**EPHX1 mutations cause a lipoatrophic diabetes syndrome due to impaired epoxide hydrolysis and increased cellular senescence**

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Abstract

Epoxide hydrolases (EHs) regulate cellular homeostasis through hydrolysis of epoxides to less reactive diols. The first discovered EH was EPHX1, also known as mEH. EH functions remain partly unknown and no pathogenic variants have been reported in humans. We identified two de novo variants located in EPHX1 catalytic site in patients with a lipoatrophic diabetes characterized by loss of adipose tissue, insulin resistance, and multiple organ dysfunction. Functional analyses revealed that these variants led to the protein aggregation within the endoplasmic reticulum and to a loss of its hydrolysis activity. CRISPR-Cas9-mediated Ephx1 knockout (KO) abolished adipocyte differentiation and decreased insulin response. This KO also promoted oxidative stress and cellular senescence, an observation confirmed in patient-derived fibroblasts. A major beneficial effect of metreleptin therapy was observed. This translational study highlights the importance of epoxide regulation for adipocyte function, and provides new insights into the physiological roles of EHs in humans.
Introduction

Epoxide hydrolases (EHs) constitute a small protein family, first characterized as a group of detoxifying enzymes (1). The first EHs were identified more than 30 years ago and, to date, five genes encoding EH have been identified in humans (2, 3). Human microsomal and soluble EHs (mEH and sEH), also named EPHX1 and EPHX2, respectively, are the best known EHs. They catalyze the rapid hydrolysis of epoxides, which are three membered cyclic ethers, to less reactive and readily excretable diols (4). In mammals, epoxides arise from cytochrome P450 (CYP450) oxidative metabolism of both xenobiotics and endogenous compounds (5). Epoxides from xenobiotics are involved in the development of cancers and organ damage through interaction with DNA, lipids, and proteins (6), and EHs are necessary for their detoxification. On the other hand, epoxides derived from fatty acids, called epoxy fatty acids (EpFAs), are important regulatory lipid mediators. They were shown to mediate several biological processes, including inflammation, angiogenesis, vasodilation, and nociception. By regulating EpFAs levels, EHs play a key role in regulating crucial signaling pathways for cellular homeostasis (7), and altered levels of EpFAs are associated with many disorders (8). Although a large amount of work has been made to characterize EHs functions, especially through the use of specific inhibitors and animal models, the full spectrum of their substrates and associated biological functions in human remain partly unknown (9). It is also a challenge to clearly define the contribution of each EH in human disorders.

Association studies suggested a role of several single nucleotide polymorphisms (SNPs) identified in \textit{EPHX1} [MIM132810] and \textit{EPHX2} [MIM132811] in multiple conditions, including liver cirrhosis, alcohol dependence, Crohn’s disease, chronic obstructive pulmonary disease, preeclampsia, diabetes mellitus, and many cancers (5, 10). This underscores the pleiotropic and crucial role of EHs in cell homeostasis, but no high-effect variants had been reported. In the present study, we identified two unrelated patients with a complex lipoatrophic and neurodevelopmental syndrome with severe metabolic manifestations and carrying a \textit{de novo} variant in \textit{EPHX1} identified by whole-exome sequencing (WES). Lipoatrophic diabetes, also known as lipodystrophic syndromes, are characterized by clinical lipoatrophy due to a defect in adipose tissue storage of triglycerides. This results in ectopic lipid infiltration of non-adipose tissues leading to insulin resistance, increased liver glucose production, hypertriglyceridemia, and liver steatosis. About 30 genes have been implicated in lipoatrophic diabetes (11). Although these disorders remain genetically unexplained in the
vast majority of cases, there is growing interest in identifying their molecular and cellular bases to improve genetic counseling and personalize treatment (12, 13).

EPHX1 is widely expressed with highest expression in the liver, adipose tissue, and adrenal glands (14). It was the first EH to be identified, and was first purified from rabbit liver in 1970s. It is an evolutionarily highly conserved biotransformation enzyme retained in microsomal membranes of the endoplasmic reticulum (ER) (14). For a long time, EPHX1 and EPHX2 were thought to simply fulfill distinct complementary roles (15). On the one hand, EPHX1 was well recognized to detoxify xenobiotic epoxides. On the other hand, EPHX2 was shown to hydrolyze endogenous terpenoid epoxides and EpFAs (5). More recently, EPHX1 was also shown in animal models to play a significant role in the hydrolysis of different endogenous EpFAs derived from polyunsaturated fatty acids (16-19). These EpFAs, including epoxyeicosatrienoic acids (EETs) and epoxyoctadecenoic acids (EpOMEs), are formed by ER-attached CYP450s and hydrolyzed by EPHX1 to their respective diols, the so-called dihydroxyeicosatrienoic acids (DHETs) and dihydroxyoctadecenoic acids (DiHOMEs), respectively.

The EPHX1 variants identified in the studied patients are localized in the catalytic domain of the enzyme and were predicted to be pathogenic. This prompted us to assess their functional consequences in several cellular models. The impact of the loss of EPHX1 activity on adipocyte differentiation and function was evaluated by developing a CRISPR-Cas9-mediated genome-editing approach.
Results

Identification of EPHX1 variants in two unrelated patients

To identify novel genetic causes responsible for lipoatrophic diabetes, WES was carried out on a parent-offspring trio. The index case was a young adult woman. Her disease phenotype was not explained by variants in genes known to be involved in lipoatrophic diabetes, as assessed by the analysis of a panel of genes used in routine genetic diagnosis (20). The analysis of exome data led to the identification of a heterozygous variant in exon 7 of EPHX1 (21), which was subsequently confirmed by Sanger sequencing: c.997A>C, p.(Thr333Pro) (Figures 1A and 1B, available by contacting the corresponding author). This variant was found to be de novo after paternity confirmation by genotyping of a set of polymorphic markers. Due to the key role of EPHX1 in cellular homeostasis and fatty acid metabolism (16), this gene was a good candidate. We then looked for additional individuals carrying a molecular defect in EPHX1. A second patient carrying a different de novo EPHX1 missense variant located in exon 9: c.1288G>C, p.(Gly430Arg) was identified through GeneMatcher (22) (Figures 1A and 1B, available by contacting the corresponding author). This patient, woman, was a teenager. She also had an insulin resistant lipoatrophic syndrome. The presence of chromosomal abnormalities was previously excluded in the two patients by karyotype and SNP chromosome microarrays. Several additional lines of evidence supported the causal role of the two variants in the disease phenotype. These variants were absent from databases reporting variants from the general population (gnomAD, ExAC, dbSNP, and Exome Variant Server), as well as from ClinVar, a database which aggregates information about genomic variations and their relationship to human diseases. The variants identified herein affected amino acids strongly conserved throughout evolution, even in zebrafish and Xenopus tropicalis (Supplemental Figure 1). The two variants predicted changes in the polarity of the corresponding amino acids, as well as in the charge for p.Gly430Arg. They were predicted pathogenic by all algorithms (PolyPhen-2, SIFT, CADD).

