FDX2 and ISCU Gene Variations Lead to Rhabdomyolysis With Distinct Severity and Iron Regulation

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Abstract

Background and Objectives
To determine common clinical and biological traits in 2 individuals with variants in ISCU and FDX2, displaying severe and recurrent rhabdomyolyses and lactic acidosis.

Methods
We performed a clinical characterization of 2 distinct individuals with biallelic ISCU or FDX2 variants from 2 separate families and a biological characterization with muscle and cells from those patients.

Results
The individual with FDX2 variants was clinically more affected than the individual with ISCU variants. Affected FDX2 individual fibroblasts and myoblasts showed reduced oxygen consumption rates and mitochondrial complex I and PDHc activities, associated with high levels of blood FGF21. ISCU individual fibroblasts showed no oxidative phosphorylation deficiency and moderate increase of blood FGF21 levels relative to controls. The severity of the FDX2 individual was not due to dysfunctional autophagy. Iron was excessively accumulated in ISCU-deficient skeletal muscle, which was accompanied by a downregulation of IRP1 and mitoferrin2 genes and an upregulation of frataxin (FXN) gene expression. This excessive iron accumulation was absent from FDX2 affected muscle and could not be correlated with variable gene expression in muscle cells.

Discussion
We conclude that FDX2 and ISCU variants result in a similar muscle phenotype, that differ in severity and skeletal muscle iron accumulation. ISCU and FDX2 are not involved in mitochondrial iron influx contrary to frataxin.

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Rhabdomyolysis (RM) is an acute injury of skeletal muscle that results from environmental causes or from inherited diseases. In the latter case, RMs are triggered by a catabolic state such as a febrile illness, fasting, and/or effort. Mitochondrial oxidative phosphorylation (OXPHOS) deficiencies are a rare cause of RM, including variants in mitochondrial DNA or in nuclear genes, especially ISCU (iron-sulfur [Fe-S] cluster scaffold homolog, OMIM #611911) and FDX2 (OMIM #614588) ensuring OXPHOS electron transfer and lipoic acid synthesis.

Deleterious variants in ISCU and FDX2 perturb the assembly of mitochondrial Fe-S clusters. Contrary to other diseases involving the Fe-S cluster pathway and leading to multisystem abnormalities, the clinical picture of patients with ISCU or FDX2 variants is mainly limited to skeletal muscle. Patients related with ISCU variants of northern Swedish descent, presented with a myopathy characterized by a progressive muscle weakness, frequent ptosis, RM since childhood, and lactic acidosis, worsened by low-level exercise. The clinical phenotype of a reported FDX2 patient is similar to ISCU phenotype. More recently, FDX2 variants were reported in several patients with optic atrophy and reversible leukoencephalopathy. Biochemical investigations in cells deficient for either ISCU or FDX2 revealed a distinctive deficiency of several mitochondrial Fe-S proteins (complex I, II, and III of the electron transport chain and mitochondrial aconitase). Skeletal muscle histology was normal in the FDX2-deficient patient having RM, whereas iron overload was shown in ISCU-deficient myofibers and in FDX2-deficient muscle fibers from the patients with neurologic presentation.

Here, we report variants in the genes FDX2 and ISCU in 2 distinct individuals who presented with recurrent episodes of severe RM. We compared biological alterations in skeletal muscle and myoblasts of both individuals, a few data of the FDX2 patient being already described. We investigated autophagy and the expression of genes involved in iron regulation.

Methods
Informed consent was obtained from both individuals after obtaining the ethics approval to work on human samples by the Comité pour la protection des personnes of the University Paris XI (2016) and the declaration of human myoblasts to the Département de la Recherche Clinique et du Développement. Myoblasts from P1 were obtained from skeletal muscle biopsy from the deltoid region and 3 control individuals from the paravertebral region. Isolation and growth of myoblasts was performed as previously described. No myoblasts were available for P2.

