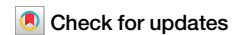


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Mitochondrial energetic failure underlies FLVCR1-related sensory neuropathy



Francesca Bertino^{1,26}, Diletta Isabella Zanin Venturini^{1,26}, Eleonora Grasso¹, Joanna Kopecka², Chiara Salio³, Barbara Gnutti⁴, Ram Manohar Basnet⁴, Stefania Bellini⁵, Luca Mignani⁴, Boxun Zhao^{6,7,8,9,10}, Felix Kleefeld¹¹, Andreas Hentschel¹², Francesca Magnani¹³, Veronica Fiorito¹, Raluca Elena Abalai¹, Livia Metani¹, Anna Lucia Allocco¹, Sara Petrillo¹⁴, Francesco De Giorgio¹, Giorgia Ammirata¹, Ettore Salsano¹⁵, Davide Pareyson¹⁵, Maja di Rocco¹⁶, Angela Abicht^{17,18}, Emily McCourt¹⁹, Rita Horvath²⁰, Heike Kölbel²¹, Austin Larson²², Andreas Roos^{21,23,24}, Timothy W. Yu^{6,7,8,9,10}, Dario Finazzi^{4,25}, Chiara Riganti², Emanuela Tolosano¹ & Deborah Chiabrando¹ ✉

Genetic pain loss disorders represent a heterogeneous group of rare diseases mainly characterized by defective nociception. Understanding the underlying molecular mechanism is fundamental to improve the treatment of patients affected by these rare disorders. Feline Leukemia Virus Subgroup C Receptor 1 (*FLVCR1*) is one of the genes previously associated with sensory neuropathy that requires further investigation. Here, we report on two additional patients with novel disease-causing variants in *FLVCR1* and introduce a zebrafish model of the disease. The analyses of patient-derived fibroblasts show that distinct *FLVCR1* variants compromised all the known functions associated with FLVCR1, thus affecting choline levels, heme biosynthesis and mitochondrial Ca^{2+} handling. Furthermore, we provide evidence that the alteration of these processes impairs the TCA cycle and OXPHOS, and induces lipid peroxidation. Our data points to the alterations of energetic metabolism as a potential driving pathomechanism in FLVCR1-associated sensory neuropathy.

Impaired nociception is the hallmark of genetic pain loss disorders, including congenital insensitivity to pain (CIP), hereditary sensory neuropathy (HSN), and hereditary sensory and autonomic neuropathy (HSAN)¹. Individuals affected by these rare disorders lack the ability to exhibit protective behaviors when exposed to noxious stimuli, thus resulting in recurrent and inadvertent self-injuries and burns associated with poor healing wounds, ulcers, and osteomyelitis. Pain insensitivity is the consequence of the alteration of sensory neurons caused by developmental defects, neurodegeneration, and/or defective excitability. The identification of pathogenetic variants in over 20 genes², coupled with studies utilizing cellular and animal models, has significantly enhanced our understanding of the molecular mechanisms underlying sensory neuron dysfunction¹. It is now evident that the dysregulation of multiple cellular processes may result in pain insensitivity, including alteration of axonal transport^{3,4}, membrane dynamics^{5–7}, sphingolipid metabolism^{8,9}, neurotrophin signaling^{10,11}, sodium channel activity^{12,13}, and epigenetic regulation¹⁴. Grasping the mechanisms that contribute to sensory dysfunctions has yielded significant impact, paving the way for preclinical approaches targeting specific HSN subtype^{15–17}. Further understanding of the disease mechanisms responsible

for pain loss disorders will undoubtedly improve the management of this heterogeneous group of rare diseases and will contribute to the definition of novel strategies for therapeutic intervention.

Feline Leukemia Virus Subgroup C Receptor 1 (*FLVCR1*) is one of the genes responsible for sensory neuropathy^{18–21} that deserves further investigation. Discovered as the receptor for Feline Leukemia virus, causing aplastic anemia in cats^{22–25}, FLVCR1 was soon recognized as an essential transporter in multiple cell types^{26–32}. From a mechanistic point of view, studies mainly focused on the characterization of FLVCR1a, the full-length isoform encoded by the *FLVCR1* gene²⁸. FLVCR1a has been initially identified as a heme exporter^{27,33}, but its function has been subsequently debated by studies claiming FLVCR1a as a choline/ethanolamine importer^{34–38}. Despite controversies in the definition of FLVCR1a's substrate specificity, substantial evidence demonstrated that modulation of FLVCR1a expression affects heme metabolism. Although the molecular mechanisms require further investigation, it has been demonstrated that FLVCR1a controls the rate of endogenous heme biosynthesis by regulating the activity of the rate-limiting enzyme of this process, δ -aminolevulinic acid synthase 1 (ALAS1)³⁹. Furthermore, the known function of FLVCR1a has been

A full list of affiliations appears at the end of the paper. ✉ e-mail: deborah.chiabrando@unito.it

recently broadened with the definition of FLVCR1a interactome⁴⁰. We recently demonstrated that FLVCR1a interacts with IP3R3-VDAC, a complex of proteins localized at mitochondria-associated membranes (MAMs) that regulates Ca²⁺ fluxes from the ER into mitochondria. Indeed, modulation of FLVCR1a expression impairs the integrity and function of MAMs⁴⁰. Therefore, it is possible to hypothesize that changes in choline, heme, and/or Ca²⁺ metabolism might play a role in the disease pathogenesis, but detailed investigations are still lacking.

Here, we describe two patients with novel disease-causing variants in the *FLVCR1* gene and demonstrate that FLVCR1a downmodulation in zebrafish impairs sensory neuron development and affects motor behaviors in response to touch. Taking advantage of primary fibroblasts derived from a cohort of four patients carrying different types of *FLVCR1* variants leading to clinical manifestation, our study suggests that perturbation of mitochondrial energetic metabolism contributes to the disease pathogenesis and that patients may benefit from therapeutic strategies boosting mitochondrial energetic metabolism. This study represents an important step toward the definition of the molecular mechanisms underlying FLVCR1-associated sensory neuropathy, possibly contributing to the identification of a therapeutic strategy for patients affected by this rare disorder.

Results

Identification of novel *FLVCR1* variants responsible for sensory neuropathy

Patient 6 (P6) was the first child of non-consanguineous parents of Caucasian origin, presented at 3 years of age with delayed motor

development, gait disturbance, frequent falls, normal psychosocial development and absent reaction to painful stimuli, multiple scars, ulcers and mutilations of hands and feet (Fig. 1A). Sensory nerve conduction in lower extremities was not measurable and was reduced (8.7 m/s) in upper extremities. At the age of 9 years, Asperger syndrome was diagnosed, and an expanded metabolism screening, including organic acid in urine, detected elevated glycolate (562 mmol/mol) and creatine (<274). Lactate, CDG, and very long-chain fatty acid (VLCFA) showed normal results. Cranial MRI and spinal MRI were normal. The child had frequent crises resembling “autonomic crises”, characterized by temperature dysregulation, frequent stool emission, and pain crises. Treatment with gabapentin due to burning feet and pain in the legs after exercise showed a good response. At the age of 15 years, the patient lost his walking ability, developed a severe scoliosis and nail clubbing. Skin biopsy did not reveal any thin nerve fibers. Now, at the age of 17 years, the patient needs non-invasive nocturnal ventilation due to severe restrictive ventilation disturbances and central apnea.

Patient 7 (P7) initially came to medical attention at 20 months of age due to gross motor delay and concern for congenital insensitivity to pain. The patient had repeated deep infections of the fingers resulting in hospitalizations and ultimately amputation of the distal tips of most fingers (Fig. 1B). At age 2 years the patient was found to have nyctalopia and retinal examination showed retinitis pigmentosa (Fig. 1C). Medical evaluations included normal MRI of the spine, MRI of the brain with bilateral symmetrical increased T2 signal in the periventricular white matter. Nerve conduction studies were consistent with severe sensory neuropathy. Lactate,

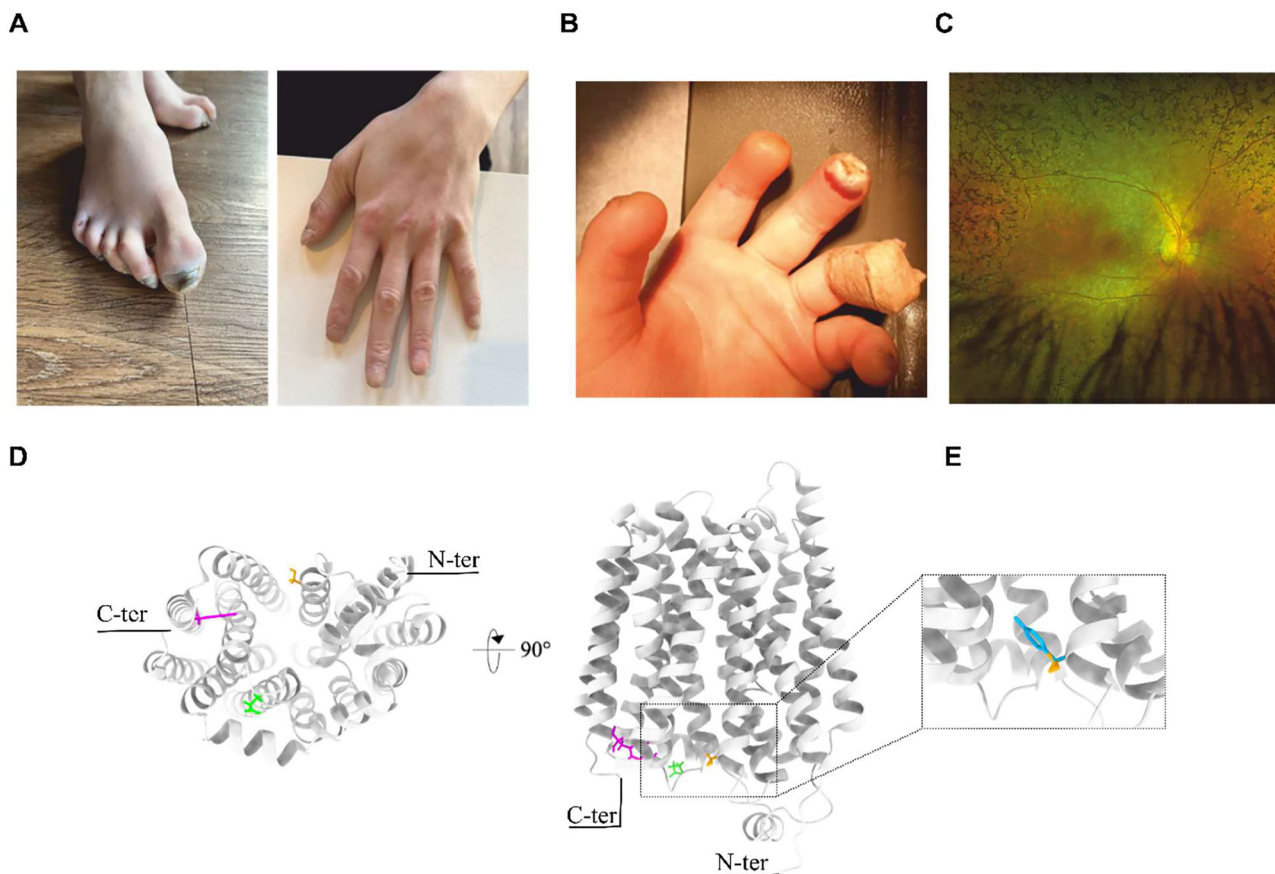


Fig. 1 | Clinical presentation of two patients carrying novel pathogenic *FLVCR1* variants. **A** Multiple scars, ulcers and mutilations of hands and feet of patient 6 carrying the c.704 C > A (p.Ser235Tyr) and c.1369 G > A (p.Glu457Lys) variants. **B** Amputation of the distal tips of most fingers due to pain insensitivity in patients 7 carrying (c.1193 A > G; p.Tyr398Cys) variant and a pathogenic pseudogene insertion in intron 8 of the *FLVCR1* gene. **C** Retinal examination of patient 7 showing

retinitis pigmentosa. **D** Cartoon representation of the AlphaFold structure prediction model of hFLVCR1a. The missense variants described in this work are highlighted in color: p.Tyr398Cys (purple), p.Ser235Tyr (orange), E457 (green). **E** Detail showing the position of the p.Ser235Tyr mutation (blue). Figures D–E were prepared using ChimeraX⁷⁶.

peroxisomal biomarkers, and transferrin glycosylation were normal. 3-methylglutaconic acid was mildly elevated twice on two occasions.

Genetic analysis of P6 and P7 revealed different heterozygous variants in the *FLVCR1* gene. Specifically, gene panel analysis of P6 showed negative results, whereas in whole-exome sequencing (WES), two variants of uncertain significance (VUS) in the *FLVCR1* (NM 014053.3) gene were detected (c.704 C > A (p.Ser235Tyr) and c.1369 G > A (p.Glu457Lys)).

Similarly, whole genome sequencing (WGS) analysis of P7 revealed that the patient is compound heterozygous for a missense variant (c.1193 A > G (p.Tyr398Cys)) and a pathogenic pseudogene insertion in intron 8 of the *FLVCR1* gene. The three amino acid substitutions identified in patients are conserved across terrestrial vertebrates, as shown in the sequence alignment generated with Clustal Omega⁴¹ (Fig. S1).

To gain insights into the impact of the variants herein reported on FLVCR1a protein conformation, we used one of the cryo-EM structures recently published³⁸ (PDB accession number 8ubw). FLVCR1a features 12 transmembrane helices (TMH) arranged into two distinct six-helix bundles and connected by a cytosolic loop, which serves as a pivotal element in the functional dynamics of transport. All the missense variants described above are in the cytosolic side of the human FLVCR1a protein. The residue Ser235 is located at the bottom of TMH 5, at the interface between the two 6-helix bundles. The amino acid substitution p.Ser235Tyr introduces a bulkier aromatic amino acid that can potentially obstruct TMH 8 movement in the conformational transition between the “inward-open” and the “outward-open” state. The amino acid substitution p.Glu457Lys in the loop between TMH 10–11 results in an inversion of charge that can destabilize electrostatic interactions, probably resulting in protein instability. Similarly, p.Tyr398-Cys results in the removal of an aromatic amino acid at the cytoplasmic side of TMH 9, affecting stability (Fig. 1D, E).

The identification of novel patients expands the spectrum of autosomal recessive FLVCR1-associated presentations.

Flvcr1a down-modulation impairs sensory neurons development in Zebrafish

Reports of individuals with *FLVCR1* mutations presenting with profound pain insensitivity, documented both in the present study and in previous publications, underscore a critical role for FLVCR1 in sensory neurons. Despite its ubiquitous expression and well-established requirement for development across multiple cell types, whether FLVCR1 function is essential for sensory neurons remains unexplored. To address this issue, we took advantage of zebrafish as a model organism. We used a splicing-blocking morpholino targeting the boundary of intron 1 and exon 2 (Mol1Ex2), that we previously reported^{42,43}. When injected in 1–2-cell embryos at the dose of 4 ng, the Mol1Ex2 largely reproduced the morphological phenotype previously described⁴² (Fig. S2). Mol1Ex2 was then injected into transgenic embryos carrying the EGFP under the control of the neurogenin1 (*ngn1*) promoter, and morphants were imaged at 48 hours post fertilization (hpf). *flvcr1a* morphants showed a reduced number of dorsal root ganglia (DRG) (Figs. 2A, B and S2). This result was confirmed upon targeting the zebrafish *flvcr1* locus by CRISPR/Cas9 (Figure S3).

To determine whether the alteration of sensory neurons morphology affects sensory perception, we evaluated motor behaviors in response to touch. Interestingly, *flvcr1a* morphants showed decreased spontaneous movements (Fig. 2C) as well as reduced distance swam after touch (Fig. 2D). The large majority of the morphants did not react or reacted very poorly to the stimulus (Fig. 2D, <5 mm), thus suggesting an alteration of sensory perception.

Taken together, these data suggest that *Flvcr1a* down-modulation in zebrafish impairs sensory neuron development and affects motor behaviors in response to touch.