Clinical features in patient 1

This patient was first referred for dysmorphic features including post-natal microcephaly (occipitofrontal circumference: 33 cm at birth; -2.5 SD), a triangular-shaped face, prominent forehead, retrognathism, irregular and high hair line, ogival palate, mandibulo-facial dysostosis including malar hypoplasia and retrognathism, teeth misalignments, arachnodactyly, camptodactyly, joint stiffness, and clitoromegaly (Figures 1C-1F and Table...
1, available by contacting the corresponding author). Patient 1 also displayed a progressive lipodystrophic phenotype with severe lipoatrophy of the face and limbs (Figures 1C and 1G, available by contacting the corresponding author). This lipoatrophic phenotype was further confirmed by dual X-ray absorptiometry (DEXA) with a total fat mass of 15%, whereas the mean normal age-matched value is 31.4 ± 8.5% (23). Her body mass index (BMI) was 20.2 kg/m². The serum leptin levels, which are strongly correlated with total body fat mass, were very low in patient 1 (4 ng/mL) and similar to those usually reported in generalized lipodystrophy (24), further confirming the lipoatrophic phenotype. Patient 1 was diagnosed with severe insulin resistant diabetes in early adolescence, with hyperglycemia (44 mmol/L), and highly elevated HbA1c (18.9%). Insulin resistance was characterized by acanthosis nigricans in the neck, axilla, and back (Figure 1E, available by contacting the corresponding author), as well as by the very high insulin requirements (15 IU/kg/day) and by the low levels of total serum adiponectin (0.5 mg/L – normal range: 3.6 - 9.6 mg/L). Mild hypertriglyceridemia was observed (2.66 mmol/L), with serum cholesterol levels around the lower limits. She had a major hepatomegaly (Figure 1D, available by contacting the corresponding author) and elevated levels of aspartate aminotransferase (AST – 120 IU/L), alanine aminotransferase (ALT – 148 IU/L), alkaline phosphatase (ALP – 170 IU/L), and gamma glutamyl transpeptidase (GGT – 320 IU/L) (Table 2). Liver computed tomography and magnetic resonance imaging (MRI) revealed liver steatosis with focal accumulation of fat depots, especially in the posterior segment (15-27%) (Figure 1D, available by contacting the corresponding author). Non-invasive FibroTest® and Acti-test® scores (25) were in favor of low-grade liver fibrosis with moderate necrosis and/or inflammation. Although pubertal development was normal, spaniomenorrhea occurred rapidly and progressed to complete amenorrhea, although FSH and LH values were normal. She progressively developed hyperandrogenism signs with severe generalized hirsutism. Total serum testosterone levels were noticed to be moderately increased when she was a young adult (2.2 nmol/L; N: 0.3-1.5 nmol/L). Over time, this patient developed major steroidogenesis abnormalities with especially highly elevated levels of dihydrotestosterone (2.3 nmol/L; N: 0.06-0.3 nmol/L) and testosterone (16.9 nmol/L; N: 0.3-1.5 nmol/L). Serum estradiol levels were within the normal range (121 pmol/L) in this woman with amenorrhea, contrasting with the high levels of androgens. MRI showed normal adrenal glands. MRI and pelvic ultrasound did not reveal any ovarian or uterine abnormalities. Neurologically, patient 1 had a delay in language acquisition and moderate intellectual disability. An axonal neuropathy, associated with a decrease in tendon reflexes and bilateral pes cavus, was further confirmed by an
electromyogram. Bilateral sensorineural hearing loss was diagnosed during childhood, leading to the use of hearing aids.

Clinical features in patient 2

This patient presented similar dysmorphic features, as compared with patient 1, including a triangular-shaped face, irregular and high hair line, frontal bossing with mid-face hypoplasia, and mandibulo-facial dysostosis (Table 1, available by contacting the corresponding author). Lipoatrophy was notable in the face. Her BMI was low (17.0 kg/m²). She had insulin resistance, as assessed by very high fasting insulin levels (284 pmol/L), which have increased over time. Her fasting glucose values remained in the normal range, and she was not diabetic at last investigation. She had severe hypertriglyceridemia (21.9 mmol/L) associated with low HDL-cholesterol levels (0.52 mmol/L). Liver enzymes were elevated: AST (71 IU/L), ALT (94 IU/L), and ALP (145 IU/L). A liver ultrasound demonstrated fatty infiltrate. She also had profound sensorineural hearing loss since birth requiring cochlear implants. Brain computerized tomography and electroencephalogram were normal. All these data demonstrate that the two affected individuals have a complex disease phenotype and share a number of clinical features including dysmorphic features, lipoatrophy, insulin resistance, hypertriglyceridemia, liver dysfunction, and bilateral sensorineural hearing loss.

Structural characterization of EPHX1 variants

EPHX1 encodes a protein of 455 residues. The enzyme is retained in microsomal membranes of the ER by a single transmembrane segment located at the N-terminus and comprising around 20 amino acids (26). The C-terminal part of the protein, containing the two variants identified, is exposed at the cytosolic membrane surface and constitute the catalytic domain (27) (Figure 2A). The EPHX1 mechanism of hydrolysis involves two chemical steps. A fast-nucleophilic attack leads to the formation of an ester intermediate, a covalent bond linking the substrate to the enzyme. Thereafter, hydrolysis of this complex to the final diol product is mediated by a molecule of water activated by a charge relay system (28) (Figure 2A). The EPHX1 active site is composed of a so-called catalytic triad consisting of Asp226, Glu404 and His431. In addition, two tyrosine residues (Tyr299 and Tyr374) provide an essential support by polarizing the epoxide (28-30). The localization of the mutated amino acids within the three-dimensional (3D) protein structure strongly supported their pathogenic effect. Although the exact structure of EPHX1 is still not available, the quaternary structure of a closely homologous enzyme was determined from the fungus Aspergillus niger (31). Glycine
430 mutated in patient 2 is located beside the crucial His431, which is directly implicated in
the water activation and hydrolytic step of the catalytic process (Figure 2B). We used a 3D
structure model from the SWISS-MODEL repository to determine the location of Thr333
(32). On the 3D-structure, this residue appears in close vicinity to Gly430, as well as to the
three critical residues of the catalytic site (Asp226, Glu404, His431) (Figure 2C). The
location of the two de novo EPHX1 variants suggests that they could affect the enzyme
activity and argues for their pathogenic effect.

EPHX1 variants dramatically alter the enzyme hydrolysis function
To investigate the functional consequences of the identified EPHX1 variants, we first
analyzed their effect on the capacity of EPHX1 to hydrolyze cis-stilbene oxide ([3H]-cSO),
one of its well-known substrates (33, 34). Overexpression studies were performed in Human
Epithelial Kidney (HEK) 293 cells, since they are of human origin, easily transfectable, and
display low endogenous levels of EPHX1. HEK 293 were transfected with plasmids encoding
wild-type (WT) and mutated forms of human EPHX1 with a C-terminal Flag tag: hEPHX1WT, hEPHX1Thr333Pro, and hEPHX1Gly430Arg (Figure 2D). The hydrolysis of cis-stilbene oxide (c-
SO) in lysates of HEK 293 cells overexpressing hEPHX1WT was high, as compared to
untransfected cells in which it was undetectable. The two variants identified in patients,
p.Thr333Pro and p.Gly430Arg, led to a near complete absence of this enzyme activity
(Figures 2D and 2E). We then analyzed the effect of two SNPs frequent in the general
population, p.Tyr113His and p.His139Arg, whose role was debated in association studies (5,
35) (Figure 2B). Neither overexpression of hEPHX1Tyr113His nor that of hEPHX1His139Arg led
to reduced c-SO hydrolysis in HEK 293 lysates as compared to hEPHX1WT (Figures 2D and
2E). We also investigated the effect of a variant affecting one of the catalytic triad residues,
p.Glu404Asp, previously proposed to result in an increased enzyme activity (30, 36) (Figure
2B). This variant did not significantly modify the hydrolysis of c-SO compared to hEPHX1WT
(Figures 2D and 2E). Immunoblot analysis against the Flag epitope was used to control the
protein level of WT and mutated EPHX1 isoforms. Although the protein expression level was
slightly diminished for the p.Gly430Arg variant, there was no significant difference in the
expression of WT and other mutated isoforms (Figure 2E). Collectively, these data showed
that the p.Thr333Pro and p.Gly430Arg variants strongly impair EPHX1 hydrolysis function
by altering its catalytic triad domain (Figure 2C).

EPHX1 variants induce the enzyme aggregation within the endoplasmic reticulum
As mentioned previously, EPHX1 is mainly localized in the microsomal fraction of the ER (14). We performed immunofluorescence staining in HEK 293 cells transiently expressing the WT and mutated forms of EPHX1 to evaluate whether missense variants alter its sub-cellular localization. Co-staining with calnexin, which is a marker of ER, confirmed that hEPHX1WT is located in the ER, as well as all mutated EPHX1 isoforms carrying the five previously mentioned missense variants (Figure 3A). However, the EPHX1 isoforms carrying the two de novo variants identified in patients (p.Thr333Pro and p.Gly430Arg) were also found to form aggregates or clumps within the ER, as compared to WT hEPHX1 and other mutated isoforms (Figure 3A). To ensure that the Flag tag did not alter EPHX1 sub-cellular localization, a new set of constructs lacking the Flag tag was generated. Of note, the two de novo variants still led to EPHX1 aggregation within the ER when the Flag tag was removed (Figure 3B). The presence of these oligomers was further confirmed by Western blot analysis since both hEPHX1Thr333Pro and hEPHX1Gly430Asp proteins were revealed as two bands, one corresponding to the protein monomer around 55 kDa, and another to an oligomer around 150 kDa (Figure 3C). When the cell lysates were enriched in EPHX1 by immunoprecipitation with an anti-Flag antibody, Western blot analysis using an anti-hEPHX1 antibody revealed an increase of these aggregates in the presence of the two p.Thr333Pro and p.Gly430Arg variants (Figure 3C). We cannot exclude that oligomers of higher molecular weight might constitute insoluble aggregates being lost during Western blotting experiments. All these data demonstrate that the p.Thr333Pro and p.Gly430Arg variants confer an aberrant conformation to EPHX1 leading to its aggregation. This likely contributes to abolish the enzyme catalytic activity through a dominant negative mechanism.

