delay, intellectual disability, spastic quadriparesis, seizures, and ultimately death within the first few years of life. Brain imaging showed gross hydrocephalus ex vacuo, effacement of the cortical surface, and cerebellar and brainstem hypoplasia and/or atrophy [5]. All three MFSD2A mutations identified were localized to the transmembrane domains of the protein. Cell-based assays showed that the mutant proteins were stably expressed and localized to the plasma membrane, comparable to wild-type protein. However, transport activity of the mutants was reduced in vitro and plasma levels of LPC were increased in affected individuals. The p.Ser339Leu mutant of the Pakistani family led to partial inactivity of the transporter, whereas the p.Thr159Met and p.Ser166Leu alterations in the Libyan and Egyptian families nearly abolished transport function [5, 13]. The hydroxyl group on Thr-159 is essential for sodium binding, and Ser-166 was found to be crucial for normal transport [11]. Accordingly, the more severe phenotype of the latter families was attributed to greater severity of the mutations. Surprisingly, the p.Pro402His mutant identified in this study renders the transporter essentially inactive and leads to increased plasma LPC levels in affected individuals; however, it leads to a non-lethal phenotype with neuroimaging similar to that of the Pakistani family. Additionally, plasma LPC levels in the father (heterozygous for the mutation) were also increased, likely reflecting the fact that LPC levels are regulated by other factors beyond MFSD2A. Consequently, dietary influences or other genetic factors may act to modify the clinical presentation by

compensating for reductions in brain DHA levels, and outcome cannot be predicted by the effect of the mutant on transport function alone.

MFSD2A belongs to the major facilitator superfamily (MFS), one of the largest families of transporters with over 70 functionally diverse subfamilies. Ligands range from simple and complex sugars and amino acids to drugs and organic anions [14, 20]. Several MFS transporters have been associated with disease phenotypes, including SLC2A1, MFSD8, and FLVCR2. Deficiency of glucose transporter 1 (GLUT1), otherwise known as solute carrier family 2, member 1 (SLC2A1) [MIM 606777], is associated with epileptic encephalopathy and other neurological phenotypes resulting from decreased glucose concentration in the central nervous system [21]. GLUT1 deficiency is treatable by a ketogenic diet which provides an alternative source of energy to the brain [22]. Mutations in the major facilitator superfamily domain-containing protein 8 (MFSD8) underlie neuronal ceroid lipofuscinosis [MIM 610951], an autosomal recessive neurodegenerative lysosomal storage disorder presenting with developmental regression, seizures, mental and motor regression, speech impairment, ataxia, visual failure, and myoclonus [23]. Finally, feline leukemia virus subgroup C receptor 2 (FLVCR2), a MFS transporter hypothesized to be involved in regulation of growth, calcium exchange, and homeostasis, is mutated in proliferative vasculopathy and hydranencephaly-hydrocephaly syndrome (PVHH; MIM 225790), a severe prenatal lethal disorder characterized by hydranencephaly and fetal akinesia deformation sequence with muscular neurogenic atrophy [24].

Transport defects may be treatable by providing supra-physiologic doses of substrates. Mutations in *SLC6A8*, encoding the creatine transporter, cause X-linked cerebral creatine deficiency syndrome [MIM 300352], manifesting with intellectual disability, developmental delay, epilepsy, and autistic behavior. Magnetic resonance spectroscopy (MRS) in such cases reveals significant reduction of the creatine signal [25, 26]. Supplementation with creatine or with its precursors L-arginine and L-glycine may lead to clinical improvement in individuals with residual protein function, provided that treatment is initiated early [27]. Mutations in *SLC1A4*, which encodes the ASCT1 transporter responsible for shuttling serine and other neutral amino acids into neuronal cells, are associated with progressive microcephaly, spastic tetraplegia, hypomyelination, and variable seizures [28–30]. Similarly, L-serine supplementation has been proposed as a therapy for serine biosynthesis and transport defects [31]. Accordingly, the role of DHA or omega-3 fatty acid dietary supplementation should be assessed in individuals with *MFSD2A* mutations, especially in cases with residual transport function of the protein.

In conclusion, we report a non-lethal presentation of syndromic microcephaly associated with an inactivating mutation in *MFSD2A*. This case illustrates the complexity of genotype-phenotype correlations, calls for additional clinical and molecular studies, and suggests the contribution of genetic or nutritional modifying factors to the disease.

## Accession numbers

The variant was submitted to ClinVar; the accession number for the DNA variant data will be available prior to publication.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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