Choline levels are reduced in patient-derived fibroblasts

FLVCR1a has been shown to function as a choline/ethanolamine transporter. The consequences of pathogenic *FLVCR1* variants on choline transport have been investigated by overexpressing several pathogenic variants in distinct cell lines^{34,36}. However, there is currently no study

investigating whether choline levels are affected in a relevant in vitro model of the disease. To address this issue, we analyzed primary fibroblasts derived from the two patients herein described (P6, P7) and the parents of P7 (HET1 and HET2: asymptomatic carriers). These cells were compared to primary fibroblasts derived from two previously reported patients (P1 and P3)^{20,21} and three healthy individuals (C1, C2, and C3)^{20,21}. Of note, this cohort of patient-derived cells is characterized by a combination of different variants in the *FLVCR1* gene (Table S1) that results in reduced FLVCR1a expression level (Fig. 3A, B).

The steady state level of choline was reduced in patient-derived fibroblasts compared to both asymptomatic carriers and healthy individuals (Fig. 3C, D). Of note, choline levels are reduced in primary fibroblasts derived from P7 compared to his healthy parents HET1 and HET2 (Fig. 3C). Given the essential role of choline for glycerophospholipid synthesis, we hypothesized that alteration of choline import may affect cell membrane composition with an impact on the physical–chemical properties of cell membranes. Thus, we measured membrane fluidity in our cohort of patient-derived cells. Interestingly, membrane fluidity was increased in patient-derived fibroblasts compared to both healthy individuals and asymptomatic carriers (Fig. 3E). To understand whether the alteration of membrane fluidity depends on a reduction of choline uptake, we performed rescue experiments by supplementing cells with supraphysiological doses of choline. Interestingly, choline supplementation fully restored membrane fluidity (Fig. 3E).

Taking together these data suggest that distinct types of pathogenic variants in the *FLVCR1* gene are associated with reduced intracellular choline levels and altered membrane fluidity, consistent with impaired choline handling.

De novo heme biosynthesis is impaired in patient-derived fibroblasts

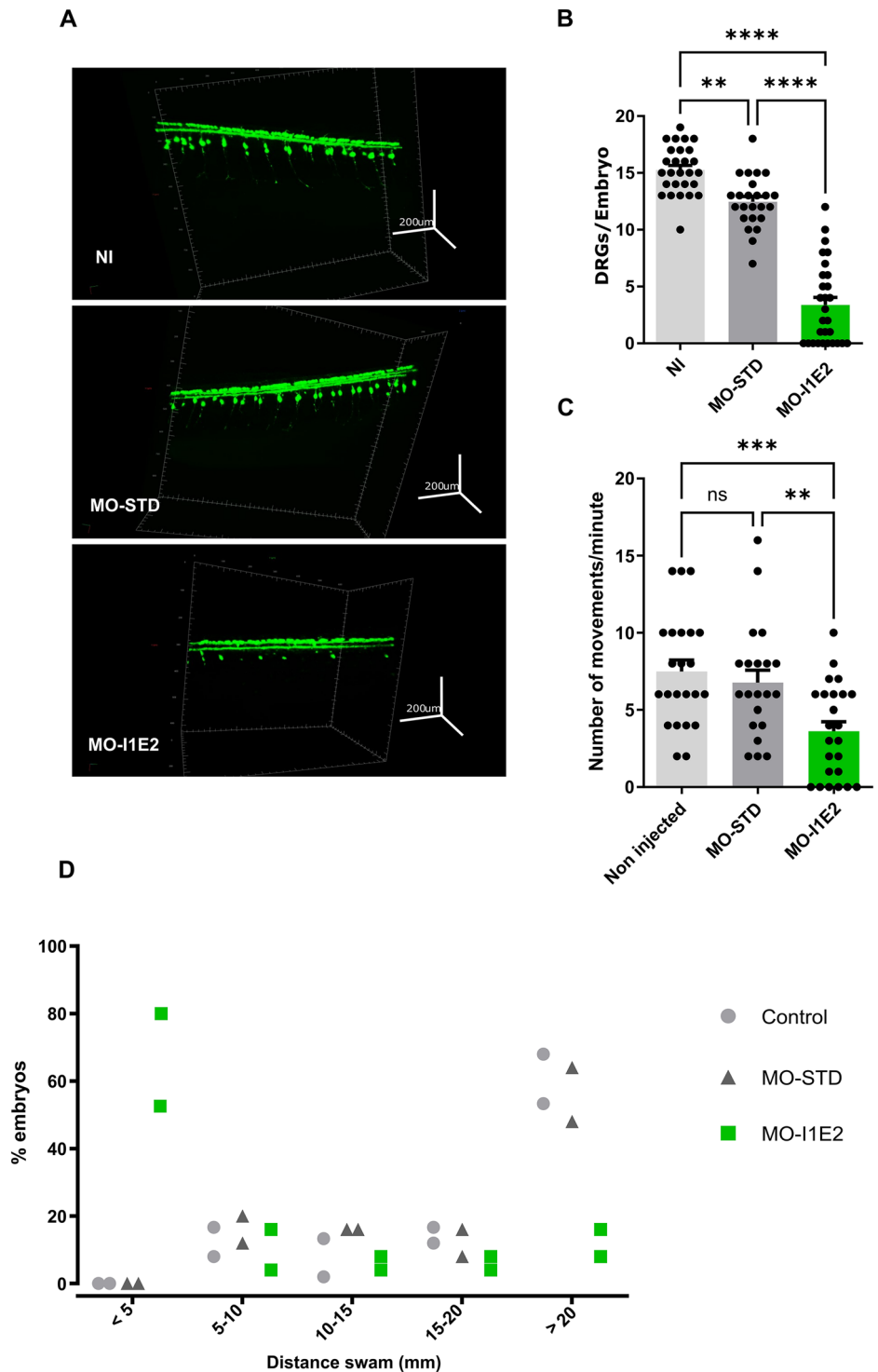
Substantial evidence indicates that modulation of FLVCR1a expression is linked to changes in heme metabolism. However, previous studies carried out on P1 fibroblasts²¹ and P3-derived lymphoblastoid cell line²⁰ failed to detect any difference in overall heme content under basal conditions. Consistently, the analysis of primary fibroblasts derived from the herein described patients showed that the overall heme content was not affected in comparison to control cells (Fig. 3F, G). However, the technique’s limited ability to detect heme cannot completely exclude subtle differences in labile heme. To gain further insight into the potential dysregulation of heme metabolism in patient cells, we assessed the activity of ALAS1 that has been demonstrated to be specifically controlled by FLVCR1a^{39,44,45}. Despite the expression level of ALAS1 being unaffected (Fig. 3H), the mitochondrial activity of ALAS1 was reduced in patients’ fibroblasts compared to both asymptomatic carriers and healthy individuals (Fig. 3I). Thus, our findings align with studies emphasizing the role of FLVCR1a in sustaining endogenous heme biosynthesis by regulating ALAS1 activity³⁹ and suggest that de novo heme synthesis rates are impaired in patients-derived fibroblasts.

Mitochondria-ER tethering and mitochondrial Ca²⁺ handling are affected in patient-derived fibroblasts

We previously reported that FLVCR1a interacts with the IP3R3-VDAC complex and regulates the structure and function of mitochondria-ER contact sites⁴⁰. We already demonstrated a reduction of the number of mitochondria-ER contact sites as well as ER-to-mitochondria Ca²⁺ influx in primary fibroblasts derived from P1 and P3⁴⁰. Here, we sought to evaluate the structure and function of MAMs in the herein identified patients. First, we assessed the number of mitochondria-ER contact sites by confocal microscopy, upon transfection of ER-targeted GFP (sec61-GFP) and mitochondria-targeted DsRed (mt-DsRed). The degree of colocalization between the two fluorophores was then quantified as an indicator of ER-mitochondria membrane contacts. Interestingly, ER-mitochondria tethering is impaired in both P6 and P7 fibroblasts compared to their respective controls (Figs. 4A, B, S4A). Furthermore, we confirmed reduced ER-mitochondria interaction in both patients by Proximity Ligation Assay

Fig. 2 | FLVCR1a down-modulation impairs sensory neurons development in Zebrafish. *Flvcr1a* expression was down-regulated in zebrafish using a splicing-blocking morpholino targeting the boundary of intron 1 and exon 2 (MO1Ex2). As controls, both non-injected (NI) and standard morpholino-injected (MO-STD) zebrafish were used.

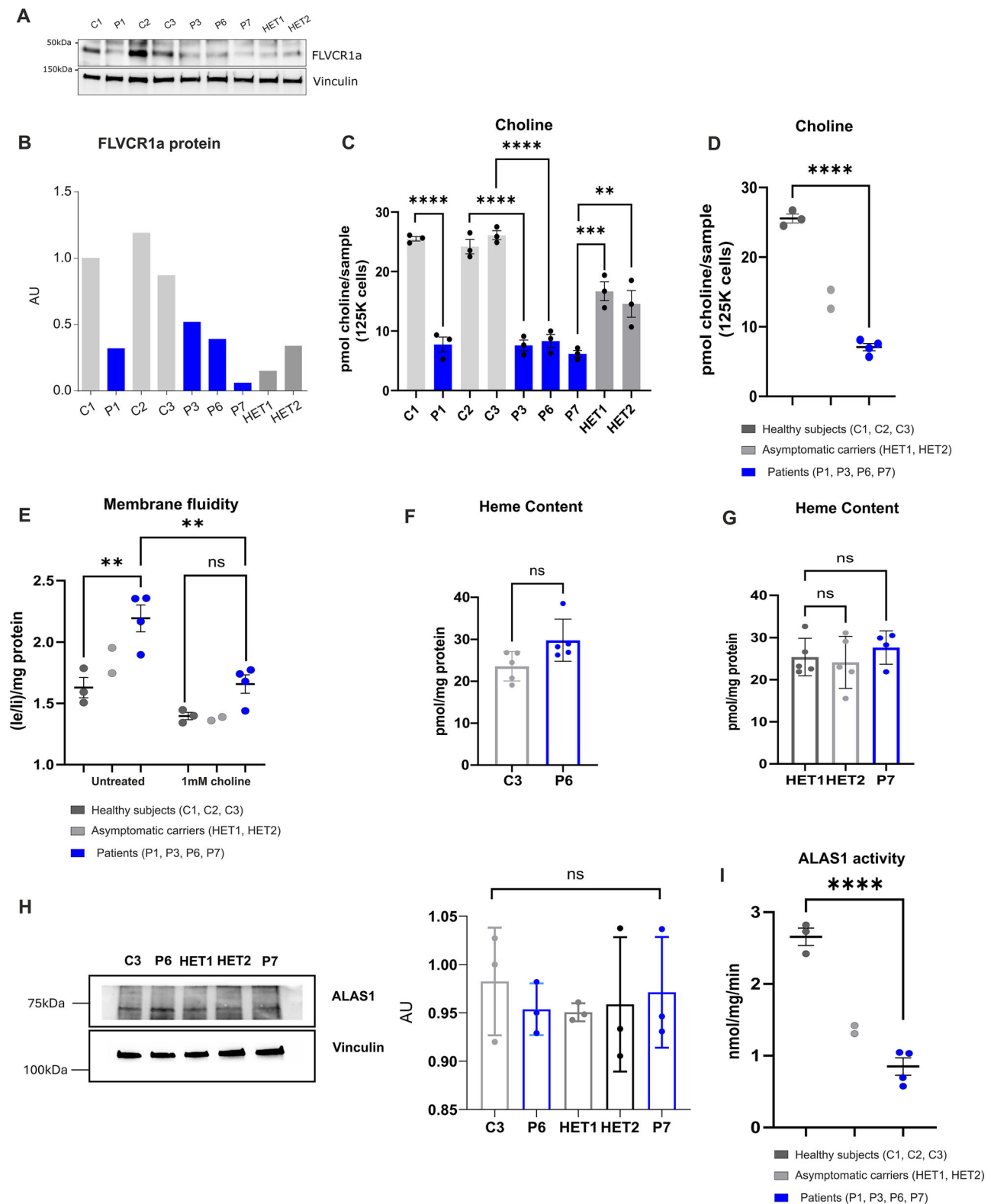
A Representative images of NI, MO-STD, and MO1Ex2 injected zebrafish. The GFP expression is under the control of the neurogenin1 (*ngn1*) promoter to visualize dorsal root ganglia (DRGs). Scale bar = 200 μ m. **B** Quantification of DRGs number in NI, MO-STD, and MO1Ex2 injected zebrafish. Each dot represents the number of DRGs per single animal. $n = 27$ biological replicates for NI; $n = 24$ biological replicates for MO-STD; $n = 29$ biological replicates for MO1Ex2. Data derived from $N = 2$ independent experiments. **C** Quantification of zebrafish spontaneous movements/minute of NI, MO-STD and MO1Ex2 injected zebrafish. Each dot represents the number of movements per minute per single animal. $n = 23$ biological replicates for NI; $n = 21$ biological replicates for MO-STD; $n = 25$ biological replicates for MO1Ex2. Data derived from $N = 2$ independent experiments. **B, C** One way ANOVA was performed. $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$; $**** = p < 0.0001$. Error bars denote the standard error of the mean (SEM). **D** Distance swam (mm) following touch stimulation in NI-, MO-STD- and MO1Ex2-injected zebrafish embryos. Data represents the percentage of embryos crossing each predetermined concentric circle. Each dot represents one independent experiment performed on 50 embryos. $N = 2$ independent experiments.



(PLA) using IP3R3-VDAC antibody pair (Fig. S4B). Notably, we observed that the expression of IP3R3 and VDAC proteins was not altered in patients' cells compared to the relative controls (Fig. S4C, D), thus confirming that the MAMs impairment is specifically due to *FLVCR1* mutation. MAMs integrity was further assessed by transmission electron microscopy to better characterize the tethering (Fig. 4C, Figure S4E). Interestingly, the analysis showed that both patient cells present a significant reduction of the contact length, measured by the ratio between the ER-mitochondria contact interface and the mitochondria perimeter, compared to their relative controls (Fig. 4D). Moreover, we observed that the percentage of mitochondria in contact with ER was significantly lower in patient-derived fibroblasts (Fig. 4E).

As a readout of MAMs' function, we evaluated Ca^{2+} fluxes from the ER into the mitochondria upon agonist stimulation, which are dependent on the IP3R3-VDAC complex. Mitochondrial Ca^{2+} influx was measured upon stimulation with the cholinergic agonist carbachol. Carbachol activates Phospholipase C (PLC) to promote phosphoinositide hydrolysis and the production of IP3, thus inducing the release of Ca^{2+} from the ER and its consequent, transient, accumulation within mitochondrial matrix⁴⁰. We observed reduced Ca^{2+} fluxes from the ER into mitochondria in both P6 and P7 compared to their controls (Fig. 4F-G).

These data demonstrated that different kinds of disease-causing variants in *FLVCR1* impair the structure and function of mitochondria-ER



contact sites, resulting in the compromise of Ca^{2+} fluxes from the ER into the mitochondrion.

Mitochondrial bioenergetics is compromised in patient-derived fibroblasts

The results described above showed that *FLVCR1* pathogenic variants impaired all the known functions described for this transporter. Of note,

choline, heme, and Ca^{2+} are all essential to sustain mitochondrial function. Indeed, choline serves as the precursor for phosphatidylcholine, a molecule essential for maintaining the integrity and function of cellular and mitochondrial membranes⁴⁶. Furthermore, heme is the cofactor of electron transport chain complexes (ETC)^{47,48}, and Ca^{2+} regulates TCA cycle dehydrogenases as well as ETC complexes⁴⁹. Thus, it is conceivable that alteration of these processes may converge on mitochondrial failure. This hypothesis

Fig. 3 | *FLVCR1* variants affect choline levels and ALAS1 activity in patient-derived fibroblasts. Primary fibroblasts derived from 4 patients carrying distinct disease-causing variants in the *FLVCR1* gene (P1–P3–P6 and P7) were analyzed and compared to primary fibroblasts derived from 3 healthy individuals (C1, C2 and C3) and 2 asymptomatic carriers (HET1 and HET2). **A, B** Western Blot analysis of *FLVCR1a* expression levels. A representative image is shown together with the relative quantification of protein abundance. **C** Measurement of choline levels in each distinct individual. Each dot represents an independent experimental replicate. $N = 3$ independent experiments. One-way ANOVA analysis was performed. **D** Measurement of choline levels in patients, asymptomatic carriers and healthy individuals is shown. Each dot represents a biological replicate. $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$ biological replicates for patients. Unpaired t-test analysis was performed to compare healthy individuals and patients. **E** Measurement of membrane fluidity under basal conditions (Untreated) and upon choline supplementation (1 mM) for 16 hours. Each dot represents a biological replicate. $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$

biological replicates for patients. Two-way ANOVA test was performed to compare healthy individuals and patients. **F** Measurement of heme content in P6 vs C3. Each dot represents an independent experimental replicate. $N = 5$ independent experiments. Unpaired t-test was performed. **G** Measurement of heme content in P7 vs HET1 and HET2. Each dot represents an independent experimental replicate. $N = 5$ independent experiments for HET1; $N = 5$ independent experiments for HET2; $N = 4$ independent experiments for P7. One-way ANOVA was performed.