**Ephx1 knockout in pre-adipocytes abolishes adipocyte differentiation and decreases insulin response**

We then sought to assess the effect of the loss of EPHX1 activity in the tissues most affected by the disease. The two patients have manifestations in adipose tissue, central nervous system, and liver. Recent studies have investigated the function of EPHX1 in liver and brain (18, 36), but there is little information on the role of EPHX1 in adipose tissue. To investigate the role of EPHX1 in adipocytes, a CRISPR-Cas9-mediated knockout (KO) approach was developed (Supplemental Figures 2 and 3). A custom-designed single-guide RNA (sgRNA)/Cas9 expression vector targeting the sixth exon of *Ephx1* was used. The murine 3T3-L1 pre-adipocytes were chosen as a cellular model due to their ability to differentiate into mature adipocytes after stimulation in vitro (Figure 4A). 3T3-L1 cells transfected with a
Cas9/scramble gRNA plasmid were used as a control (CTL). KO efficiency was confirmed by Western blot analysis. A near complete loss of Ephx1 expression, which remained stable over time during adipocyte differentiation, was indeed observed (Figure 4B). The efficiency of adipocyte differentiation was confirmed by progressive lipid accumulation, as revealed both by the appearance of refractive droplets in optical microscopy, and by an increase in Oil Red O staining, which is a marker of intracellular lipids (Figures 4A and 4C). WT and control 3T3-L1 pre-adipocytes differentiated into adipocytes within 12 days and displayed strong accumulation of lipid droplets in the cytoplasm (Figures 4C and 4D). In contrast, Ephx1 KO led to strong and significant decrease in lipid droplet formation (p < 0.0001) (Figures 4C and 4D). The expression study of adipocyte markers constitutes another way to evaluate adipocyte differentiation. As compared to WT and control cells submitted to in vitro adipocyte differentiation, Ephx1 KO cells displayed a significantly reduced expression of adipogenic markers, including peroxisome proliferator activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), SREBP-1c, as well as the expression of mature adipocyte markers, such as fatty acid synthase (FAS), and adiponectin (Figure 4E).

We next investigated the effect of the deletion of Ephx1 on insulin sensitivity. In WT and control 3T3-L1 adipocytes stimulated with insulin, Western blot analysis revealed a strong increase in the phosphorylation of several signaling intermediates from the mitotic and metabolic pathways including insulin receptor β subunit (IRβ), insulin receptor substrate-1 (IRS1), AKT, and extracellular-regulated kinase (ERK) (Figure 4F). In contrast, the Ephx1 KO cells were resistant to insulin, as shown by the lack or strong decrease in the phosphorylation of these intermediates upon insulin stimulation (Figure 4F). Taken together, these results show that Ephx1 deficiency alters adipogenesis and inhibits insulin signaling, consistent with the lipoatrophic and insulin resistant phenotype.

Ephx1 knockout in pre-adipocytes promotes oxidative stress and senescence

A previous study has shown that EPHX1 might protect cells from oxidative stress (37). In addition, increased cellular senescence has been functionally linked to fat-related metabolic dysfunction (38) and has been observed in a few lipodystrophic syndromes (39, 40). Cellular aging has also been associated with an increased production of reactive oxygen species (ROS) (41). Consequently, we wondered if Ephx1 deficiency might promote ROS production and senescence. To test this hypothesis, oxidative stress was first evaluated. Ephx1 KO cells displayed higher levels of ROS in cell lysates, compared to either WT or control 3T3-L1 cells (p < 0.0001) (Figure 5A). The proliferative capacity and biochemical markers of cellular
senescence was then evaluated in edited 3T3-L1 pre-adipocytes. Bromodeoxyuridine (BrdU) incorporation was lower in Ephx1 KO cells compared to WT and control cells ($p < 0.0001$), consistent with a reduced proliferation rate (Figure 5B). In parallel, the levels of P21 and P16, two cell cycle cyclin-dependent kinase inhibitors were significantly increased in KO cells, consistent with increased senescence (Figure 5C). Additionally, compared to WT and control 3T3-L1 cells, Ephx1 KO cells were characterized by a significant increase in senescence-associated β-galactosidase (SA-β-gal) activity ($p < 0.0001$), which is another marker of cellular senescence (Figures 5D and 5E) (42). Furthermore, enhanced levels of phosphorylated P53 were observed in KO cells (Figure 5C), further underlining the senescent cellular phenotype (43). Altogether, these data strongly suggest a functional link between EPHX1 dysfunction, oxidative stress, and cellular senescence.

Fibroblasts from patient 1 display a senescent phenotype

Although skin is not a tissue in which EPHX1 is highly expressed, the protein was detected by Western blot in cultured fibroblasts from skin biopsies of two normal individuals (T1 and T2) and patient 1 (Supplemental Figure 4). These immunoblot analyses using several anti-EPHX1 polyclonal antibodies allowed us to detect only the monomeric EPHX1 form (55 kDa) but not the aggregates. We next assessed oxidative stress. The mutant fibroblasts showed increased levels of ROS in cell lysates compared with controls ($p < 0.0001$) (Figure 6A). Regarding the impact of the EPHX1 variant on cellular senescence, the patient 1-derived fibroblasts displayed an altered morphology with an enlarged, flattened, and irregular shape, as compared to spindle-shaped control fibroblasts (Supplemental Figure 5). BrdU incorporation was significantly reduced in mutant fibroblasts ($p < 0.0001$), which was correlated with increased levels of P21 and P16 (Figures 6B and 6C). Furthermore, SA-β-gal activity was markedly increased in the mutant fibroblasts ($p < 0.0001$), even though these fibroblasts were at an earlier passage than controls (Figures 6D and 6E). This increased SA-β-gal activity was accompanied by enhanced levels of phosphorylated P53 in mutant fibroblasts (Figure 6C). Together, these results were similar to those obtained using Ephx1-KO pre-adipocytes, confirming ex vivo the key role of EPHX1 in controlling senescence-associated oxidative stress.

Treatment of patient 1 with metreleptin is very beneficial
Since patient 1 suffered from severe diabetes, insulin therapy was started in adolescence. High-doses (up to 15 IU/kg/day) of rapid acting insulin analogs and then Humulin Regular U-500® were required through a basal-bolus regimen then an insulin pump. However, this did not provide suitable glycemic control (HbA1c: 10-16.5 %). Other anti-diabetic treatments, including metformin and exenatide, did not bring additional effectiveness, and the patient’s hyperphagia hampered the compliance with diet. Treatment with metreleptin, a recombinant form of leptin, was initiated in early adulthood at the initial dose of 5 mg, then readjusted after 6 months at the dose of 7.5 mg. The treatment efficacy was evaluated 3 and 6 months after its introduction (Table 2). There was a major beneficial effect on metabolic manifestations since it led to a decrease of HbA1c to 7.3 % after six months of treatment, allowing to reduce daily insulin doses by almost 50 % (Table 2). Metreleptin effectiveness was also evidenced by a decrease in liver enzymes, and an improvement of liver scores for steatosis, fibrosis, and necrosis/inflammation (Table 2).
Discussion

This translational study presents the first example of a monogenic disorder related to a gene in the EH family. The data demonstrate the pleiotropic effect of EPHX1 and its key role in cell homeostasis. This is consistent with the high evolutionary conservation of EHs across multiple organisms, including animals, insects, plants, fungi and bacteria (44) and underscores their essential biological function.