H Western Blot analysis of ALAS1 protein. A representative image is shown together with the relative quantification of protein abundance. Each dot represents an independent experimental replicate. $N = 3$ independent experiments. One-way ANOVA was performed. **I** Mitochondrial ALAS1 activity. Each dot represents a biological replicate. $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$ biological replicates for patients. T-test analysis was performed to compare healthy individuals and patients. $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$; $**** = p < 0.0001$. Error bars denote the standard error of the mean (SEM).

was supported by the results of unbiased proteomic profiling performed on patient-derived fibroblasts compared to healthy donors. This analytical approach revealed the statistically significant dysregulation of 79 proteins (46 are increased and 33 are decreased in abundance) out of 4641 quantifiable proteins (1.7% dysregulated proteins of the entire quantified proteome represented by 72707 unique peptides) (Fig. S5). Proteomaps-based in silico analysis of the dysregulated proteins indicates effects on metabolism (including glycolysis and amino acid metabolism), on signaling pathways (including PPAR-, FoxO-, and PI3K-Akt signaling), and on RNA-processing, among others (Figure S5).

To specifically investigate the impact of *FLVCR1* variants on mitochondrial bioenergetics, we performed metabolic assays on patient-derived fibroblasts. Patients' fibroblasts showed reduced TCA cycle flux and reduced activity of TCA cycle enzymes compared to controls (Fig. 5A, B). Furthermore, the overall ETC activity and mitochondrial ATP content were reduced in patients' fibroblasts compared to controls (Fig. 5C, D). Interestingly, asymptomatic carriers did not differ from healthy subjects in TCA cycle enzyme activity and flux, but they showed a decrease in ETC flux and mitochondrial ATP, although at a lesser extent than patients. Of note, ETC dysfunction is a well-known mechanism inducing the production of mitochondrial reactive oxygen species (ROS), which can result in lipid peroxidation. Here, we demonstrated increased peroxidation of lipids, both in mitochondrial and cytosolic extracts, in patient-derived fibroblasts (Fig. 5E, F), a molecular finding in line with previous studies performed in P1 and P3 fibroblasts^{20,21}. Interestingly, defective energetic metabolism and increased lipid peroxidation were also observed in *flvcr1a* morphants (Fig. S6).

To confirm the results of functional assays, we analyzed cellular respiration by measuring oxygen consumption rate on fibroblasts derived from patient 1 (P1). This showed a significant decrease in basal and maximal mitochondrial oxygen consumption rates (OCR) in P1-derived fibroblasts compared to control C1 (Fig. 5G). To further investigate the metabolic profile of patients' cells, we measured the glycolytic rate by using Seahorse XF Glycolytic Rate Assay in P1 and C1-derived fibroblasts. This analysis showed an increase in basal and compensatory glycolysis. Indeed, the GlycoPER parameter, representing the proton efflux rate (PER) in the media predominantly due to glycolysis, is significantly increased (Fig. 5H).

Altered energetic states of cells are often associated with different mitochondrial morphologies^{50,51}. However, after our electron microscopy analysis, no marked ultrastructural abnormalities were observed in the patient's mitochondria, principally characterized by cristae with a regular pattern of organization. Only in a few cases, we observed patients' mitochondria with shorter and wider cristae. To analyze the mitochondrial network, P1-derived fibroblasts were stained with MitoTracker, and the mitochondrial area, number of objects, average size, circularity, perimeter, and branching were analyzed. Interestingly, the total mitochondrial area in P1 fibroblasts is not altered if compared to the healthy control C1 (Fig. S7).

However, the number of individual mitochondrial objects is reduced, while the size of each object is increased. Additionally, mitochondria appear to be more elongated and interconnected (Fig. S7). To further characterize mitochondrial branching, we employed the MiNa ImageJ Toolset. MiNa analysis confirmed that P1 mitochondria exhibit significantly higher levels of branching within their networks (Fig. S7). Finally, we assessed mitochondrial DNA copy number and found no significant differences among patients, asymptomatic carriers, and healthy controls (Fig. 5I). Collectively, these findings suggest that patients' cells display an altered mitochondrial network, characterized by 'hyper-fusion'. As it has been proposed that hyperfused mitochondria networks are more efficient at producing energy⁵⁰, we can speculate that hyper-fusion observed in patients likely acts as a compensatory mechanism to counteract the metabolic defect and maintain cellular integrity.

Taken together, these data demonstrate that different kinds of disease-causing *FLVCR1* variants impair mitochondrial function by reducing mitochondrial respiration and enhancing glycolysis rates.

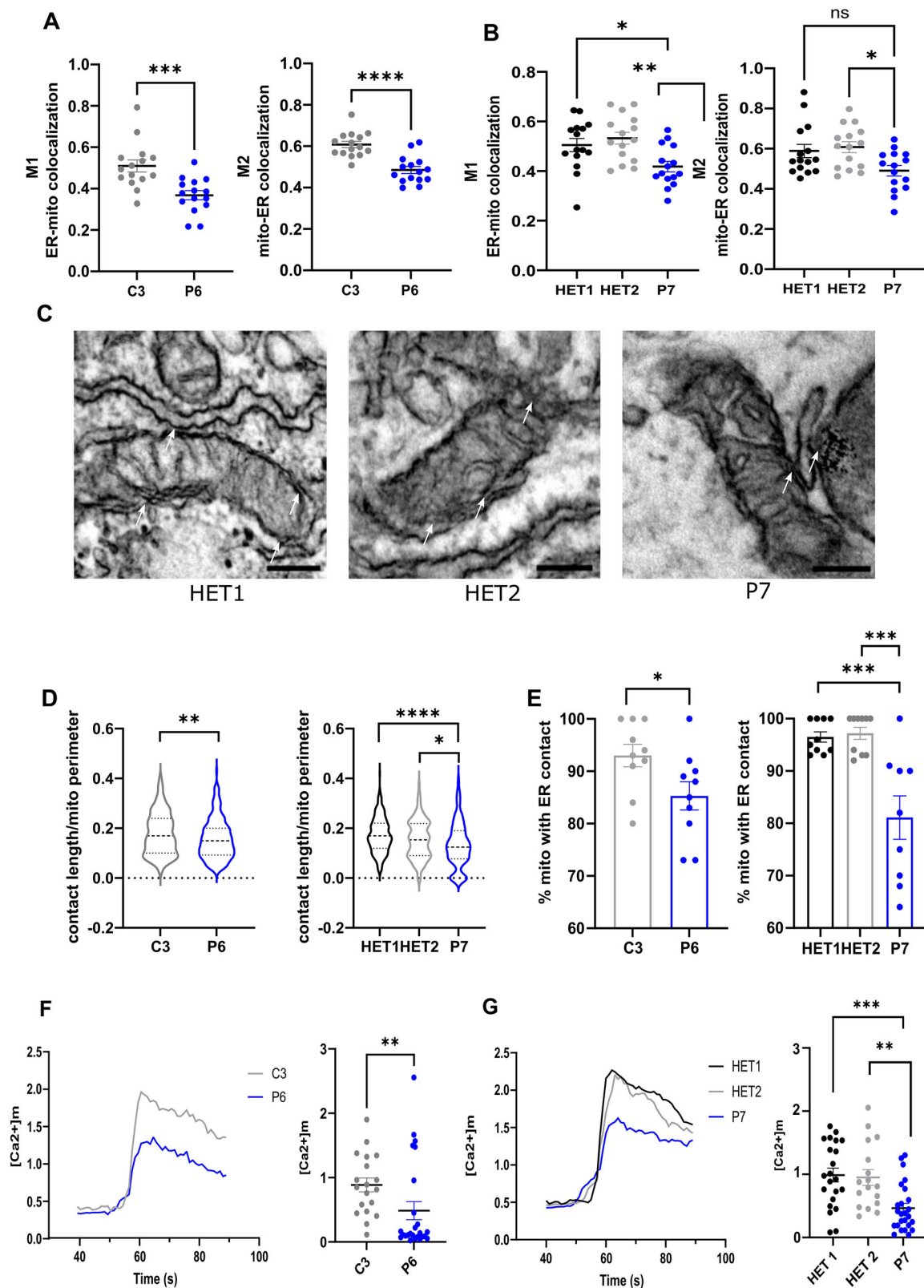
Choline, heme, and calcium dysregulation contribute to bioenergetic failure in patient fibroblasts

The data reported above suggest that decreased choline uptake, reduced ALAS1 activity, and disrupted mitochondrial Ca^{2+} handling ultimately impair mitochondrial energetic metabolism in patient-derived fibroblasts. To assess the relative contribution of these pathways to the observed bioenergetic defect, we evaluated their impact using targeted rescue strategies.

We already demonstrated the dependency of bioenergetics failure on the disruption of mitochondria-ER contact sites, as mitochondrial Ca^{2+} loading fully restored energetic metabolism in P1 and P3 fibroblasts⁴⁰. Here, the analysis of P6 and P7 fibroblasts showed a consistent trend. Indeed, the overexpression of the mitochondrial calcium uniporter (MCU) to increase mitochondrial Ca^{2+} levels restored the activity of Ca^{2+} -dependent dehydrogenases (Figs. 6A–C and S8), enhanced total (Fig. 6D) and complex-specific ETC function (Fig. S8B–E), increased mitochondrial ATP production (Fig. 6E), and reduced mitochondrial lipid peroxidation levels (Fig. 6F).

To determine whether reduced ALAS1 activity contributes to the compromise of energetic metabolism, we took advantage of 5-aminolevulinic acid (ALA) that stimulates endogenous heme synthesis, bypassing ALAS1 inhibition. Interestingly, ALA administration promoted the activity of TCA cycle enzymes, the TCA cycle flux, the ETC activity, mitochondrial ATP content, and lipid peroxidation in patients' fibroblasts (Fig. 7).

Finally, we evaluated whether choline supplementation is effective in restoring energetic metabolism. Interestingly, short-term choline supplementation did not significantly affect the TCA cycle and OXPHOS but modestly improved mitochondrial ATP content and fully decreased lipid peroxidation (Fig. 8).



Altogether, these data highlight a complex relationship between choline, heme, and calcium metabolism that likely converges on the disruption of mitochondrial energetic metabolism in patient-derived cells, thereby implicating the alteration of mitochondrial energetic metabolism as a potential driving mechanism in *FLVCR1*-associated sensory neuropathy.

Discussion

The identification of two additional individuals with *FLVCR1*-associated sensory neuropathy reinforces the role of *FLVCR1* in the maintenance of nociceptive sensory neurons. Consistent with this observation, we demonstrated that *flvcr1a* down-modulation affects sensory neurons'

Fig. 4 | Alteration of Mitochondria-ER tethering and Ca²⁺ fluxes in patient-derived fibroblasts. **A, B** Quantification of Manders coefficients (M1 and M2) on confocal images of fibroblasts expressing mt-DsRed and sec61-GFP, as a readout of the number of mitochondria-ER contact sites. Each dot represents a single cell. $n = 15$ biological replicates. Data derived from $N = 2$ independent experiments. An unpaired t -test was performed to compare P6 vs C3; one-way ANOVA was performed to compare P7 vs HET1 and HET2. **C** Representative images of transmission electron microscopy ER-mitochondria tethering details. White arrows indicate representative contact sites. Scale bars: 200 nm. **D** Violin plot of normalized ER-mitochondria contact length. The relative length is expressed as a ratio of contact length per mitochondrial perimeter. $n = 260$ biological replicates for C3; $n = 240$ biological replicates for P6; $n = 221$ biological replicates for HET1; $n = 130$ biological replicates for HET2; $n = 174$ biological replicates for P7; Data derived from $N = 2$ independent experiments. An unpaired t -test was performed to compare P6 vs C3; one-way ANOVA was performed to compare P7 vs HET1 and HET2. **E** Percentage

of mitochondria in contact with ER. Each dot represents a biological replicate. $n = 10$ biological replicates for C3, P6, HET1 and HET2. $n = 9$ for P7. Data derived from $N = 2$ independent experiments. An unpaired t -test was performed to compare P6 vs C3; one-way ANOVA was performed to compare P7 vs HET1 and HET2. **F, G** Mitochondrial calcium uptake measured as Ca²⁺ responses to agonist stimulation with the cholinergic agonist carbachol in fibroblasts expressing mitochondria-GECO1. Representative calcium traces are shown together with the quantification of peak mitochondrial Ca²⁺ amplitude in fibroblasts upon agonist stimulation. Each dot represents the peak amplitude of single cells. $n = 19$ biological replicates for C3; $n = 25$ biological replicates for P6; $n = 22$ biological replicates for HET1; $n = 17$ biological replicates for HET2; $n = 31$ biological replicates for P7. Data derived from $N = 2$ independent experiments. An unpaired t -test was performed to compare P6 vs C3; one-way ANOVA was performed to compare P7 vs HET1 and HET2. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. Error bars denote the standard error of the mean (SEM).

morphology and motor behaviors in response to touch in zebrafish. Although these data did not demonstrate a cell-autonomous role for FLVCR1a in sensory neurons, our results indicate that reduction of FLVCR1a expression may directly or indirectly affect the survival of sensory neurons, and potentially nociception. Of note, *flvcr1a* morphants and *flvcr1* crispants represent the first animal models exhibiting sensory neuron alterations, as previously generated knockout mice^{26,28} displayed precocious lethality, preventing the study of sensory neurons. This marks a significant advancement in the study of FLVCR1-related sensory neuropathy, providing a valuable tool for further mechanistic investigations and therapeutic exploration.

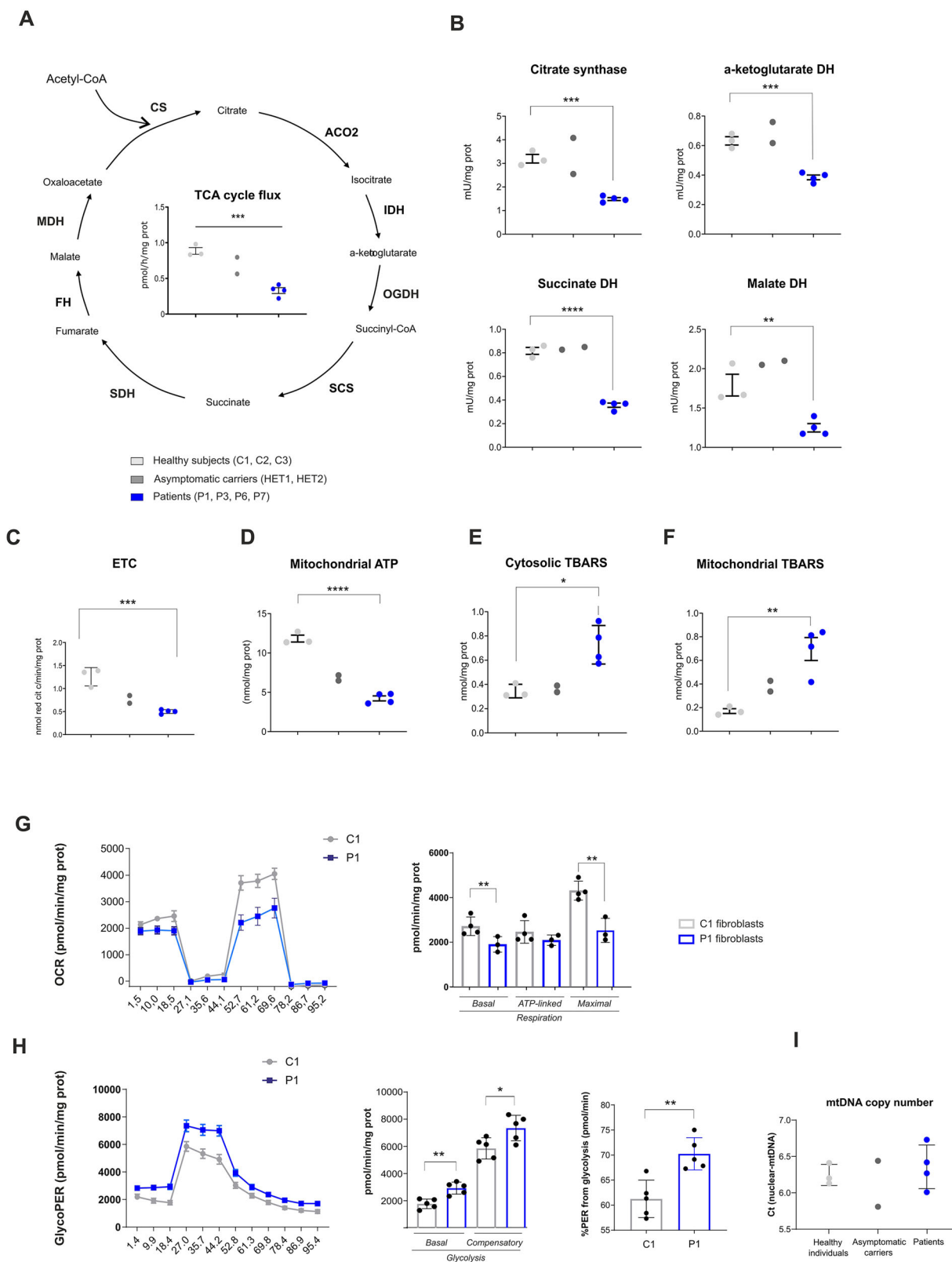
Our study demonstrates that *FLVCR1* variants compromise mitochondrial energetic metabolism in patient-derived fibroblasts. Indeed, patients' fibroblasts showed decreased oxygen consumption rates compared to controls. Remarkably, the alteration of energetic metabolism is independent of the specific type of disease-causing variant. Furthermore, defective energetic metabolism was also observed in *flvcr1a* morphants, thus suggesting that alteration of energetic metabolism may play a role in the degeneration of sensory neurons.

Mechanistically, we have provided evidence that disease-causing *FLVCR1* variants impair all the known functions associated with this transporter, ultimately leading to mitochondrial failure (Fig. 9). Indeed, reduced choline levels, decreased heme biosynthesis rates, and reduced mitochondrial-ER contacts were observed in patient-derived fibroblasts. We propose that the alteration of all these processes may contribute to mitochondrial dysfunction through distinct mechanisms. Reduced choline uptake may affect the synthesis of phospholipids and sphingolipids, leading to the alteration of cell membrane composition, as suggested by the observation of increased membrane fluidity of patient-derived fibroblasts. Notably, OXPHOS critically depends on the unique lipid composition of mitochondrial membranes⁴⁶. Thus, it is plausible that defective choline uptake may contribute to the compromise of OXPHOS by affecting the lipid composition and structures of mitochondrial membranes. Interestingly, choline supplementation fully rescued membrane fluidity but modestly impacted energetic metabolism. We cannot exclude that prolonged choline supplementation will be necessary to fully restore OXPHOS. Additionally, we observed reduced activity of ALAS1, the rate-limiting enzyme in heme biosynthesis, as well as the alteration of mitochondria-ER contact sites structure and function. The alteration of these processes also contributes to the energetic failure by reducing the level of both heme and Ca²⁺, which crucially regulates the TCA cycle and OXPHOS⁴⁷. We previously demonstrated that increasing mitochondrial Ca²⁺ levels is sufficient to completely restore mitochondrial energy metabolism in primary fibroblasts derived from P1 and P3⁴⁰. Here, we confirmed these findings in primary fibroblast cultures established from P6 and P7. Furthermore, we demonstrated that the stimulation of heme synthesis also promoted mitochondrial energy metabolism in patient-derived fibroblasts. Although further studies are required to elucidate the functional link between altered choline uptake,

ALAS1 activity, and mitochondrial Ca²⁺ handling, we demonstrated that dysregulation of these processes contributes to mitochondrial failure in patient-derived fibroblasts and suggest that patients could benefit from therapeutic strategies aimed at enhancing mitochondrial energy metabolism. Based on these results, we proposed that the development of future therapeutic approaches for FLVCR1-associated sensory neuropathy should consider combinatorial approaches to target the multiple biological processes that are affected in this rare disorder.

Choline supplementation has been proposed as a potentially safe and effective therapeutic approach³⁵. Supporting this hypothesis, we demonstrated that supraphysiological doses of choline fully restored membrane fluidity and modestly improved mitochondrial function in cells. Although we cannot exclude the possibility that chronic treatment may further enhance mitochondrial function, our findings also highlight additional potential therapeutic targets to stimulate mitochondrial energetic metabolism. Indeed, we observed an improved mitochondrial function upon ALA administration and MCU overexpression. Although ALA is already an FDA-approved drug for photodynamic therapy in cancer, its therapeutic implications for FLVCR1-linked sensory neuropathy are probably limited, as we already showed that the prolonged treatment with ALA induces cell death in patient-derived cells^{20,21}. On the other hand, the ability of MCU overexpression to fully restore bioenergetic hold significant therapeutic implications, as MCU enhancers have already been proposed for the treatment of other neurodegenerative disorders^{52,53}. Therefore, this study provides proof of concept of the feasibility to restore energetic metabolism in an in vitro model of the disease. Future studies in relevant animal models that recapitulate the full spectrum of patient clinical features will be essential to evaluate the therapeutic efficacy of choline supplementation alone or in combination with other strategies aimed at boosting energetic metabolism.

Biallelic *FLVCR1* pathogenetic variants have been associated with multiple clinical presentations. Most severe cases showed profound neurodevelopmental defects often associated with premature death^{40,54} whereas patients affected by pain insensitivity might also develop sensory ataxia and retinitis pigmentosa^{55,56}. Proper regulation of energy metabolism is critical during central nervous system development, and we have previously shown that mitochondrial defects are associated with impaired neurogenesis in a mouse model phenocopying the most severe cases⁴⁰. Notably, sensory neurons are particularly sensitive to defects in energetic metabolism because of the long dimension of their axons⁵⁷. Proper energy supply is required not only to restore the membrane potential upon depolarization but also for other energy-demanding processes like neurotransmitter recycling, intracellular signaling, maintenance of cytoskeletal structure, and axonal transport⁵⁸. Interestingly, the alteration of these processes is common in several sensory neuropathies^{1,57}. Based on these observations, we hypothesized that mitochondrial dysfunction may be responsible for the progressive degeneration of sensory neurons in FLVCR1-related sensory neuropathy.



Beyond peripheral neurons, photoreceptors also have strong energetic requirements and rely on proper mitochondrial functions^{59,60}. Indeed, posterior column involvement, sensory ataxia, and retinopathy are not infrequent manifestations of mitochondrial disorders, as highlighted in Sensory Ataxic Neuropathy with Dysarthria and Ophthalmoplegia (SANDO)⁶¹ or Neuropathy Ataxia and Retinitis Pigmentosa (NARP)⁶²

syndromes. Therefore, mitochondrial failure may contribute to the multiple clinical presentations associated with *FLVCR1* mutations, and promoting mitochondrial function and maintenance might represent a promising starting point for therapeutic intervention. Along this line, as demonstrated in this study, patient-derived fibroblasts and a zebrafish model might serve as suitable systems for pre-clinical intervention studies.

Fig. 5 | Impairment of cell bioenergetics in patient-derived fibroblasts. Primary fibroblasts derived from 4 patients carrying distinct disease-causing variants in the *FLVCR1* gene (P1–P3–P6 and P7), 3 healthy individuals (C1, C2 and C3) and 2 asymptomatic carriers (HET1 and HET2) were analyzed. **A** Graphical representations of TCA cycle flux and enzymes. In the middle, TCA cycle measurement. **B** Activities of the main TCA cycle enzymes. **C** Total activity of the ETC measured as nmol of reduced cytochrome C /minute. **D** Mitochondria ATP content. **E, F** Measurement of cytosolic and mitochondrial Thiobarbituric Acid Reactive Substance (TBARS) as index of lipid peroxidation. Each dot in the graphs (**A–F**) represents a biological replicate. $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$ biological replicates for patients. An unpaired t-test was performed to compare healthy individuals and patients. $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$; $**** = p < 0.0001$. Error bars in the graphs denote the standard deviation (SD). **G** Oxygen Consumption Rate (OCR) was measured in C1 and P1 derived fibroblasts using the Seahorse Mito Stress Test. The OCR was monitored during sequential injection of 1 μ M oligomycin, 2 μ M

FCCP, and 1 μ M rotenone plus 1 μ M antimycin A. Calculated values for respiratory parameters are shown on the right. $N = 4$ independent experiments for C1; $N = 3$ independent experiments for P1. Basal: basal respiration. ATP-linked: ATP-linked respiration. Maximal: maximal respiratory capacity. **H** The glycolytic proton efflux rate (GlycoPER) was measured in C1 and P1-derived fibroblasts using the Seahorse Glycolytic Rate Assay. Calculated values for basal and compensatory glycolysis as well as the percentage of proton efflux rate (%PER) derived from glycolysis only, are shown on the right. $n = 5$ independent experiments. **G, H** One-way ANOVA analysis of variance or unpaired t-test was performed. $* = p < 0.5$; $** = p < 0.01$; $*** = p < 0.001$; $**** = p < 0.0001$. Error bars in the graphs denote the standard deviation (SD). **I** Mitochondria copy number assessed in controls, asymptomatic carriers, and patients derived fibroblasts. Each dot in the graph represents a biological replicate. $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$ biological replicates for patients. An unpaired t-test was performed to compare healthy individuals and patients.

Materials and methods

Patients

Patient 6. First, gene panel analysis (including *ATL1*, *ATL3*, *DNMT1*, *FAM134B*, *KIF1A*, *RAB7A*, *SCN9A*, *SPTLC1*, *SPTLC2*, *WNK1*) was carried out as custom-made target enrichment (Agilent SureSelect). Two years later, whole exome sequencing (Human All Exon V7, NextSeq 500 system, Illumina, San Diego, CA) was carried out.

Patient 7. Blood samples were collected from the proband, and genomic DNA was isolated using DNeasy Blood & Tissue Kit (QIAGEN; cat. no. 69504). WGS library construction, sequencing, variant calling, and annotation for SNVs, CNVs, and SVs were performed by Novogene, Inc. Skin biopsy was collected from the proband for the establishment of patient-derived fibroblast cultures.

All ethical regulations relevant to human research participants were followed.

Cell culture

Patient 1 (P1) and Patient 3 (P3) have been described previously²¹. In brief, P1 had compound heterozygous variants in the *FLVCR1* gene (NM_014053.4 c.574 T > C; p.(Cys192Arg) and c.610del; p.(Met204-Cysfs*56)) and exhibited early-onset pain insensitivity characteristic of hereditary sensory and autonomic neuropathy (HSAN)²¹. P3 had a homozygous missense variant c.661 C > T, p.Pro221Ser in the *FLVCR1* gene (NM_014053.4) and displayed a combination of typical PCARP and sensory-autonomic neuropathy²⁰.

Primary fibroblasts from P1 and P3 have been previously described^{21,40}. P6, P7, and HET1, HET2 (the heterozygous parents of P7) were isolated from skin biopsy upon informed consent. As control, we used primary fibroblasts derived from healthy donors (C1, C2, and C3) that were obtained from the “Cell Line and DNA Biobank” from patients affected by Genetic Diseases (Istituto Giannina Gaslini), member of Telethon Network of Genetic Biobanks (project no. GTB12001). Control and patient-derived primary fibroblasts were propagated in DMEM medium (ThermoFisher Scientific) supplemented with 10% heat-inactivated low-endotoxin fetal bovine serum (ThermoFisher Scientific), 100 U/ml penicillin, 100 mg/ml streptomycin, and 1% NEAA (ThermoFisher Scientific).

Cells were maintained at 37 °C under a 5% CO₂ atmosphere. To stimulate the endogenous heme synthesis, cells were treated with 5 mM ALA (A3785; Sigma-Aldrich). Choline supplementation was performed with 1 mM Choline Chloride (67481; Sigma-Aldrich). All these treatments were performed for 16 hours under starved conditions.

Western blot

To assess FLVCR1a expression, human-derived primary fibroblasts were lysed by rotation for 30 min at 4 °C in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA). The buffer was freshly supplemented with 1 mM

phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA, catalog no. P0044), 1 mM PMSF (Sigma Aldrich, St. Louis, MO, USA, catalog n° 93482-50ML-F), and protease inhibitor cocktail (La Roche, Basel, CH, catalog n° 04693116001). The cell lysate was clarified by centrifugation for 10 min at 4 °C. Protein concentration in the supernatant was assessed by Bradford assay. For FLVCR1a protein detection, 10 μ g of protein extracts was incubated 10 min at 37 °C with 1 μ L of PNGase-F from Elizabethkingia meningoseptica (Sigma Aldrich, St. Louis, MO, USA, catalog n° P-7367) to remove protein glycosylation. Before loading on 4–15% mini-PROTEAN TGX precast gel (Bio-Rad, Hercules, CA USA, catalog n° 4568084), samples were incubated 5 min at 37 °C (FLVCR1) or 10 min at 95 °C (Vinculin) in 1 $\times$ Laemmli buffer freshly supplemented with 8% 2-mercaptoethanol. The primary antibodies and dilutions are as follows: FLVCR1 (C-4) (Santa Cruz Biotechnology, Dallas, TX, USA, catalog n° sc-390100; 1:500); ALAS1 (Santa Cruz Biotechnology, Dallas, TX, USA, catalog n° sc-137093 # C0112; 1:1000); Vinculin (home-made, 1:8000).

TCA cycle flux and enzymatic activities

The flux of glucose through the TCA cycle was determined by radiolabeling cells with 2 μ Ci/ml [6-¹⁴C]-glucose (55 mCi/mmol; PerkinElmer, Waltham, MA, USA). A 1-hour incubation of the cell suspensions was performed in a closed experimental apparatus to trap the ¹⁴CO₂ developed from [¹⁴C]-glucose. The reaction was terminated by injecting 0.5 ml of 0.8 N HClO₄. The quantity of glucose converted into CO₂ through the TCA cycle was expressed as nmoles CO₂/h/mg protein. The activities of citrate synthase, α -ketoglutarate dehydrogenase, succinic dehydrogenase, malate dehydrogenase, were measured spectrophotometrically using the Citrate Synthase Assay Kit (Sigma Aldrich, St. Louis, MO; cat. no. CS0720), the Alpha Ketoglutarate (alpha KG) Assay Kit (Abcam, Cambridge, UK; ab83431), the Succinate Dehydrogenase Activity Colorimetric Assay Kit (BioVision, Waltham, MA; AB228560), Malate Dehydrogenase Assay Kit (Sigma Aldrich; MAK196), as per manufacturer's instructions. Results were expressed as U/mg proteins (citrate synthase), nmoles NADH/min/mg proteins (α -ketoglutarate dehydrogenase, malate dehydrogenase), nmoles FADH₂/min/mg proteins (succinic dehydrogenase).