In this study, two different EPHX1 de novo missense variants were identified in patients with a progressive multisystemic disorder. According to the American College of Medical Genetics and Genomics (ACMG) criteria (45), these variants can be classified as “pathogenic” with the inclusion of our functional data. The two patients share a number of clinical characteristics including dysmorphic features and manifestations affecting the liver, adipose tissue, and nervous system. The broad expression of EPHX1 explains how germline variants in this gene result in this multisystemic phenotype. As an example, EPHX1 transcripts have been detected in various areas of the brain (46), and EPHX1 was also recently shown to play a complementary role to EPHX2 in the brain metabolism of naturally occurring EETs, which constitute a major class of EpFAs (36, 47). The involvement of the EPHX1 de novo variants identified herein in diabetes and liver dysfunction is also consistent with a number of previous observations. Firstly, the expression of EPHX1 is regulated by various transcriptional factors including GATA4 and HNF4A (48, 49), two genes implicated in other forms of monogenic diabetes. In regard to systemic hormonal regulation, it was demonstrated in primary hepatocytes that insulin positively and glucagon negatively regulate EPHX1 expression (50). A previous study also showed that a common polymorphism in EPHX1 (p.Tyr113His) was associated with an increased risk of type 2 diabetes mellitus and insulin resistance (51), as well as liver cirrhosis (52). It was shown that EPHX1 plays a key role in the liver metabolism of endogenous lipids (18). Regarding hormonal pathophysiology, patient 1 developed amenorrhea associated with steroidogenesis alterations. The extremely high testosterone levels in the absence of a tumor and in the presence of normal estradiol levels argue for a direct or indirect blockade of the aromatase activity. A potential role of EPHX1 in reproductive physiology was suggested previously. EPHX1 is expressed in ovarian follicle cells (53) and is regulated by progesterone during the menstrual cycle (54). Several endogenous biologically active epoxide mediators of the steroidogenic pathway were found to be EPHX1 substrates, such as androstene oxide (16α,17α-epoxyandrosten-3-one) and estroside (epoxyestriatrienol) (55). EPHX1 protects cells from oxidative stress in oviducts...
A decrease in estradiol formation from testosterone was seen in human ovaries upon treatment with an EPHX1 inhibitor (56). Polycystic ovary syndrome, characterized by hyperandrogenism and elevated serum testosterone levels, is observed in patients taking sodium valproate (57), an anti-epileptic and anti-convulsant drug known to inhibit EPHX1 activity (58). This observation is reminiscent of what is seen in our patient, who displays signs of hyperandrogenism in association with a loss of EPHX1 activity. The role of EPHX1 on the reproductive function could also be illustrated by the reported link between EPHX1 polymorphisms and spontaneous abortion (59) or preeclampsia (60). Consequently, further functional experiments in estrogen-producing granulosa cell models would be helpful to understand how EPHX1 could modulate aromatase activity or other steps of the steroidogenic pathway.

There were few data on the role of EPHX1 in adipose tissue and its function in adipocytes remained elusive. The current study supports a role for EPHX1 in adipogenesis and adipocyte functions. We observed a defect in adipocyte differentiation in 3T3-L1 cells with CRISPR-Cas9 KO of Ephx1. This cellular model also revealed decreased expression of mature adipocyte markers and altered insulin signaling. This is consistent with the lipoatrophic phenotype observed in the two patients carrying de novo EPHX1 pathogenic variants. Such an adipocyte differentiation defect has been reported in several other lipoatrophic diabetes of various genetic origins (61-63). Lipoatrophic diabetes are indeed characterized by an incapacity of adipose tissue to store triglycerides, leading to ectopic fat depots and insulin resistance. The profound serum leptin and adiponectin deficiency observed in patients further confirms an endocrine defect of adipose tissue, since these hormones are secreted by mature adipocytes. Notably, it has been shown that oxylipins, which are EPHX1 substrates, target peroxisome proliferator-activated receptors (PPARs) to modify adipocyte formation and function (64). It has also been reported that EETs decrease mesenchymal stem cells (MSC)-derived adipocyte differentiation by inhibiting PPARγ, C/EBPα, and FAS (65). Altogether, these data show that EPHX1, with its crucial role in epoxide reactive species biotransformation, stands at the crossroad of several signaling pathways and thereby plays a key role in cell metabolism and homeostasis.

EpFAs are endogenous lipid mediators functionally regulated in part by their hydrolysis by EPHX1 and EPHX2. Strategies stabilizing or mimicking EpFAs are commonly reported to contribute to cell homeostasis maintenance (66-68). In this regard, it has been proposed that EpFAs prevent mitochondrial dysfunction, reduce ROS formation, and alleviate ER stress (69). EETs exhibit numerous beneficial effects, such as anti-inflammatory,
analgesic, vasodilatory, angiogenic, fibrinolytic, tissue-regenerating and cytoprotective effects (18, 70, 71). This has been deeply investigated for EPHX2, and specific inhibitors have shown beneficial effects on a wide range of apparently unrelated conditions, including diabetes, fibrosis, chronic pain, cardiovascular and neurodegenerative diseases (72), so that several of these EPHX2 inhibitors were tested in phase I clinical trials (8, 73). In contrast, the current study shows that a loss of EPHX1 activity can be associated with a severe multisystemic phenotype. How to reconcile these apparently conflicting data? Like numerous other signaling molecules, the function of EpFAs strongly depend on the biological context (69). The absence of a measurable effect of EpFAs under basal conditions is consistently observed across different laboratories and is in stark contrast to their strong efficacy under pathological situations. EpFAs seem to function with fine tuning to regulate and maintain cell homeostasis. This point is best illustrated by the ability of EPHX2 inhibitors to shift both hypertension and hypotension towards normotension in rodent models (74, 75).

At the cellular level, the pathogenic variants identified herein might result in a dominant negative due to EPHX1 aggregation within the ER. In any case, it finally results in a loss of the enzyme activity, consistent with the localization of the variants at the center of EPHX1 catalytic site. Ephx1 KO in 3T3L1-preadipocytes revealed a high rate of ROS production, an observation confirmed in patient 1-derived fibroblasts. These data reinforce the proposed role of EHs as regulators of oxidative stress (37, 69, 75). Patient 1-derived fibroblasts displayed an altered morphology, senescent features such as an increase in the SA-β-gal marker, as well as a reduced proliferative rate. This senescent phenotype was reproduced by removing Ephx1 in 3T3-L1 pre-adipocytes, which subsequently loss their capacity to differentiate into adipocytes. Our findings support the notion that cellular senescence is an important player in adipogenesis, but also argues that EPHX1 may directly regulate adipocyte differentiation.

In this study, we were also interested in improving the therapeutic management of this novel clinical entity, which justifies careful clinical evaluation and requires multidisciplinary care. Patient 1 suffered from very severe diabetes with persistent glycemic imbalance despite very high insulin doses. Metreleptin was shown to reduce hyperphagia leading to weight loss, to improve insulin sensitivity and secretion, to reduce hypertriglyceridemia, hyperglycemia, and fatty liver disease in some patients with lipoatrophic diabetes (76, 77). All these beneficial effects were rapidly observed in patient 1 after treatment initiation. Besides, regarding the specific role of EPHX1 as a xenobiotic detoxifying enzyme, another practical implication of this study for the patient is the contraindication to all drugs known to be
metabolized by EPHX1, especially carbamazepine, phenobarbital and phenytoin (5). They would not be properly metabolized by EPHX1 and eliminated by the patient leading to toxicity.

The data presented here emphasize that lipid mediator regulation by EHs is essential for homeostasis and that its alteration is a newly discovered mechanism in monogenic insulin resistant lipoatrophic diabetes. The field of monogenic diabetes is quickly advancing, and knowledge gained in recent years led to great improvements in understanding of their molecular and cellular bases. This allows to improve overall quality of life for patients and their families, with earlier diagnoses and personalized treatments. Continued efforts at gene discovery may help reveal mechanistic pathways implicated in the more common forms of type 1 and type 2 diabetes and lead to better treatments and outcomes.
## Materials and methods

### Key resources table

| Reagent type (species) or resource | Designation | Source and reference | Identifiers | Additional information |
|-----------------------------------|-------------|----------------------|-------------|------------------------|
| Cell line (*Homo sapiens*)        | HEK 293     | ATCC                 | CRL-1573    | Embryonic kidney       |
| Cell line (*Mus musculus*)        | 3T3-L1      | ATCC                 | CL-173      | The cells undergo a pre-adipose to adipose like conversion as they progress from a rapidly dividing to a confluent state |
| Primary fibroblasts               | T1          | Pr. Fève lab at CRSA, Paris | N/A         | Non-obese and non-diabetic female, skin biopsy |
| Primary fibroblasts               | T2          | Pr. Fève lab at CRSA, Paris | N/A         | Non-obese and non-diabetic female, skin biopsy |
| Primary fibroblasts               | Patient 1   | Pr. Fève lab at CRSA, Paris | N/A         | Patient 1, female, skin biopsy |
| Antibody                          | Anti-adiponectin | Thermo Fisher Scientific | Cat# MA1-054 | WB (1:1000) |
| Antibody                          | Anti-AKT    | Santa Cruz Biotechnology | Cat# sc-8312 | WB (1:1000) |
| Antibody                          | Anti-β-actin | Sigma Aldrich        | Cat# A2228  | WB (1:10000) |
| Antibody                          | Anti-Calnexin | ENZO Life Science   | Cat# AD0-SPA-860 | IF (1:200) |
| Antibody                          | Anti-C/EPBα | Protein Tech         | Cat# 18311-1-1P | WB (1:1000) |
| Antibody                          | Anti-EFHX1  | Novus                | Cat# NB1-3301 | WB (1:1000) - IF (1:1000) |
| Antibody                          | Anti-ERK    | Cell Signaling Technology | Cat# 9102   | WB (1:1000) |
| Antibody                          | Anti-FAS    | Cell Signaling Technology | Cat# 3180   | WB (1:1000) |
| Antibody                          | Anti-Flag   | Origene              | Cat# TA50011-100 | WB (1:1000) - IF (1:1000) - IP (1:200) |
| Antibody                          | Anti-IRB    | Cell Signaling Technology | Cat# 3025   | WB (1:1000) |
| Antibody                          | Anti-IRS1   | Protein Tech         | Cat# 17509-1-AP | WB (1:1000) |
| Antibody                          | Anti-P16    | Protein Tech         | Cat# 10883-1-AP | WB (1:1000) |
| Antibody                          | Anti-P21    | Protein Tech         | Cat# 10355-1-AP | WB (1:1000) |
| Antibody                          | Anti-P53    | Abcam                | Cat# ab1101  | WB (1:1000) |
| Antibody                          | Anti-P-AKT  | Santa Cruz Biotechnology | Cat# sc-7985-R | WB (1:1000) |
| Antibody                          | Anti-P-ERK  | Cell Signaling Technology | Cat# 9101   | WB (1:1000) |
| Antibody                          | Anti-P-P53  | Abcam                | Cat# ab38497 | WB (1:1000) |
| Antibody                          | Anti-P-PPARg | Protein Tech        | Cat# 16643-1-AP | WB (1:1000) |
| Antibody                          | Anti-SREBP-1 | Santa Cruz Biotechnology | Cat# sc-366  | WB (1:1000) |
| Antibody                          | Anti-Tubulin | Protein Tech        | Cat# 66031-1-lg | WB (1:10000) |
| Antibody                          | Anti-P-Tyr  | Santa Cruz Biotechnology | Cat# sc-7020 | WB (1:1000) |
| Antibody                          | Anti-rabbit- | GE Healthcare       | Cat# NA934V  | WB (1:2000) |
| Antibody | Recombinant DNA reagent (plasmid) | Cat# | WB (1:2000) |
|----------|----------------------------------|------|-------------|
| Anti-mouse-HRP | pCMV-entry-Flag | NA931V | GE Healthcare |
| | pCMV-EPHX1 WT-Flag or without Flag | PS100001 | Origene |
| | pCMV-EPHX1 c.337T>C -Flag or without Flag | N/A | This paper |
| | pCMV-EPHX1 c.416A>G -Flag or without Flag | N/A | This paper |
| | pCMV-EPHX1 c.997A>C -Flag or without Flag | N/A | This paper |
| | pCMV-EPHX1 c.1212G>C -Flag or without Flag | N/A | This paper |
| | pCMV-EPHX1 c.1288G>C -Flag or without Flag | N/A | This paper |
| | pSpCas9(BB)-2A-GFP (PX458) | 48138 | Addgene |
| | | | | |

**Recombinant DNA reagent (plasmid)**

Recombinant DNA reagent (plasmid) for EPHX1 variants are used in this study. Cat# PS100001 for Origene and Cat# 48138 for Addgene. These reagents are publicly available.

**Software algorithm**

Software algorithms used in this study include FIJI software, GraphPad, and Prism. These tools are available under a CC-BY-NC-ND 4.0 International license.

**Genetic studies**

Diagnostic laboratories performed genetic analyses on genomic blood DNA extracted from peripheral blood leukocytes using standard procedures. Exons and flanking intronic sequences of a panel of genes involved in lipoatrophic diabetes (20) were captured from fragmented DNA with the SeqCapEZ enrichment protocol (Roche NimbleGen, USA). Paired-end
massively parallel sequencing was achieved on a MiSeq platform (Illumina, USA). Bioinformatic analysis was performed using the Sophia DDM pipeline® (Sophia Genetics, Switzerland). Identification of EPHX1 variants was obtained by WES. For patient 1, library preparation, exome capture, sequencing and variant calling and annotation were performed by IntegraGen SA (Evry, France). Genomic DNA was captured using Twist Human Core Exome Enrichment System (Twist Bioscience, USA) and IntegraGen Custom, followed by paired-end 75 bases massively parallel sequencing on Illumina HiSeq4000. Identification of the potentially-pathogenic variants were determined using Sirius software (IntegraGen SA, France). For patient 2, exome sequencing was performed on the proband and both parents as previously described (21). EPHX1 variants were confirmed by Sanger sequencing with the Big Dye Terminator v3.1 sequencing kit (Thermo Fisher Scientific, MS, USA) after PCR amplification and analyzed on a 3500xL Dx device with the SeqScape v2.7 software (Thermo Fisher Scientific, MS, USA). EPHX1 variants were described based on the longest isoform (NM_000120.4) using Alamut 2.11 (Sophia Genetics, Switzerland) and Human Genome Variation Society guidelines. Variants were classified according to the ACMG recommendations (45). Protein sequences were aligned using the Clustal Omega software and the degree of conservation was presented with help of the BoxShade software. EPHX1 variants were queried in human populations using gnomAD.

Plasmids and Transfection

EPHX1 cDNA was amplified by RT-qPCR using RNA from HepG2 cells and inserted into the pCMV6-entry mammalian expression vector containing a C-terminal Flag Tag (#PS100001; Origene, MD, USA). EPHX1 variants (c.337T>C, c.416A>G, c.997A>C, c.1212G>C, and c.1288G>C) were introduced using the Quikchange II Site-directed mutagenesis kit (#200523; Agilent Technologies, CA, USA) and constructs were checked by Sanger sequencing. To remove the C-terminal Flag Tag of the different plasmids, a nonsense variant was introduced by site-directed mutagenesis. Transient transfection of the different cell lines was carried out in six-well plates with TurboFect™ Transfection Reagent (#R0532; Thermo Fisher Scientific, MS, USA) according to the manufacturer’s instructions.

Cell culture

HEK 293 cells were cultured in high-glucose (4.5 g/L) Dulbecco’s Modified Eagle’s Medium (DMEM; #11960085; Thermo Fisher Scientific) containing 10% fetal calf serum (FCS) (#F7524; Sigma-Aldrich, MI, USA), 1 % penicillin/streptomycin (PS). 3T3-L1 pre-adipocytes
were maintained in an undifferentiated state in high-glucose (4.5 g/L) DMEM supplemented with 10 % newborn calf serum and 1 % PS (#CA-1151500; Biosera, MI, USA), and the medium was changed every 2 days without ever reaching confluence. Adipocyte differentiation was induced by treating 2-day post-confluent cultures with high-glucose (4.5 g/L) DMEM supplemented with 10% FCS, 1% PS, 1µM dexamethasone (#D4902; Sigma-Aldrich), 250 µM 3-isobutyl-1-methyl xanthine (IBMX) (#I7018; Sigma-Aldrich) and 0.17 µM insulin (#I0516; Sigma-Aldrich) for three days. The medium was then replaced with high-glucose DMEM supplemented with 10% FCS, 1% PS and 0.17 µM insulin, and changed to fresh medium every 2 days until the 12th day. Primary fibroblast cultures were established using skin biopsies from two healthy non-obese non-diabetic women, named T1 and T2, as well as from patient 1. Fibroblasts were grown in low glucose (1 g/L) DMEM with pyruvate (#31885049; Thermo Fisher Scientific) and supplemented with 10% FCS, 1% PS, and 2 mM glutamine. Fibroblasts were stained at a low passage number (i.e., passage 4 for patient 1, passage 9 for T1 and T2). All culture conditions were kept constant throughout the experiments.