Mitochondrial extraction

Mitochondria were extracted as previously reported⁶³. Cells were lysed in 0.5 ml mitochondria lysis buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM Mg Cl₂, 1.8 mM ATP, 1 mM EDTA, pH 7.2), supplemented with Protease Inhibitor Cocktail III (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 250 mM NaF. Zebrafish tissues were homogenized using a Tissue Lyser II (Qiagen), filtered through a 60 μ m strainer to obtain a single-cell suspension, centrifuged at 1200 g for 2 min at room temperature, and re-suspended in 0.5 ml mitochondria lysis buffer. All samples were clarified by centrifugation at 650 g for 3 min at 4 °C. Supernatants were collected and centrifuged at 13000 g for 5 min at 4 °C. The new supernatants, corresponding to the cytosolic fraction, were used for cytosolic ROS

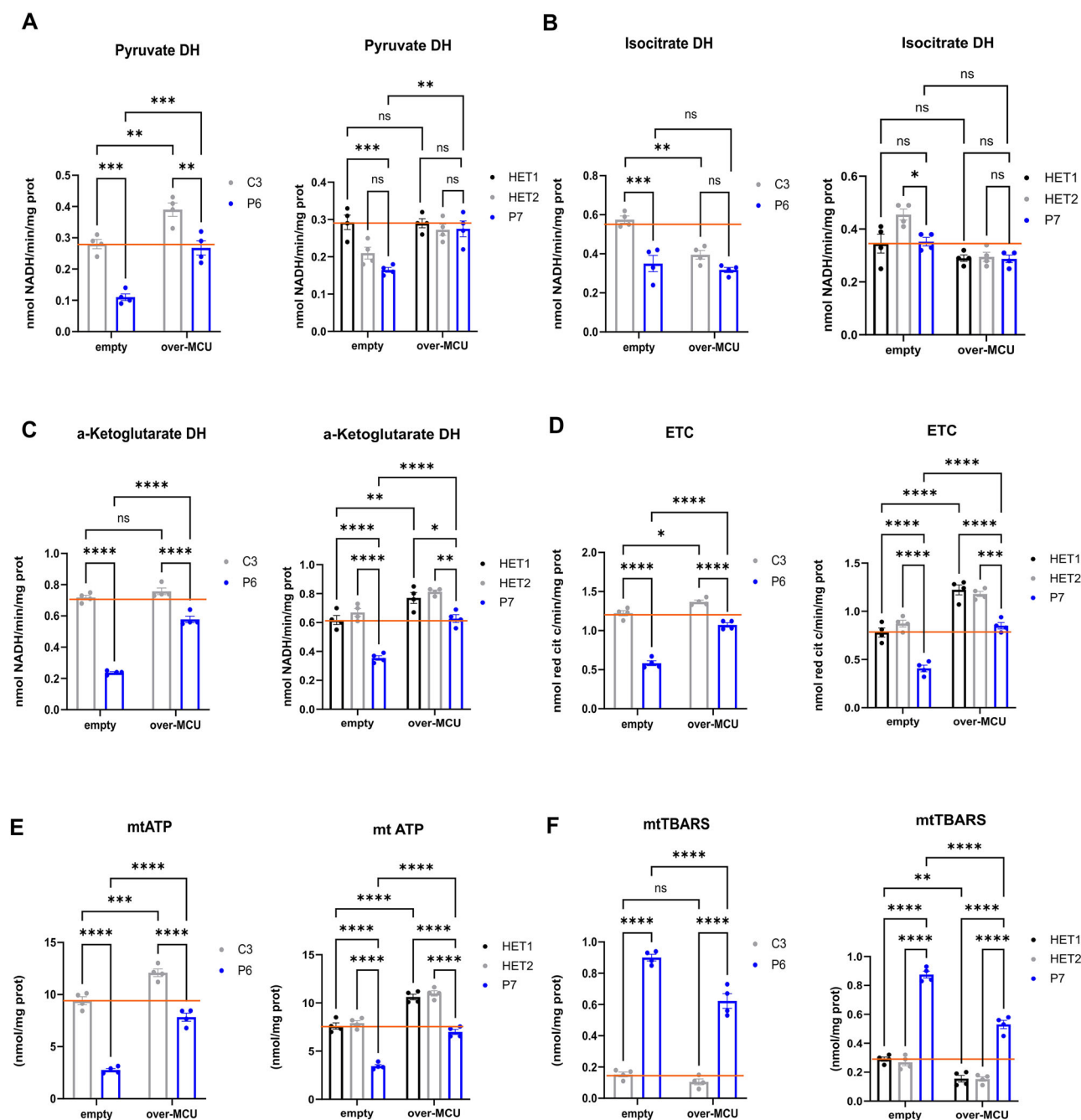


Fig. 6 | MCU overexpression rescues defective bioenergetic in patient-derived fibroblasts. Primary fibroblasts from patient P6 compared to control C3 and from patient P7 compared to HET1 and HET2. Cells overexpressing MCU (over-MCU) were compared to cells transfected with the control vector (empty). Each dot in the graphs represents an independent experimental replicate. **A** Pyruvate dehydrogenase activity. **B** Isocitrate dehydrogenase activity. **C** a-ketoglutarate

dehydrogenase activity. **D** Total electron transport chain activity. **E** Mitochondrial ATP levels. **F** Mitochondrial thiobarbituric acid reactive substance (TBARS) levels used as index of lipid peroxidation. **A–F** $N = 4$ independent experiments. Two-way ANOVA analysis of variance was performed. * = $P < 0.5$; ** = $P < 0.01$; *** $P < 0.001$; **** $P = 0.0001$. Error bars denote the standard error of the mean (SEM).

measurements. Pellets, containing mitochondria, were washed once with lysis buffer and resuspended in 0.25 ml mitochondria resuspension buffer (250 mM sucrose, 15 mM K_2HPO_4 , 2 mM $MgCl_2$, 0.5 mM EDTA). 50 μ l aliquots were sonicated and used for the measurement of protein content by the BCA Protein Assay kit (Sigma Aldrich; 71285-M) and for quality control: 10 μ g of each sonicated sample were analyzed by SDS-PAGE and immunoblotting with an anti-porin antibody (Abcam; clone 20B12AF2) to confirm the presence of mitochondrial proteins in the extracts⁴⁰. The remaining 200 μ l were used for ALAS1, ETC, ATP, and TBARS measurement.

Measurement of Choline levels

Total Choline was measured with a sensitive fluorescence-based assay (Total Choline Detection Kit; ab219944 from Abcam) according to manufacturer instructions. The choline quantity correlates with the concentration of hydrogen peroxide generated in the choline oxidase-mediated enzyme coupling reaction cycle, subsequently detected with the proper kit indicator. The rise in fluorescence is detected at an optimal Ex/Em = 540/590 nm. Fluorescence was converted into choline concentration using the Standard Curve obtained in the same assay. Picomols of choline per sample (12.5×10^4 cells) were calculated.

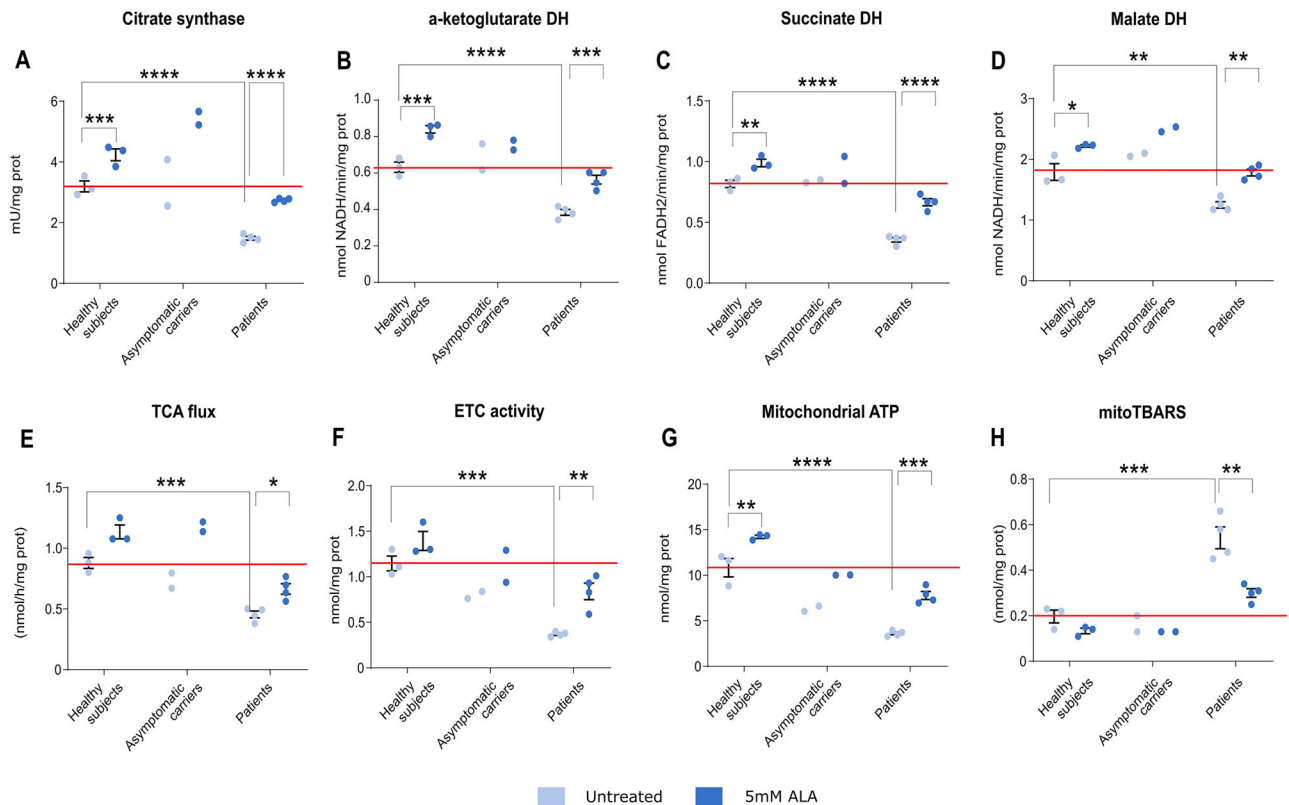


Fig. 7 | The stimulation of heme synthesis improves bioenergetic in patient-derived fibroblasts. Primary fibroblasts derived from 4 patients carrying distinct pathogenic variants in the *FLVCR1* gene (P1-P3-P6 and P7), from 3 healthy individuals (C1, C2, and C3) and from 2 asymptomatic carriers (HET1 and HET2) were used. Primary fibroblasts were treated for 16 hours with 5 mM δ -aminolevulinic acid (ALA) alone to restore heme synthesis. Each dot in the graphs represents a biological replicate. Error bars in the graphs denote the standard error of the mean (SEM). **A** Citrate synthase activity upon ALA treatment. **B** α -ketoglutarate dehydrogenase activity upon ALA treatment. **C** Succinate dehydrogenase activity

upon ALA treatment. **D** Malate dehydrogenase activity upon ALA treatment. **E** TCA cycle flux measurement upon ALA treatment. **F** Total activity of the ETC measured as nmol of reduced cytochrome C /minute upon ALA treatment. **G** Mitochondria ATP content upon ALA treatment. **H** Measurement of mitochondrial Thiobarbituric Acid Reactive Substance (TBARS) as an index of lipid peroxidation upon ALA treatment. **A–H** $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$ biological replicates for patients. Two-way ANOVA analysis of variance was performed to compare healthy individuals and patients. * = $P < 0.5$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$.

Membrane fluidity measurement

Membrane fluidity was measured in triplicate using a membrane fluidity kit (Marker Gene Technologies M0271) according to the manufacturer's protocol. In brief, we incubated the samples (adherent or suspension cells) with 2 μ M pyrenedecanoic acid (PDA) and 0.08% pluronic F127 (to increase labeling) in either Perfusion buffer or cell culture medium (supplement-free) for 30 min at 22 °C with mild shaking, then washed twice with PBS. In PBS, we recorded fluorescence emissions between 392–450 nm in 2 nm steps after excitation at 360 nm with a Tecan microplate reader (Infinite® 200 PRO NanoQuant). We subtracted a background sample where the cells were processed without PDA. With increased membrane fluidity, the lipophilic pyrene probe forms excimers upon interaction. The ratio of excimer (peak around 450 nm) to monomer (peak around 394–398 nm) IE/IM was calculated as a quantitative measure of membrane fluidity⁶⁴.

ALA synthase (ALAS) activity

ALAS activity was determined on 100 μ g of mitochondrial extracts, following the previously reported procedure⁶⁵. 10 μ l of the reaction's product was injected into a Waters Acquity ultra performance liquid chromatography (UPLC), equipped with a binary solvent manager, sample manager, photodiode array detector (PDA), fluorescence detector, column heater, and an Acquity UPLC BEH C18, 1.7 μ M, 2.1 $\times$ 100 mm column. The detection of ALA-derivatives was performed by setting the detector with λ excitation 370 nm and λ emission 460 nm, and the range of the PDA scanner between 210 and 500 nm. The results were converted into

nmol/min according to a titration curve previously set and expressed as nmol/min/mg mitochondrial proteins⁴⁴.

Endoplasmic reticulum (ER)- mitochondria contact sites measurement

ER-mitochondria contacts were measured as previously described⁴⁰. Fibroblasts were transfected with a plasmid encoding a mitochondria-targeting peptide fused with the fluorescent protein RFP (mt-DsRed) and another plasmid encoding the ER resident protein Sec61 fused with GFP (Sec61-GFP), using Lipofectamine™ 3000 Transfection Reagent (ThermoFisher Scientific, L3000001) following manufacturer instructions. Live cell imaging for mt-DsRed and Sec61-GFP expression was performed using Leica TCS SP8 confocal scanning system (Leica Microsystems) equipped with 405-nm Diode, 488-nm Ar, and 561-nm DPSS lasers. A 60X HC PL APO oil immersion objective was used to take images at 1024 $\times$ 1024 resolution. Colocalization of the two signals was quantified with the ImageJ Colocalization plugin JACOP, after applying the TOP HAT (BOX) filter.

Quantification of mitochondrial DNA copy number (mtDNA CN)

The relative mtDNA copy number per cell was quantified by a multiplex Taqman qPCR assay (Bio-Rad 4369510), following a previously published protocol⁶⁶. B2M was used as a nuclear encoded reference gene, and MT-ND1 as a mitochondrial encoded reference gene. The primers used for the qPCR reaction were as follows: B2M Fw-CACTGAAAAAGATGAGTAT GCC, Rv-AACATTCCTGACAATCCC; MT-ND1 Fw-ACGCCATA

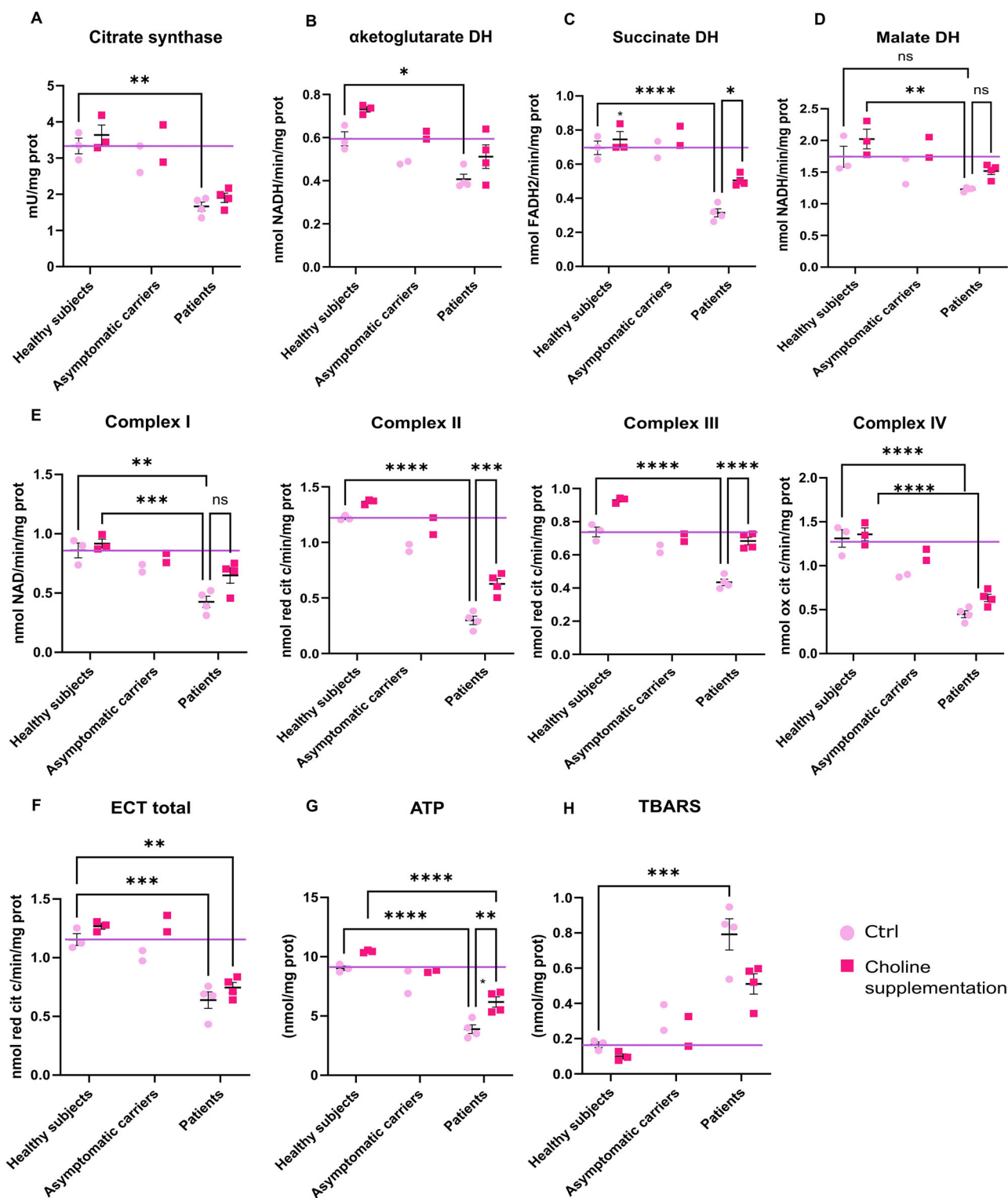


Fig. 8 | Choline supplementation improves bioenergetics in patient-derived fibroblasts. Primary fibroblasts derived from 4 patients carrying distinct pathogenic variants in the *FLVCR1* gene (P1-P3-P6 and P7), from 2 healthy individuals (C1 and C2), and from 2 asymptomatic carriers (HET1 and HET2) were analyzed. Choline supplementation was achieved by treating the cells with 1 mM choline chloride for 16 hours. Each dot in the graphs represents a biological replicate. Error bars in the graphs denote the standard error of the mean (SEM). **A** Citrate synthase activity upon choline supplementation. **B** α -ketoglutarate dehydrogenase activity upon choline supplementation. **C** Succinate dehydrogenase activity upon choline supplementation. **D** Malate dehydrogenase activity upon choline supplementation.

E Activity of the individual ETC complexes I-IV upon choline supplementation. **F** Total activity of the ETC measured as nmol of reduced cytochrome C /minute upon choline supplementation. **G** Mitochondria ATP content upon choline supplementation. **H** Measurement of mitochondrial Thiobarbituric Acid Reactive Substance (TBARS), as index of lipid peroxidation upon choline supplementation. **A-H** $n = 3$ biological replicates for healthy individuals; $n = 2$ biological replicates for asymptomatic carriers; $n = 4$ biological replicates for patients. Two-way ANOVA analysis of variance was performed to compare healthy individuals and patients. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$.

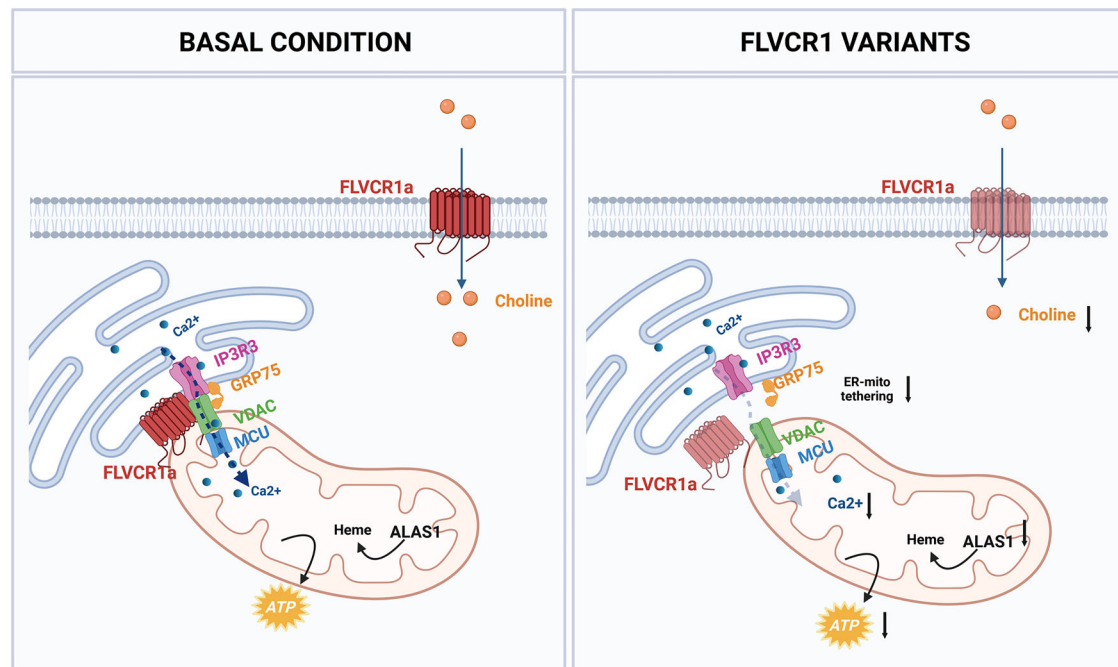


Fig. 9 | Functional consequences of pathogenic *FLVCR1* variants. Schematic model showing that disease-causing *FLVCR1* variants impair all the known functions associated with this transporter (reduced choline levels, reduced ALAS1

activity, impaired ER-mitochondria tethering, and mitochondria calcium uptake), ultimately leading to mitochondrial failure. Created in BioRender.

AAACTCTTACCAAAG, Rv-GGGTTCATAGTAGAAGAGCGATGG. The probes used for the qPCR reaction were as follows: MT-ND1 probe HEX-5'-ACCCGCCACATCTACCATACCCTC and 5'-B2M probe 6FAM-3'-CCGTGTGAACCATGTGACTTTGTC. All samples were run in triplicates and replicates with greater than 0.5 Ct differences were removed. The relative amount of mtDNA was calculated at the Ct value difference of B2M and ND1, where Delta Ct (Δ Ct) equals the sample Ct of the mitochondrial gene (MT-ND1) subtracted from the sample Ct of the nuclear reference gene (B2M). Statistical analysis was performed in the GraphPad Prism software (v. 9.5.0)⁶⁷.

Ca²⁺ imaging and quantification

For ratiometric imaging of calcium, we used previously published protocols⁴⁰. Briefly, fibroblasts were transfected with the plasmid pCMV-mito-GEM-GECCO1 (Addgene plasmid #32461) encoding the mito-GEM-GECCO1 dye, with Lipofectamine[™] 3000 Transfection Reagent (ThermoFisher Scientific, L3000001) following manufacturer instructions. Images were acquired with a 405-nm diode laser on a Leica TCS SP8 confocal scanning system (Leica Microsystems). Emission windows were configured for 427–467 nm (blue channel) and 502–537 nm (green channel). Eight-bit digital images were captured from a single optical plane using a 63x HC PL APO CS2 oil-immersion objective (numerical aperture 1.40) from Leica. The settings for laser intensity, pinhole (1 Airy unit), emission window range, electronic zoom, gain and offset of the photomultiplier, field format, and scanning speed were optimized at the start and maintained consistently throughout the study to ensure uniform digitization of all cells. Mitochondrial signals from the blue and green channels were segmented, and their ratio was calculated and measured using the Analyze Particles command in FIJI/ImageJ. To induce calcium release from the Endoplasmic Reticulum and subsequent rapid calcium uptake by mitochondria, primary fibroblasts were treated with Carbachol at a concentration of 500 μ M.

Transmission electron microscopy and quantification of ER-mitochondrial contact sites

Fibroblast cell cultures (at 1×10^6 cells/ml density) from 2 asymptomatic carriers (HET1, HET2), 1 control (C3), and 2 patients (P6 and P7) were

harvested, and the pellet was fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 1 h at 4 °C. The pellet was washed in cacodylate buffer $3 \times 10'$, postfixed in osmium ferrocyanide (1 volume of 2% aqueous osmium tetroxide: 1 volume of 3% potassium ferrocyanide) for 1 hour at 4 °C, and dehydrated in increasing concentrations of acetone (30%, 60%, 90%, 100%). The pellet was then incubated in acetone/Spurr resin (1:1, 30 min; 1:2, 30 min) and in Spurr resin overnight at room temperature. Finally, the pellet was embedded in Spurr resin in 0.5 ml Eppendorf tubes for 24 hours at 70 °C. Fibroblast cells were pelleted by centrifugation at 100 g for 15 min in all steps up to resin.

Ultrathin sections (70 nm thickness) were cut with an ultramicrotome (EM UC6, Leica Microsystems), collected on uncoated nickel grids (100 mesh), and counterstained for 30 s with UranylLess EM Stain and 30 s with lead citrate (Electron Microscopy Sciences).

Grids were observed with a JEM-1400 Flash transmission electron microscope (Jeol, Tokyo, Japan), and a Jeol Matataki Flash camera's automated montage system was employed to obtain electron micrographs.

For the analysis, we have considered as ER-mito contacts only those presenting a distance between the organelles inferior or equal to 100 nm⁶⁸. To analyze ER-mitochondria interaction, we evaluated both MERCS interface, considered as the contact length of ER-OMM extension, normalized over the mitochondria perimeter, and the % of mitochondria with ER contact. All the measurements were manually made using the ImageJ software (NIH, Bethesda, MD).

Mitochondrial electron transfer chain (ETC) and complex activity

To measure electron flux from complex I to complex III, taken as an index of the mitochondrial respiratory activity⁶³, 50 μ g of non-sonicated mitochondrial samples, isolated as indicated above, were re-suspended in 0.2 ml buffer A (5 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v bovine serum albumin, BSA; pH 7.2) to which 0.1 ml buffer B (25% w/v saponin, 50 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v BSA, 0.12 mM oxidized cytochrome c, 0.2 mM NaN₃, which blocks complex IV allowing the accumulation of reduced cytochrome c; pH 7.5) was added for 5 min at room temperature. The cytochrome c

reduction reaction was started by adding 0.15 mM NADH and was followed for 5 min at 37 °C, reading the absorbance at 550 nm by a Packard microplate reader EL340 (Bio-Tek Instruments, Winooski, VT). The results were expressed as nanomoles of reduced cytochrome c /min/mg mitochondrial proteins⁴⁰.

More specific spectrophotometric determination of electron flux through complex I, complex II, complex III, and complex IV was measured as detailed in (Xu, 2021). To measure complex I activity, 20 µg of non-sonicated mitochondrial samples were re-suspended in 0.2 ml buffer 1 A (5 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v BSA), incubated 1 min at room temperature followed by 7 min in 0.1 ml buffer 1B (25% w/v saponin, 50 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v BSA, 0.12 mM oxidized ubiquinone, which acts as electrons shuttle from complex I to complex III, 2.5 mM antimycin A, which inhibits complex III, 0.2 mM NaN₃, which blocks complex IV; pH 7.5). 1.5 mM NADH, as an electron donor, was added to the mix. The rate of NADH oxidation was followed for 5 min at 37 °C, reading the absorbance at 340 nm. The results were expressed as nanomoles of NAD⁺/min/mg mitochondrial proteins. Complex II activity was measured as the rate of electron transfer between complex II and complex III. 20 µg of non-sonicated mitochondrial samples were re-suspended in 0.1 ml buffer 2 A (50 mM KH₂PO₄, 7.5 mM MgCl₂, 25% w/v saponin, 20 mM succinic acid; pH 7.2) and incubated for 30 min at room temperature. 0.2 ml buffer 2B (50 mM KH₂PO₄, 7.5 mM MgCl₂, 5% w/v BSA, 30 mM succinic acid as substrate of complex II, 0.12 mM oxidized ubiquinone as electrons shuttle from complex II to complex III, 0.12 mM oxidized cytochrome c as acceptor of electrons flowing from complex II to complex III, 5 mM rotenone to prevent electron flux from complex I, 0.2 mM NaN₃, to block complex IV) were added. The rate of reduction of cytochrome c was measured for 5 min at 37 °C, reading the absorbance at 550 nm. The results were expressed as nanomoles of reduced cytochrome c/min/mg mitochondrial proteins. The activity of complex III was measured in the same samples where the electron flux from complex I to complex III was evaluated. After 1 min from the addition of NADH, as an inducer of electron flow, 5 mM rotenone, which blocks the activity of complex I, was added. The rate of reduction of cytochrome c, which is dependent on the activity of complex III only in the presence of rotenone, was followed for 5 min at 37 °C, reading the absorbance at 550 nm. The results were expressed as nanomoles of reduced cytochrome c/min/mg mitochondrial proteins. To measure the activity of complex IV, the rate of oxidation of cytochrome c (reduced form, generated by complex III) was measured. 20 µg of non-sonicated mitochondrial samples were re-suspended in 0.1 ml buffer 4 A (50 mM KH₂PO₄, 20 mM succinic acid, 25% w/v saponin; pH 7.2) and incubated 30 min at room temperature. 0.2 ml buffer 4B (50 mM KH₂PO₄, 5 mM rotenone, which prevents electron flux from complex I to complex III, 30 mM succinic acid as substrate of complex II and electron generator, 0.03 mM reduced cytochrome c as acceptor of electrons flowing from complex III to complex IV) was added. The rate of oxidation of cytochrome c was followed for 5 min at 37 °C, reading the absorbance at 550 nm. The results were expressed as nanomoles of oxidized cytochrome c/min/mg mitochondrial proteins⁴⁰.

ATP levels

ATP amounts in mitochondrial extracts were measured with the ATP Bioluminescent Assay Kit (Millipore Sigma; FLAA), as per the manufacturer's instructions. Results were expressed as nmol/mg mitochondrial proteins⁴⁰.

TBARS (thiobarbituric reactive substances)

Oxidative damage was evaluated in mitochondrial and cytosolic extracts, obtained as reported above, by using the Lipid Peroxidation (4-HNE) Assay Kit (Abcam; ab238538) that evaluates the 4-hydroxy-nonenal, one of the

thiobarbituric reactive substances used as an indicator of lipid peroxidation. Results were expressed as nmol/mg mitochondrial protein.

Measurement of heme concentration

Intracellular heme concentration was measured using a fluorescence assay, as previously reported^{28,69}. Briefly, cells were washed twice with PBS 1X and lysed on ice using TBS buffer with 1% Triton X-100 for 15 minutes. Then, 50–100 µg of total protein was resuspended in 100 µl of PBS 1X and mixed with 1 mL of 2 M oxalic acid at room temperature. The resultant protoporphyrin was measured by fluorescence using a Glomax Multi Detection System (excitation: 405 nm, emission: 580–640 nm). Data were normalized to the endogenous protoporphyrin content (by measuring the fluorescence of unheated samples) and to total protein concentration⁴⁰. Results were expressed as pmol of heme/mg total protein.

Seahorse

Real time-measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed using the Seahorse XF, both the Cell Mito Stress Test Kit (103015-100) and the Glycolytic Rate Assay Kit (103344-100), on a XFe24 Seahorse Analyzer (Agilent Technologies, Santa Clara, CA). Fibroblasts were plated at a density of 35,000 cells/well in 24-well assay plates (Agilent Technologies) and cultured in complete DMEM medium as described. After 18 hours, culture medium was replaced by XF DMEM medium supplemented with 10 mM glucose, 1 mM pyruvic acid, and 2 mM L-glutamine (pH 7.4, Agilent Technologies), and cells were incubated at 37 °C in a non-CO₂ incubator for 45 minutes prior to the assay. To assess mitochondrial function, OCR was measured at baseline and after sequential addition of 1 µM oligomycin, 2 µM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), and 0.5 µM rotenone/antimycin A (Rot/AA). To evaluate glycolytic rates, both OCR and ECAR were measured at baseline and after sequential addition of 0.5 µM Rot/AA, and 50 µM 2-deoxy-D-glucose (2-DG). Data were acquired through the WAVE software (Agilent Technologies) and normalized by total protein concentration/well. Metabolic parameters of interest, including both basal and maximal mitochondrial respiration and glycolytic proton efflux rate (glycoPER) were calculated using the XF Mito Stress Test and Glycolytic Rate Assay Report Generators (Agilent Technologies).