CRISPR/Cas9-mediated deletion of Ephx1

pSpCas9(BB)-2A-GFP (PX458) was a gift from Zhang lab (Addgene, MA, USA; plasmid #48138) and was used to transfect 3T3-L1 cells with Cas9 along with the targeting guide RNAs (gRNAs). gRNAs were designed and checked for efficiency (http://cistrome.org/SSC) and specificity (http://crispr.mit.edu). Subsequently, they were cloned in the plasmid and transfected into cells using TurboFect (#R0532; Thermo Fisher Scientific) according to the manufacturer’s instructions. 48 hr post transfection, cells were sorted by flow cytometry (Cell Sorting Core Facility, Centre de Recherche Saint-Antoine) and cells with the highest GFP positivity were transferred into a 24-well plate and propagated. The gRNA sequences used in this study to target Ephx1 were the following: gRNA sense 5’ TCTTAGAGAAGTTCTCCACC 3’; gRNA antisense 5’ GGTGGAGAACTTCTCTAAGA 3’.

Immunofluorescence

For indirect immunofluorescence, HEK 293 cells were grown on glass coverslips and after transfection, they were fixed for 15 min in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and then permeabilized for 15 min with 0.1 % Triton X-100 in PBS at room temperature. Cells were washed three times with a blocking solution containing PBS.
with 5% fatty acid free bovine serum albumin. Primary antibodies used for immunostaining were as follow: mouse anti-Flag (Origene) (1/1000), rabbit anti-EPHX1 (Novus) (1/1000), rabbit anti-Calnexin (#ADO-SPA-860; Enzo Life Sciences, France) (1/200). Cells were incubated 1 hr at 37°C, rinsed, and then incubated for 45 min at room temperature with the appropriate Alexa-conjugated isotype-specific secondary antibodies (Thermo Fisher Scientific) and 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). The coverslips were mounted in DAKO fluorescence mounting media (#S3023; Agilent Technologies, CA, USA). Images were acquired using a SP2 inverted confocal microscope (Leica Biosystems, Germany), equipped with an HCX PL APO CS 63X/1.32 oil immersion objective, and analyzed using Leica Confocal Software and FIJI Software. For each experiment, all images were acquired with constant settings (acquisition time and correction of signal intensities).

**Measurement of EPHX1 activity**

EPHX1 activity in lysates of transfected HEK 293 cells was determined using [3H]-cis stilbene oxide ([3H]-cSO), as described previously (35). After thawing on ice, cells were diluted with chilled sodium phosphate buffer (20 mM, pH 7.4) containing 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then broken with a 10 s ultrasonic pulse. The mixture was centrifuged at 10,000 g for 20 min at 4°C. Supernatants were collected and flashed frozen, before being used for further analysis. Protein concentration was quantified using the Pierce BCA assay (Pierce, IL, USA), using Fraction V bovine serum albumin (BSA) as the calibrating standard. After thawing on ice, supernatants were diluted (5 to 20-folds) with Tris-HCl buffer (0.1 M, pH 9.0) containing freshly added BSA (0.1 mg/mL). In glass tubes containing 100 μL of the diluted extract, the reaction was started by adding 1μL of 5 mM [3H]-cSO in ethanol (10,000 cpm, [S]final = 50 μM). The mixture was incubated at 37°C for 5 to 90 min. The reaction was then quenched by the addition of 250 μL of isoctane, which extracts the remaining epoxide from the aqueous phase. The activity was followed by measuring the quantity of radioactive diols formed in the aqueous phase using a scintillation counter (TriCarb 2810 TR, Perkin Elmer, Shelton, CT). Assays were performed in triplicates.

**Western blot**

Cells were homogenized in NP-40 lysis buffer to obtain protein lysates. Thirty micrograms of protein extracts were separated by sodium dodecyl sulfate - polyacrylamide gel
electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane and analyzed by immunoblotting.

**Immunoprecipitation**

Transfected HEK 293 cells were recovered in 1 mL of PBS. They were centrifuged at 4,025 g for 1 min in a microfuge and resuspended in 50-100 µL of TNT buffer (20 mM Tris-HCl - pH 7.5, 200 mM NaCl, 1 % Triton X-100). After incubation on ice for 10 min and centrifugation at 16,099 g for 10 min, supernatants were recovered and protein concentrations determined. Protein lysates (100-200 mg in 200 µL of TNT) were either directly analyzed by Western blotting or first immunoprecipitated. In this latter case, extracts were incubated under rotation for 2 hr at 4°C with the relevant antibody. Protein G Sepharose (Sigma) was then added and the mixture incubated for a further 1 hr at 4°C. Sepharose beads were quickly centrifuged in a microfuge (30 s at 11,180 g) and washed three times with TNT. After final wash, the beads were resuspended in 30 mL of buffer A (10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10 mM Hepes - pH 7.8) complemented with loading dye, before being processed as described in Western blot analysis.

**Oil Red-O staining, image processing, and quantification**

Intracellular lipids were stained by Oil Red-O (#O0625; Sigma-Aldrich). Cells were washed with PBS and fixed with 4 % PFA in PBS, for 10 min. Fixed cells were incubated with Oil Red-O solution for 1 h at room temperature and then with DAPI (Thermo Fischer Scientific) for 5 min. Fluorescence images were generated with IX83 Olympus microscope, acquired with Cell-Sens V1.6 and analyzed with FIJI software. Images of 8-10 different areas per condition were visualized by fluorescence microscopy using mCherry filter, followed by computer image analysis using FIJI software. Briefly, analysis was performed by threshold converting the 8-bit Red-Green-Blue image into a binary image, which consists only of pixels representing lipid droplets (i.e., red). Importantly, after separation, the binary image was manually compared with the original image for consistency and correct binary conversion. The area occupied by lipid droplets in the image was displayed by FIJI software as surface area in µm² and normalized to cell number by semi-automated counting of DAPI-stained nuclei.

**Cell proliferation assay**
3T3-L1 cells and fibroblasts were seeded in a 12-well plate (5000 per well) and incubated overnight at 37°C in DMEM supplemented with 10% FCS and 1% PS. Cell proliferation was evaluated by BrdU incorporation using a colorimetric ELISA assay (#QIA58; Sigma-Aldrich) 16 h after seeding, according to the manufacturer’s instructions.

Oxidative Stress and Cellular Senescence
The oxidation of the fluorogenic probe 2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFHDA) (2 µg/mL, #C6827; Sigma-Aldrich) was used to evaluate intracellular levels of ROS on a 200-plate fluorescence reader (Tecan Infinite, Switzerland) at 520-595 nm. The blue staining of β-galactosidase (β-gal) at pH 6 was used as a biomarker of cellular senescence. Cells were fixed with 4% PFA in PBS for 5 min at room temperature. Cells were washed twice with PBS and incubated overnight in fresh SA-β-gal staining solution containing 1 mg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside) (#3117073001; Sigma-Aldrich), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂, and 0.4 mM phosphate buffer, pH 6.0, in darkness at 37 °C without CO₂. For positive staining controls, fixed cells were treated with the same solution, but at pH 4.0. After imaging with an IX83 Olympus microscope, stained cells were resuspended with 2% SDS, scratched, and sonicated. Finally, the absorbance (630 nm) was read with a Tecan Infinite 200-plate reader and the staining ratio at pH 6.0/pH 4.0 was calculated.

Statistics
Data are presented as means ± SEM (standard error of the mean). GraphPad Prism software (GraphPad Software) was used to calculate statistical significance. Gaussian distribution was tested with the Kolmogorov–Smirnov test. Multiple comparisons were conducted by one-way analysis of variance (ANOVA) with Bonferroni-test or Kruskal-Wallis test for post hoc analysis. \( P < 0.05 \) was considered statistically significant.

Study Approval
We obtained written informed consent for all genetic studies as well as for the use of photographs shown in Figure 1. The study was approved by the CPP Ile de France 5 research ethics board (Paris, France) and the Columbia institutional review board (New York, United States).
**Figure legends**

**Figure 1.** *EPHX1* pathogenic variants in a newly-characterized lipoatrophic diabetes syndrome. (A) Genealogical trees and segregation analysis for the two *EPHX1* variants identified in this study. Arrows indicate probands. p.Thr333Pro and p.Gly430Arg were absent from both parents of each proband, indicating that they occurred *de novo.* +, normal allele; M, mutant allele. (B) Schematic of *EPHX1* transcript displaying the location of the two variants identified. (C) Characteristics of the patient’s head. Left panel: Black shadow of the patient’s profile over a grayscale photo. Black arrows point to frontal bossing and retrognathism. The white dotted line indicates the base of the scalp showing high hair line. Top right panel: Front photo of the patient’s face showing lipoatrophy and retrognathism. Bottom right panel: Profile radiography of the skull showing teeth misalignments and mandibulo-facial dysostosis. (D) Top panel: Frontal photo of the patient’s abdomen showing prominent abdomen with umbilical herniation and hirsutism. Bottom panel: axial computed tomography slice of the abdomen showing hepatomegaly and liver steatosis. (E) Picture of the armpit showing *acanthosis nigricans* and *molluscum pendulum.* (F) Picture (left panel) and radiography (right panel) of the left-hand showing arachnodactyly with tapered fingers and thickening of proximal interphalangeal joints. (G) Front picture of the legs showing distal lipoatrophy. This figure can be made available by contacting the corresponding author.

**Figure 2.** Loss of *EPHX1* hydrolysis activity due to the p.Thr333Pro and p.Gly430Arg variants. (A) Schematic representation of the *EPHX1* protein, showing its subcellular localization and function. Epoxide hydrolases open three membered cyclic ethers, known as epoxides, by the addition of water to yield 1,2-diols. The location of the amino acids affected by the missense variants identified in this study are indicated by stars. (B) Schematic representation of the variants used in functional tests. Residues of the catalytic triad are shown above the protein structure. Variants used in functional assays are depicted below. Variants identified in patients are displayed in red. (C) Model of the 3D structure of *EPHX1*, based on the quaternary structure of the closely homologous EH enzyme from the *Aspergillus niger* fungus (31). On the left panel, the location of the two variants identified in patients are indicated by a star. On the right panel, the two variants found in patients are depicted in red and the three key residues of the catalytic site in blue. (D) c-SO (*cis*-stilbene oxide) hydrolysis assay performed in HEK 293 cells transiently expressing Flag-tagged wild-type (WT) and mutated forms of human *EPHX1*, as indicated. Results are expressed as means ± SEM of two
independent biological experiments, each of them being performed in duplicates. # indicates that hydrolysis activity of EPHX1 carrying the p.Thr333Pro and p.Gly430Arg de novo variants was abolished, compared with WT and other variants. (E) Western blot analysis aimed at controlling the expression of WT and mutant forms of EPHX1 in protein extracts used in c-SO hydrolysis assays presented in (D), using antibodies as indicated. Numbers on the left correspond to molecular weight markers (kDa). Western blot images are representative of two independent experiments.

Figure 3. The p.Thr333Pro and p.Gly430Arg variants induce the formation of EPHX1 aggregates within the endoplasmic reticulum. (A) HEK 293 cells transiently expressing Flag-tagged wild-type (WT) and mutated isoforms of human EPHX1 were grown on coverslips, fixed, permeabilized, and stained with an anti-Flag antibody followed by an anti-Calnexin antibody. They were then incubated with Alexa Fluor® 594-and 488-conjugated secondary antibodies and visualized by confocal microscopy. Nuclei were counterstained with DAPI (Blue). Red arrows point to EPHX1 aggregates. Representative pictures of three independent experiments are presented. Scale bar is 10 μm. (B) Immunofluorescence staining of HEK 293 cells transiently expressing WT and mutated isoforms of human EPHX1 using an anti-EPHX1 antibody and visualized using an Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody. Nuclei were counterstained with DAPI (Blue). Representative pictures of two independent experiments are presented. Scale bar is 10 μm. (C) HEK 293 cells were transiently transfected with Flag-tagged WT and mutated isoforms of human EPHX1. Whole-cell extracts were prepared 24 hr later, immunoprecipitated with an anti-Flag antibody and analyzed by Western blotting using an anti-EPHX1 antibody. The formation of EPHX1 aggregates in the presence of the p.Thr333Pro and p.Gly430Arg variants is shown by red arrows. The asterisk indicates a non-specific band present only in direct immunoblotting using anti-EPHX1 antibody. Numbers on the left correspond to molecular weight markers (kDa). Western blot images are representative of three independent experiments.

Figure 4. Ephx1 deficiency suppresses adipocyte differentiation of 3T3-L1 cells and alters insulin signaling. Data were obtained in 3T3-L1 pre-adipocytes from ATCC, 3T3-L1 cells with a CRISPR-Cas9-mediated Ephx1-knockout (KO), and 3T3-L1 cells transfected with a Cas9/scramble gRNA plasmid corresponding to control (CTL) cells. (A) Timeline representation of the 3T3-L1 pre-adipocyte differentiation process using a hormonal cocktail. Dexa: dexamethasone; IBMX: 3-isobutyl-1-methylxanthine; D0-D12: Day 0 to Day 12.
Validation of Ephx1 KO in 3T3-L1 pre-adipocytes and study of its expression during adipocyte differentiation. Numbers on the left correspond to molecular weight markers (kDa). Western blot images are representative of three independent experiments. (C) Adipocyte differentiation assessed by Oil Red-O lipid staining. 3T3-L1 pre-adipocytes were studied during adipocyte differentiation for 12 days. First and third lines: Pictures of dishes stained by Oil Red-O. Images are representative of three independent experiments. Second and fourth lines: representative images of fluorescence microscopy after staining of intracellular lipids (Oil Red-O, red) and nuclei (DAPI, blue). Images are representative of five independent experiments. (D) Quantification of Oil Red-O fluorescence normalized to DNA content (DAPI). Results are expressed as means ± SEM of five independent experiments. ****: p < 0.0001. p-values were determined by analysis of variance (ANOVA) with Kruskal-Wallis post hoc multiple comparison test. (E) Protein expression of adipocyte markers obtained by Western blotting during in vitro adipocyte differentiation of 3T3-L1 pre-adipocytes. Numbers on the left correspond to molecular weight markers (kDa). Western blot images are representative of three independent experiments. PPARγ: peroxisome proliferator-activated receptor-gamma; C/EBPα: CCAAT/enhancer-binding protein-alpha; SREBP-1c: sterol regulatory element-binding protein-1c; FAS: fatty acid synthase. (F) Activation of insulin signaling in 3T3-L1 pre-adipocytes after 10 days of adipocyte differentiation. The 3T3-L1 cells from ATCC, CTL and Ephx1-KO cells were deprived of serum for 6 hr, stimulated with 20 nM insulin for 5 min or left untreated, and subjected to immunoblotting with antibodies against total and phospho-insulin receptor β-subunit (IRβ), insulin receptor substrate-1 (IRS1), AKT, and extracellular-regulated kinase (ERK)1/2. Numbers on the left correspond to molecular weight markers (kDa). Western blot images are representative of three independent experiments.

Figure 5. Ephx1 deficiency causes oxidative stress and cellular senescence in 3T3-L1 pre-adipocytes. Data were obtained in 3T3-L1 pre-adipocytes from ATCC, 3T3-L1 cells with a CRISPR-Cas9-mediated Ephx1-knockout (KO), and 3T3-L1 cells transfected with a Cas9/scramble gRNA plasmid corresponding to control (CTL) cells. Differences between the three cell lines were determined by analysis of variance (ANOVA) with Bonferroni’s post hoc multiple comparison test. All results are expressed as means ± SEM of three independent experiments. (A) Reactive oxygen species (ROS) production assessed by oxidation of 5-6-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate (CM-H2DCFDA) in 3T3-L1 pre-
adipocytes. Results were normalized to DNA content measured by DAPI. ****: \( p < 0.0001 \).

(B) Evaluation of cellular proliferation by BrDU incorporation. ****: \( p < 0.0001 \). (C) Evaluation of cellular senescence by Western blotting using antibodies against the indicated proteins. Numbers on the left correspond to molecular weight markers (kDa). (D) Representative immunofluorescence images of senescence (SA-\( \beta \)-gal) after staining at pH4 and pH6. Scale bar is 100 \( \mu m \). (E) The SA-\( \beta \)-gal staining ratio at pH 6.0/pH 4.0 was calculated. ****: \( p < 0.0001 \).