Zebrafish maintenance and egg collection

Wild-type (AB line; ZFIN: *ZDB-GENO-960809-7*) and transgenic *Tg(-3.1neurog1:GFP)*⁷⁰ (ZDB-ALT-030904-6) zebrafish (shortened to *Tg(ngn1:GFP)* in the manuscript) were originally sourced from the KIT-European Zebrafish Resource Centre (EZRC; <https://www.ezrc.kit.edu/>). The *Tg(-3.1neurog1:GFP)* line is maintained on an AB genetic background.

Zebrafish were raised and maintained in a 14:10 h light: dark cycle at 28 °C in a circulating aquatic system maintained at pH 7.0 and conductivity between 400 and 500 µS. Zebrafish were fed three times daily with a combination of granular food (GEMMA from Skretting) in the morning and afternoon and freshly prepared artemia in the evening (cysts bought from SDS, Witham, UK). Adult fluorescent *Tg(ngn1:GFP)* fish were crossed with AB fish. Fluorescent embryos were selected and used in experiments or grown to adulthood to maintain the line. Males and females were put in the breeding tank overnight, and the next morning, fresh spawned embryos were collected. The collected eggs were placed in 100 mm Petri dishes in fish water with methylene blue (0.1 g/L Instant Ocean Salt; 0.1 g/L NaHCO₃; 0.19 g/L CaSO₄; methylene blue 0.5 ppm in sterile deionized water). Fish water was added with 0.0045% PTU (1-phenyl-2-thiourea, Sigma) to prevent melanin formation and maintain the optical clarity in the zebrafish embryos. Any unfertilized eggs, dead or bad embryos, were removed, and the good-quality embryos at one cell stage were selected for microinjection.

The study did not require any ethics oversight since we used only embryos and larvae within 120 hpf.

We have complied with all relevant ethical regulations for animal use.

Morpholino microinjection

Morpholino stock solution (1 mM) was purchased from Gene Tool. AB wild-type and transgenic zebrafish embryos at one-cell stage were injected, by an Eppendorf FemtoJet Micromanipulator 5171, with 4 nanolitres (nl) of splicing-blocking *flvcr1a* morpholino targeting the boundary of intron 1 and exon 2, hereafter written as *Flvcr1a* MO IIE2 (Sequence: ACCAAGCTGACGGGAAATAAGAGA). 4 ng of standard morpholino (STD-Mo) injected zebrafish embryos were used as a control, and non-injected embryos were used as an internal control. The morpholinos were co-injected with 0.05% phenol red as a tracer. After microinjection, any defective or injured zebrafish embryos were immediately discarded, and the correctly injected healthy zebrafish embryos were collected in a petri dish and maintained in fish water at 28 °C.

sgRNAs design, microinjection, and sequencing

In silico analyses were performed to identify candidate sgRNAs targeting the zebrafish *flvcr1* locus. All synthetic sgRNAs were synthesized by IDT. SpCas9 Plus Protein was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Before injections, 100 ng/μL sgRNAs were complexed with 150 ng/μL Cas9 and incubated for 10 minutes at room temperature. A mix of 4 nL of Cas9 and synthetic sgRNAs was injected into the cell at the one-cell stage zebrafish embryos. The final concentrations were 150 pg/nL for Cas9 and 100 pg/nL for sgRNAs. The sgRNAs used are listed in Supplementary Table S2.

10 injected embryos per pool were collected at 24 hpf to evaluate sgRNAs editing efficiency. Genomic DNA was extracted using an alkaline lysis method: samples were incubated in 10 μL of NaOH (50 mM) at 95 °C for 15 minutes, followed by neutralization with 2 μL of Tris-HCl (pH 7.5, 100 mM). The crude lysate was then used directly as a template for PCR amplification. Genomic DNA samples were stored at 4 °C for short-term use or at -20 °C for long-term storage. Primers used are listed in Supplementary Table S2. Sanger sequencing results (Figure S9) were analyzed using TIDE and ICE analysis tools, and the 3 sgRNAs with editing efficiency higher than 40% were selected to generate *flvcr1* crispants. For the subsequent analyses, PCR was performed on DNA extracted from embryos' tail clips. Only F0 crispants were analyzed.

Survival and morphological evaluation in *flvcr1a* morphants

After microinjection, the survival of the *flvcr1a* morphants was recorded every 24 hours. Embryos injected with STD-Mo and non-injected embryos were used as controls. Any embryo with coagulation or no movement and heartbeat was scored dead. The survival was documented till 144 hpf, and at least 235 embryos per group were used. The data are presented as percentages of alive embryos at 24, 48, 72, and 144 hpf.

The morphological evaluation of the *flvcr1a* morphants, STD-Mo-injected and non-injected embryos, was performed at 48 hpf, coinciding with the peak time of morpholino action. The morphology was categorized into normal, abnormal/mild, and severe phenotypes. Embryos were examined for several morphological abnormalities such as hydrocephalus, small eyes, ear deformation, decreased tail detachment, irregular heartbeat, abnormal blood circulation, pericardial edema, yolk deformation, abnormal yolk extension, hyperextension or flexion of the trunk, and lateral, upward, and downward bending of the tail. Normal embryos were defined according to Kimmel et al.⁷¹ by proper stage-specific morphology, including normal axis formation, correct somite number and organization, and appropriate development of major embryonic structures.

Any embryos with up to 2 morphological deformities were considered a mild phenotype, and embryos with more than 2 were considered a severe phenotype. As the *flvcr1a* morphants had typical phenotypes such as thinning of yolk, elongation, and hydrocephalus, we also documented the percentage of embryos with yolk elongation, thinning, and hydrocephalus in the morphants at 48 hpf. At 48 hpf, representative brightfield images of

flvcr1a morphants, STD-mo, and non-injected embryos were obtained at 40X magnification with Zeiss Axiozoom V16 (Zeiss, Jena, Germany) equipped with PlanNeoFluar Z 1×/0.25 FWD 56 mm lens and connected to a workstation with Zen Pro software. Embryo images were acquired using a compound Axio Zoom.V16 stereo zoom microscope from Zeiss, equipped with an Axiocam 506 color digital camera with ZEN Pro software from Zeiss. Fluorescence in the transgenic lines was imaged with an illuminator HXP 200 C from Carl Zeiss. For in vivo analyses, embryos and larvae were anaesthetized with tricaine 0.16 mg/mL.

Zebrafish lightsheet microscopy

For lightsheet imaging, embryos were anaesthetized using tricaine (0.02%) and included in a low-melting agarose matrix 0.5% (Top Vision Low Melting Point Agarose, Thermo Fisher Scientific), then put in the instrument holder. Images were acquired using the Zeiss LightSheet microscope V1, supported by ZenPro software. EGFP acquisition was performed using a 488–30 nm laser and a 505–545 nm filter. Images within the same experiment were acquired using identical laser intensity and exposure settings to ensure comparability. After the acquisition, 3D images were generated and manipulated using Arivis software. 3D reconstructions were exported as a single snap with the same compression setting.

Zebrafish DRG counting

Tg (ngn1:GFP) zebrafish embryos were microinjected with 4 ng *flvcr1a* Mo-IIE2 4 ng STD-Mo at 1 cell stage, or with scramble-sgRNA and FLVCR1-sgRNA. Non-injected embryos were used as an internal control. Following microinjection, embryos were raised in fish water with 0.0045% PTU (1-phenyl-2-thiourea, Sigma). At 48 hpf, embryos were anesthetized with tricaine 0.16 mg/ml and placed in a lateral position in a 100 mm petri dish with 1% agarose base. Then, the fluorescence images of the zebrafish embryos were obtained for each of the three groups at 40× magnification with a Zeiss Axiozoom V16 (Zeiss, Jena, Germany) fluorescence microscope with GFP filter cube equipped with PlanNeoFluar Z 1×/0.25 FWD 56 mm lens and connected to work station with Zen Pro software. The number of inter-somitic neurons in each zebrafish embryo was counted from the images obtained, and a bar diagram was plotted. A total of 25–30 embryos were used for each group.

Zebrafish behavioral assays

To investigate if loss of DRG in the *flvcr1a* knockdown embryos is associated with the abnormal movement behavior, we performed behavioral assays in the *flvcr1a* morphants along with Mo-STD-injected embryos and non-injected embryos. The movement behaviour tests were performed according to the scheme described by Basnet et al.⁷².

Zebrafish spontaneous head-tail coil movement

At 24 hpf, spontaneous head-tail coil movement was counted for one minute under the Leica MZ16F stereomicroscope (Leica Microsystems Ltd.). The total count of head-tail coil movements was determined for 20–25 embryos for each group in three independent experiments, and a bar diagram was plotted.

Measurement of the distance swam by zebrafish embryos

Touch-response test in zebrafish embryos was performed at 48 hpf as described in Basnet et al. Briefly, embryos injected with Mo-*flvcr1a* IIE2, Mo-STD, and non-injected embryos were touched at the tail, following which the distance swam was recorded with a motility wheel containing 5 concentric circles of 5 mm, 10 mm, 15 mm, and 20 mm diameters. Each embryo was placed in the centre of the motility wheel and then gently touched at the tail with an embryo poker. The escape response of each embryo was quantified as the distance traveled within the predefined concentric circles. For each experiment group, a touch-response test was performed on 50 embryos. The data was calculated as the percentage of embryos that crossed each predetermined concentric circle.

Sample preparation for proteomic analysis

Sample processing on whole protein extracts of fibroblasts derived from patients and controls for LC-MS/MS analysis was carried out as described previously⁷³.

LC-MS/MS measurements

All samples were measured in data-independent mode (DIA). Analysis was performed using an Ultimate 3000 nano RSLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (all Thermo Scientific). For each measurement, 1 µg of peptides from each sample was pre-concentrated on a 100 µm × 2 cm C18 trapping column for 10 minutes with 0.1% TFA (v/v) at a flow rate of 20 µL/min, followed by separation on a 75 µm × 50 cm C18 main column (both Pepmap, Thermo Scientific) with a 120-minute LC gradient of 3 to 35% B (84% ACN in 0.1% FA) at a flow rate of 250 nL/min. An appropriate amount of iRT standard peptides (Biognosys) was added to each sample before starting the measurement. MS survey scans were acquired from 300 to 1100 m/z at a resolution of 60,000 FWHM, followed by MS/MS using 24 DIA windows, each covering a range of 25 m/z (with 1 m/z overlap) at a resolution of 30,000. The polysiloxane ion at 445.12 m/z was used as a lock mass. The CID spectra were recorded with a normalized collision energy of 32 and an activation time of 10 ms⁷⁴. Each biological sample was measured in technical triplicate.

Analysis of proteomic data

For reliable label-free quantification, only proteins identified with two or more unique peptides were included in the subsequent analysis to ensure robustness and minimize false-positive identifications. The average normalized abundances, as determined by Spectronaut, were calculated for each protein and used to derive the abundance ratios between patient samples and their corresponding controls. To ensure reproducibility, the mean of all technical triplicates was calculated and used for final ratio determination.

The resulting ratios were log₂-transformed, and Student's *t*-tests were performed for each protein using Microsoft Excel to assess statistical significance. Proteins were considered differentially regulated if they exhibited a *p*-value ≤ 0.05 and an abundance ratio ≥ 1.9 or ≤ 0.54, corresponding to approximately a twofold change in expression (up- and down-regulated, respectively). These stringent ratio thresholds were deliberately chosen to minimize the likelihood that observed changes represent biological noise or naturally occurring abundance variations rather than true differential regulation. No correction for multiple hypothesis testing (e.g., Benjamini–Hochberg correction) was applied. This decision was based on the objective of the study, which was not solely the identification of potential biomarkers, but rather the exploration of biological processes and molecular functions affected or regulated by the different treatments. While applying such corrections would increase the confidence in individual protein regulations, it would also reduce the sensitivity for detecting the broader regulatory patterns and pathways of interest. The use of high fold-change thresholds (≥ 1.9 or ≤ 0.54) was therefore implemented as an alternative means to maintain stringency while retaining biological interpretability.

Statistics and reproducibility

All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software). Error bars in the graphs indicate the standard error of the mean (SEM) or standard deviation (SD) as indicated in the figure legend. Normality of the data was assessed using the Shapiro–Wilk test. Depending on the distribution, parametric tests (Student's *t*-test, one-way ANOVA, or two-way ANOVA) were used for normally distributed data, whereas non-parametric tests were applied to non-normally distributed data. Statistical significance was defined as follows: * = *p* < 0.05; ** = *p* < 0.01; *** = *p* < 0.001; **** = *p* = 0.0001. A minimum of 3 biological replicates was chosen, as well as a minimum of 2 independent experiments, to give statistical significance at data collection.

Ethics approval and consent to participate

Patient 6. The patient was enrolled at the Department of Pediatric Neurology (University Hospital Essen, Essen, Germany) and approved by the local ethical committee of the University of Duisburg-Essen (19-9011-BO). Written informed consent was obtained from the patients' caretakers.

Patient 7. The proband and her parents (HET1 and HET2) were enrolled at The Manton Center for Orphan Disease Research, approved by the Institutional Review Boards (protocol ID: 10-02-0053) at Boston Children's Hospital. Written informed consents were obtained from the patient and family members.

All ethical regulations relevant to human research participants were followed.

The experiments on primary fibroblasts were also approved by the local ethics committee of the University of Torino (Prot. n. 0254821).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source Data underlying the graphs and charts in the manuscript can be found in Supplementary Data 1. Full, unedited blots are shown in Fig. S10. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁷⁵ partner repository with the dataset identifier PXD071580. All other data are available from the corresponding author on reasonable request.

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References

- Lischka, A. et al. Genetic pain loss disorders. *Nat. Rev. Dis. Prim.* **8**, 41 (2022).
- Benarroch, L., Bonne, G., Rivier, F. & Hamroun, D. The 2024 version of the gene table of neuromuscular disorders (nuclear genome). *Neuromuscul. Disord.* **34**, 126–170 (2024).
- Rivière, J. B. et al. KIF1A, an axonal transporter of synaptic vesicles, is mutated in hereditary sensory and autonomic neuropathy type 2. *Am. J. Hum. Genet.* **89**, 219–230 (2011).
- Verhoeven, K. et al. Mutations in the small GTP-ase late endosomal protein RAB7 cause Charcot-Marie-Tooth type 2B neuropathy. *Am. J. Hum. Genet.* **72**, 722–727 (2003).
- Guelly, C. et al. Targeted high-throughput sequencing identifies mutations in atlastin-1 as a cause of hereditary sensory neuropathy type I. *Am. J. Hum. Genet.* **88**, 99–105 (2011).
- Kornak, U. et al. Sensory neuropathy with bone destruction due to a mutation in the membrane-shaping atlastin GTPase 3. *Brain* **137**, 683–692 (2014).
- Kurth, I. et al. Mutations in FAM134B, encoding a newly identified Golgi protein, cause severe sensory and autonomic neuropathy. *Nat. Genet.* **41**, 1179–1181 (2009).
- Dawkins, J. L., Hulme, D. J., Brahmabhatt, S. B., Auer-Grumbach, M. & Nicholson, G. A. Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. *Nat. Genet.* **27**, 309–312 (2001).
- Rotthier, A. et al. Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type I. *Am. J. Hum. Genet.* **87**, 513–522 (2010).
- Einarsdottir, E. et al. A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Hum. Mol. Genet.* **13**, 799–805 (2004).
- Indo, Y. et al. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat. Genet.* **13**, 485–488 (1996).

12. Cox, J. J. et al. An SCN9A channelopathy causes congenital inability to experience pain. *Nature* **444**, 894–898 (2006).
13. Leipold, E. et al. A de novo gain-of-function mutation in SCN11A causes loss of pain perception. *Nat. Genet.* **45**, 1399–1404 (2013).
14. Baets, J. et al. Defects of mutant DNMT1 are linked to a spectrum of neurological disorders. *Brain* **138**, 845–861 (2015).
15. Garofalo, K. et al. Oral L-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1. *J. Clin. Invest.* **121**, 4735–4745 (2011).
16. Fridman, V. et al. Randomized trial of L-serine in patients with hereditary sensory and autonomic neuropathy type 1. *Neurology* **92**, e359–e370 (2019).
17. Auranen, M. et al. Clinical and metabolic consequences of L-serine supplementation in hereditary sensory and autonomic neuropathy type 1C. *Cold Spring Harb. Mol. Case Stud.* **3**. <https://doi.org/10.1101/mcs.a002212> (2017).
18. Lischka, A. et al. Genetic landscape of congenital insensitivity to pain and hereditary sensory and autonomic neuropathies. *Brain*. <https://doi.org/10.1093/brain/awad328> (2023).
19. Bertino, F. et al. Heme and sensory neuropathy: insights from novel mutations in the heme exporter feline leukemia virus subgroup C receptor 1. *Pain* **160**, 2766–2775 (2019).
20. Castori, M. et al. Posterior column ataxia with retinitis pigmentosa coexisting with sensory-autonomic neuropathy and leukemia due to the homozygous p.Pro221Ser FLVCR1 mutation. *Am. J. Med Genet B Neuropsychiatr. Genet.* **174**, 732–739 (2017).
21. Chiabrando, D. et al. Mutations in the heme exporter FLVCR1 cause sensory neurodegeneration with loss of pain perception. *PLoS Genet* **12**, e1006461 (2016).
22. Lipovich, L., Hughes, A. L., King, M. C., Abkowitz, J. L. & Quigley, J. G. Genomic structure and evolutionary context of the human feline leukemia virus subgroup C receptor (hFLVCR) gene: evidence for block duplications and de novo gene formation within duplicons of the hFLVCR locus. *Gene* **286**, 203–213 (2002).
23. Quigley, J. G. et al. Cloning of the cellular receptor for feline leukemia virus subgroup C (FeLV-C), a retrovirus that induces red cell aplasia. *Blood* **95**, 1093–1099 (2000).
24. Abkowitz, J. L., Holly, R. D. & Grant, C. K. Retrovirus-induced feline pure red cell aplasia. Hematopoietic progenitors are infected with feline leukemia virus and erythroid burst-forming cells are uniquely sensitive to heterologous complement. *J. Clin. Invest.* **80**, 1056–1063 (1987).
25. Yang, Z. et al. Delayed globin synthesis leads to excess heme and the macrocytic anemia of Diamond Blackfan anemia and del(5q) myelodysplastic syndrome. *Sci. Transl. Med.* **8**, 338ra367 (2016).
26. Keel, S. B. et al. A heme export protein is required for red blood cell differentiation and iron homeostasis. *Science* **319**, 825–828 (2008).
27. Quigley, J. G. et al. Identification of a human heme exporter that is essential for erythropoiesis. *Cell* **118**, 757–766 (2004).
28. Chiabrando, D. et al. The mitochondrial heme exporter FLVCR1b mediates erythroid differentiation. *J. Clin. Invest.* **122**, 4569–4579 (2012).
29. Vinchi, F. et al. Heme exporter FLVCR1a regulates heme synthesis and degradation and controls activity of cytochromes P450. *Gastroenterology* **146**, 1325–1338 (2014).
30. Philip, M. et al. Heme exporter FLVCR is required for T cell development and peripheral survival. *J. Immunol.* **194**, 1677–1685 (2015).
31. Fiorito, V., Forni, M., Silengo, L., Altruda, F. & Tolosano, E. Crucial Role of FLVCR1a in the maintenance of intestinal heme homeostasis. *Antioxid. Redox Signal* **23**, 1410–1423 (2015).
32. Petrillo, S. et al. Heme accumulation in endothelial cells impairs angiogenesis by triggering paraptosis. *Cell Death Differ.* **25**, 573–588 (2018).
33. Yang, Z. et al. Kinetics and specificity of feline leukemia virus subgroup C receptor (FLVCR) export function and its dependence on hemopexin. *J. Biol. Chem.* **285**, 28874–28882 (2010).
34. Tsuchiya, M., Tachibana, N., Nagao, K., Tamura, T. & Hamachi, I. Organelle-selective click labeling coupled with flow cytometry allows pooled CRISPR screening of genes involved in phosphatidylcholine metabolism. *Cell Metab.* **35**, 1072–1083.e1079 (2023).
35. Kenny, T. C. et al. Integrative genetic analysis identifies FLVCR1 as a plasma-membrane choline transporter in mammals. *Cell Metab.* **35**, 1057–1071.e1012 (2023).
36. Ha, H. T. T. et al. Mfsd7b facilitates choline transport and missense mutations affect choline transport function. *Cell Mol. Life Sci.* **81**, 3 (2023).
37. Ri, K. et al. Molecular mechanism of choline and ethanolamine transport in humans. *Nature*. <https://doi.org/10.1038/s41586-024-07444-7> (2024).
38. Son, Y., Kenny, T. C., Khan, A., Birsoy, K. & Hite, R. K. Structural basis of lipid head group entry to the Kennedy pathway by FLVCR1. *Nature* **629**, 710–716 (2024).
39. Fiorito, V. et al. The heme synthesis-export system regulates the tricarboxylic acid cycle flux and oxidative phosphorylation. *Cell Rep.* **35**, 109252 (2021).
40. Bertino, F. et al. Dysregulation of FLVCR1a-dependent mitochondrial calcium handling in neural progenitors causes congenital hydrocephalus. *Cell Rep. Med.* **5**, 101647 (2024).
41. Sievers, F. et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
42. Mercurio, S. et al. The heme exporter Flvcr1 regulates expansion and differentiation of committed erythroid progenitors by controlling intracellular heme accumulation. *Haematologica* **100**, 720–729 (2015).
43. Petrillo, S. et al. Endothelial cells require functional FLVCR1a during developmental and adult angiogenesis. *Angiogenesis*. <https://doi.org/10.1007/s10456-023-09865-w> (2023).
44. Manco, M. et al. FLVCR1a Controls Cellular Cholesterol Levels through the Regulation of Heme Biosynthesis and Tricarboxylic Acid Cycle Flux in Endothelial Cells. *Biomolecules* **14**, <https://doi.org/10.3390/biom14020149> (2024).
45. Mistretta, M. et al. Flvcr1a deficiency promotes heme-based energy metabolism dysfunction in skeletal muscle. *Cell Rep.* **43**, 113854 (2024).
46. Decker, S. T. & Funai, K. Mitochondrial membrane lipids in the regulation of bioenergetic flux. *Cell Metab.* <https://doi.org/10.1016/j.cmet.2024.07.024> (2024).
47. Chiabrando, D., Fiorito, V., Petrillo, S. & Tolosano, E. Unraveling the Role of Heme in Neurodegeneration. *Front Neurosci.* **12**, 712 (2018).
48. Chiabrando, D., Bertino, F. & Tolosano, E. Hereditary Ataxia: a focus on heme metabolism and Fe-S cluster biogenesis. *Int. J. Mol. Sci.* **21**, <https://doi.org/10.3390/ijms21113760> (2020).
49. Glancy, B. & Balaban, R. S. Role of mitochondrial Ca²⁺ in the regulation of cellular energetics. *Biochemistry* **51**, 2959–2973 (2012).
50. Mishra, P. & Chan, D. C. Metabolic regulation of mitochondrial dynamics. *J. Cell Biol.* **212**, 379–387 (2016).
51. Liu, Y. J., McIntyre, R. L., Janssens, G. E. & Houtkooper, R. H. Mitochondrial fission and fusion: A dynamic role in aging and potential target for age-related disease. *Mech. Ageing Dev.* **186**, 111212 (2020).
52. Rodríguez, L. R. et al. Therapeutic Strategies Targeting Mitochondrial Calcium Signaling: A New Hope for Neurological Diseases? *Antioxidants* **11**, <https://doi.org/10.3390/antiox11010165> (2022).
53. De Mario, A. et al. Identification and functional validation of FDA-approved positive and negative modulators of the mitochondrial calcium uniporter. *Cell Rep.* **35**, 109275 (2021).

54. Calame, D. G. et al. Biallelic variation in the choline and ethanolamine transporter *FLVCR1* underlies a severe developmental disorder spectrum. *Genet. Med.* **101273** (2024).
55. Rajadhyaksha, A. M. et al. Mutations in *FLVCR1* cause posterior column ataxia and retinitis pigmentosa. *Am. J. Hum. Genet.* **87**, 643–654 (2010).
56. Tiwari, A. et al. Next generation sequencing based identification of disease-associated mutations in Swiss patients with retinal dystrophies. *Sci. Rep.* **6**, 28755 (2016).
57. Prior, R., Van Helleputte, L., Benoy, V. & Van Den Bosch, L. Defective axonal transport: A common pathological mechanism in inherited and acquired peripheral neuropathies. *Neurobiol. Dis.* **105**, 300–320 (2017).
58. Cunneane, S. C. et al. Brain energy rescue: an emerging therapeutic concept for neurodegenerative disorders of ageing. *Nat. Rev. Drug Discov.* **19**, 609–633 (2020).
59. Wang, W. et al. Metabolic deregulation of the blood-outer retinal barrier in retinitis Pigmentosa. *Cell Rep.* **28**, 1323–1334.e1324 (2019).
60. Petit, L. et al. Aerobic glycolysis is essential for normal rod function and controls secondary cone death in Retinitis Pigmentosa. *Cell Rep.* **23**, 2629–2642 (2018).
61. Li, L. X. et al. Clinical and molecular features of *POLG*-related sensory ataxic neuropathy with Dysarthria and Ophthalmoparesis. *J. Mol. Neurosci.* **71**, 2462–2467 (2021).
62. Finsterer, J. Neuropathy, Ataxia, and Retinitis Pigmentosa Syndrome. *J. Clin. Neuromuscul. Dis.* **24**, 140–146 (2023).
63. Salaroglio, I. C. et al. Mitochondrial ROS drive resistance to chemotherapy and immune-killing in hypoxic non-small cell lung cancer. *J. Exp. Clin. Cancer Res.* **41**, 243 (2022).
64. Centonze, G. et al. p140Cap modulates the mevalonate pathway decreasing cell migration and enhancing drug sensitivity in breast cancer cells. *Cell Death Dis.* **14**, 849 (2023).
65. Bergonia, H. A., Franklin, M. R., Kushner, J. P. & Phillips, J. D. A method for determining δ -aminolevulinic acid synthase activity in homogenized cells and tissues. *Clin. Biochem.* **48**, 788–795 (2015).
66. Bartsakoulia, M. et al. Cysteine supplementation may be beneficial in a subgroup of mitochondrial translation deficiencies. *J. Neuromuscul. Dis.* **3**, 363–379 (2016).
67. Kleefeld, F. et al. Multi-level profiling unravels mitochondrial dysfunction in myotonic dystrophy type 2. *Acta Neuropathol.* **147**, 19 (2024).
68. Dematteis, G. et al. ER-mitochondria distance is a critical parameter for efficient mitochondrial Ca. *Commun. Biol.* **7**, 1294 (2024).
69. Sinclair, P. R., Gorman, N. & Jacobs, J. M. Measurement of heme concentration. *Curr. Protoc. Toxicol.* **Chapter 8**, Unit 8.3. <https://doi.org/10.1002/0471140856.tx0803s00> (2001).
70. Blader, P., Plessy, C. & Strähle, U. Multiple regulatory elements with spatially and temporally distinct activities control neurogenin1 expression in primary neurons of the zebrafish embryo. *Mech. Dev.* **120**, 211–218 (2003).
71. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995).
72. Basnet, R. M., Guarienti, M. & Memo, M. Zebrafish embryo as an in vivo model for behavioral and pharmacological characterization of Methylxanthine Drugs. *Int. J. Mol. Sci.* **18** <https://doi.org/10.3390/ijms18030596> (2017).
73. Hentschel, A. et al. Protein signature of human skin fibroblasts allows the study of the molecular etiology of rare neurological diseases. *Orphanet J. Rare Dis.* **16**, 73 (2021).
74. Pugliese, A. et al. Mutations in *PTPN11* could lead to a congenital myasthenic syndrome phenotype: a Noonan syndrome case series. *J. Neurol.* **271**, 1331–1341 (2024).
75. Perez-Riverol, Y. et al. The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res.* **53**, D543–D553 (2025).
76. Goddard, T. D. et al. UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci.* **27**, 14–25 (2018).

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Author contributions

F.B., D.I.Z.V., E.G., J.K., B.G., R.M.B., conducted the experiments, acquired the data, and analyzed the data. C.S. conducted electron microscopy analyses. S.B., L.Mi., F.K., A.H., F.M., R.E.A., and L.Me. conducted some experiments. V.F. and E.T. reviewed and edited the manuscript. A.L.A., S.P., F.D.G., and G.A. reviewed the manuscript. E.S., D.P., M.R., A.A., E.M.C., H.K., A.L., B.Z., and T.W.Y. provided patient-derived cells and/or clinical data. A.R., R.H., D.F., C.R., and E.T. designed some experiments and reviewed the manuscript. F.B., D.I.Z.V., and D.C. designed the experiments and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Deborah Chiabrando.

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¹Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center “Guido Tarone”, University of Torino, Turin, Italy. ²Department of Oncology, Molecular Biotechnology Center, University of Torino, Turin, Italy. ³Department of Veterinary Sciences, University of Torino, Largo Braccini 2, Grugliasco, Turin, Italy. ⁴Section of Biotechnology, Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy. ⁵Department of Medical Sciences, University of Torino, Turin, Italy. ⁶Division of Genetics and Genomics, Boston Children's Hospital, Boston, MA, USA. ⁷Manton Center for Orphan Disease Research, Boston Children's Hospital, Boston, MA, USA. ⁸Department of Pediatrics, Boston Children's Hospital, Boston, MA, USA. ⁹Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA, USA. ¹⁰Harvard Medical School, Boston, MA, USA. ¹¹Charité-Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt Universität zu Berlin, and Berlin Institute of Health (BIH), Department of Neurology, Charitéplatz 1, Berlin, Germany. ¹²Leibniz-Institut für Analytische Wissenschaften-ISAS-e.V., Dortmund, Germany. ¹³Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Pavia, Italy. ¹⁴Department of Life Sciences and Systems Biology, Molecular Biotechnology Center “Guido Tarone”, University of Torino, Turin, Italy. ¹⁵Unit of Rare Neurological Diseases, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milano, Italy. ¹⁶Department of Pediatrics, Unit of Rare Diseases, Giannina Gaslini Institute, Genova, Italy. ¹⁷Medical Genetics Center, Munich, Germany. ¹⁸Friedrich-Baur-Institute, Department of Neurology, Klinikum der Universität, Ludwig-Maximilians-Universität, Munich, Germany. ¹⁹University of Colorado, Department of ophthalmology 1675 Aurora ct., Aurora, CO, USA. ²⁰Department of Clinical Neurosciences, University of Cambridge, Cambridge, UK. ²¹Department of Paediatric Neurology, Center for Neuromuscular Disorders in Children and Adolescents, University Clinic Essen, Essen, Germany. ²²Department of Pediatrics, Section of Clinical Genetics and Metabolism, University of Colorado School of Medicine, Aurora, CO, USA. ²³Brain and Mind Research Institute, Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON, Canada. ²⁴Department of Neurology, Medical Faculty and University Hospital Düsseldorf, Heinrich Heine University, Düsseldorf, Germany. ²⁵Laboratory of Clinical Chemistry, ASST-Spedali Civili di Brescia, Brescia, Italy. ²⁶These authors contributed equally: Francesca Bertino, Diletta Isabella Zanin Venturini. ✉ e-mail: deborah.chiabrando@unito.it