**Figure 6.** The p.Thr333Pro variant causes oxidative stress and cellular senescence in patient 1-derived fibroblasts. Data were obtained using cultured fibroblasts from skin biopsies of two normal individuals (T1 and T2) and patient 1. Differences between the three fibroblast cultures were determined by analysis of variance (ANOVA) with Bonferroni’s post hoc multiple comparison test. All results are expressed as means ± SEM of three independent experiments. (A) Reactive oxygen species (ROS) production assessed by oxidation of 5,6-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate (CM-H\(_2\)DCFDA) in fibroblasts derived from T1, T2 and patient 1. Results were normalized to DNA content measured by DAPI. ****: \( p < 0.0001 \). (B) Evaluation of cellular proliferation by BrDU incorporation. ****: \( p < 0.0001 \). (C) Evaluation of cellular senescence by Western blotting using antibodies against the indicated proteins. Numbers on the left indicate molecular weight markers (kDa). (D) Representative immunofluorescence images of senescence (SA-\( \beta \)-gal) after staining at pH4 and pH6. Scale bar is 100 \( \mu m \). (E) The SA-\( \beta \)-gal staining ratio at pH 6.0/pH 4.0 was calculated. ****: \( p < 0.0001 \).
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Author contributions

Jérémie Gautheron and Isabelle Jéru, Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Validation, Writing - original draft, Writing - review and editing; Christophe Morisseau and Wendy K. Chung, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Validation, Writing - review and editing; Jamila Zammouri, Geneviève Baujat, Emilie Capel, Célia Moulin, Yuxin Wang and Jun Wang, Data Curation, Formal Analysis, Investigation, Writing - review and editing; Martine Auclair, Data Curation, Formal Analysis, Investigation, Methodology, Writing - review and editing; Bruce Hammock, Barbara Cerame and Frank Phan, Data Curation, Formal Analysis, Writing - review and editing; Bruno Fève, Corinne Vigouroux and Fabrizio Andreelli, Conceptualization, Formal Analysis, Investigation, Validation, Writing - review and editing.

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| Institute of Cardiometabolism and Nutrition (ICAN) | PAP17NECJG                     | Jérémie Gautheron        |
| Fondation pour la Recherche Médicale (FRM)  | ARF2017938613 and EQU202000310517 | Jérémie Gautheron        |
| National Institute of Environmental Health Sciences (NIEHSS) | R35ES030443 and P42 ES004699 | Christophe Morisseau    |
| National Institutes of Health (NIH)         | DK52431                          | Wendy K. Chung           |
| Fondation pour la Recherche Médicale (FRM)  | EQU201903007868                  | Bruno Fève, Corinne Vigouroux, Isabelle Jéru |

Declaration of interests

The authors have declared that no conflict of interest exists.
Web resources

Boxshade, https://embnet.vital-it.ch/software/BOX_form.html
CADD, https://cadd.gs.washington.edu
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar
Clustal Omega program, https://www.ebi.ac.uk/Tools/msa/clustalo/
dbSNP, https://www.ncbi.nlm.nih.gov/snp
Exome Variant Server, https://evs.gs.washington.edu/EVS
Human Genome Variation Society guidelines, http://www.hgvs.org/mutnomen
GeneMatcher, https://genematcher.org
GnomAD, https://gnomad.broadinstitute.org
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2
SWISS-MODEL, https://swissmodel.expasy.org

Supplemental data

Supporting information includes five Supplementary Figures.
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Figure 1. EPHX1 pathogenic variants in a newly-characterized lipoatrophic diabetes syndrome.

This Figure can be made available by contacting the corresponding author.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Table 1. Clinical and biological features in patients with EPHX1 de novo variants.
This Table can be made available by contacting the corresponding author.
Table 2. Evolution of metabolic markers in patient 1 over a period of 6 months of metreleptin treatment. For data before treatment, values are given as the ranges observed over the last past three years. AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GGT: gamma glutamyl transpeptidase.

|                               | Before metreleptin | After 3 month-metreleptin therapy (5 mg/day) | After 6 month-metreleptin therapy (7.5 mg/day) |
|-------------------------------|-------------------|---------------------------------------------|-----------------------------------------------|
| **Anthropometric markers**    |                   |                                             |                                               |
| Weight                        | 53                | 51                                          | 50                                            |
| BMI                           | 20.2              | 19.4                                        | 18.9                                          |
| **Glucose homeostasis**       |                   |                                             |                                               |
| HbA1c (%) (N: 4-6%)           | 11.6-16.5         | 7.9                                         | 7.3                                           |
| **Liver assessment**          |                   |                                             |                                               |
| AST (IU/L)                    | 83-120            | 57                                          | 54                                            |
| N: 17-27 IU/L                 |                   |                                             |                                               |
| ALT (IU/L)                    | 81-148            | 50                                          | 59                                            |
| N: 11-26 IU/L                 |                   |                                             |                                               |
| ALP (IU/L)                    | 100-110           | 92                                          | 102                                           |
| N: 35-105 IU/L                |                   |                                             |                                               |
| GGT (IU/L)                    | 170-320           | 58                                          | 67                                            |
| N: 8-36 IU/L                  |                   |                                             |                                               |
| Steatosis (SteatoTest®)       | low-grade (S1)    | not detectable (S0)                         | low-grade (S1)                                |
| Fibrosis (FibroTest®)         | intermediate grade (F1-F2) | not detectable (F0) | not detectable (F0) |
| Necrotic and inflammatory activity (ActiTest®) | intermediate grade (A1-A2) | very low grade (A0-A1) | low grade (A1) |
| **Lipid profile**             |                   |                                             |                                               |
| Triglycerides (mmol/L) (N: 0.4-1.7 mmol/L) | 1.3 – 2.7         | 2.0                                         | 1.6                                           |
| **Insulin requirement**       |                   |                                             |                                               |
| Human insulin (daily doses – IU/kg) | 2.9       | 2                                           | 1.65                                          |
Supplemental data for

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Figure S1. Partial protein alignment of human EPHX1 across species.

Figure S2. Sorting of GFP+ 3T3-L1 pre-adipocytes transfected with Cas9/scramble gRNA expression vector.

Figure S3. Sorting of GFP+ 3T3-L1 pre-adipocytes transfected with Cas9/Ephx1 gRNA expression vector.

Figure S4. Expression of EPHX1 in primary fibroblasts from patient 1.

Figure S5. Comparative morphology analysis of primary fibroblasts from patient 1 and controls.
Figure S1. Partial protein alignment of human EPHX1 across species. Partial protein alignment of human EPHX1 (GenBank accession NP_001129490.1) with corresponding sequences in other species: macaque, NP_001245016.1; cat, XP_011289115.1; mouse, NP_034275.1; zebrafish, NP_957362.1; Xenopus, NP_001121449.1). Residues Thr333 and Arg430 are indicated by a star. Black shading indicates identical residues, and gray shading indicates semi-conserved residues.
Figure S2. Sorting of GFP+ 3T3-L1 pre-adipocytes transfected with Cas9/scramble gRNA expression vector. (A) Gate based of light scattering forward area and side area was first established on all particles detected graph. (B-C) Two sequential doublet exclusion gating were performed on 3T3-L1 gate. (D-E) Two representative graphs of GFP+ cells. The sorting was performed on GFP+ gate (3T3-L1/doublet exclusion) of the forward area and 488-526/52 height-Log and frequencies of GFP+ cells are depicted.
Figure S3. Sorting of GFP+ 3T3-L1 pre-adipocytes transfected with Cas9/Ephx1 gRNA expression vector. (A) Gate based of light scattering forward area and side area was first established on all particles detected graph. (B-C) Two sequential doublet exclusion gating were performed on 3T3-L1 gate. (D-E) Two representative graphs of GFP+ cells. The sorting was performed on GFP+ gate (3T3-L1/doublet exclusion) of the forward area and 488-526/52 height-Log and frequencies of GFP+ cells are depicted.
Figure S4. Expression of EPHX1 in primary fibroblasts from patient 1. EPHX1 expression was evaluated by Western blotting in cultured fibroblasts from patient 1 and two controls (T1 and T2). Tubulin was used as a loading control.
Figure S5. Comparative morphology analysis of primary fibroblasts from patient 1 and controls. Fibroblasts from patient 1 and two controls (T1 and T2) were cultured and observed at a low passage number. Patient 1-derived fibroblasts exhibited abnormal morphology compared with controls, as shown by their enlarged size and irregular shape. The bottom panel displays magnifications of the framed areas of the top panel.