Genetic Analysis
White blood cells from the affected individuals and their parents were used a source of DNA after informed consent. Genomic DNA (1 μg) extracted from individual (P1) and his parents was analyzed by whole-exome sequencing as previously reported. For P2, and to exclude known metabolic and calcic genes involved in RM for P1, a panel of genes dedicated to RM was studied as previously described. Bioinformatic analysis was configured for identification of qualitative and quantitative variations.

Pathogenic variants in FDX2 and ISCU were confirmed by using the Sanger method with specific primers. The PCR products were sequenced and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1; Applied Biosystems, Foster City, CA) and then analyzed with SeqScape software (version 3.0; Applied Biosystems).

Myopathologic Study
For each patient, 7-μm cryostat cross-section from deltoid muscle samples was performed for routine histologic staining (hematoxylin-eosin, oil red, periodic acid–Schiff, Gomori trichrome, succinyl dehydrogenase [SDH], and cytochrome c oxidase). In addition, Perl’s staining was used for iron (Fe(II)) visualization. Automated immunochemistry for regenerating myofibers (CD56/NCAM) (clone #NCL-CD56-1B6, 1/100; Novocastra, Antony, France) and complement activation (MAC/C5b-9) (clone #ab5811, 1/50; Dako, Glostrup, Denmark) were performed using Bond-III automaton (Leica, Nanterre, France).

Electron Microscopy
Samples were fixed in glutaraldehyde (2.5%, pH 7.4), followed by postfixation in osmium tetroxide (2%) and embedded in resin (EMBed-812; Electron Microscopy Sciences, Hatfield, PA). Ultra-thin sections were stained with lead citrate and uranyl acetate, observed using a Philips CM120 electron microscope (Philips, Amsterdam, the Netherlands) and recorded with a Morada camera (Soft Imaging System, Münster, Germany).

Gene Expression Analysis
Total RNA from skeletal muscle was obtained by TRIzol extraction (Invitrogen, Waltham, MA). Complementary DNA was synthesized from 1 μg of total RNA by means of the High Capacity RNA-to-cDNA Kit (Applied Biosystems) after translation.
depleting genomic DNA. The expression of IRP1, mitoferrin2, and frataxin (FXN) genes in skeletal muscle was assessed by quantitative reverse transcription PCR using Power SYBR Green PCR Master Mix, using β-actin as a housekeeping gene. The RQ value was equal to 2^ΔΔct where ΔΔct is calculated by (Ct target − Ct β-actin) test sample − (Ct target − Ct β-actin) calibrator sample. Each value was derived from 3 technical replicates. The probes were as follows: FXN: FW: 5'-cctgcagacaagcataca-3', RV: 5'-ccactg-gatggagaagatag-3'; IRP1: FW: 5'-tgcttcctcaggtgattggctaca-3', RV: 5'-tagctcggtcagcaatggacaact-3'; mitoferrin2: FW: 5'-ctgcgtgatgtaccccatcg-3', RV: 5'-cctgttgctgtgacgttcag-3'; and β-actin FW: 5'-cagcggaaccgctcattgccaatgg-3', RV: 5'-tcacccacactgtgcccatctacga-3'.

**OXPHOS Measurement**

Mitochondrial respiratory chain complex activities were measured in skeletal fibroblasts by standard spectrophotometric assays.24

**Oxygen Consumption Rate Measurements**

The assays were performed as previously described,19 using 2 x 10^6 myoblasts/well in XF96 plates (Seahorse Bioscience) to evaluate the cellular O2 consumption in a Seahorse Bioscience XF96 extracellular flux analyzer.

**Mitochondrial Enrichment of Myoblasts and Fibroblasts**

Approximately 2 x 10^6 cells were trypsinized, washed in cold 1x phosphate-buffered saline, and lysed in buffer A (250 mM saccharose, 20 mM Tris-base, 40 mM KCl, and 2 mM EGTA pH 7.2) supplemented with 1 mg/mL bovine serum albumin (BSA), 10% wt/vol Percoll, and 0.01% wt/vol digitonin for 10 minutes on ice. Cells were spun down 5 minutes at 5,000g in a tabletop centrifuge. The pellet was harvested and washed in buffer A + 1 mg/mL BSA, further spun down, and finally lysed in buffer M (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 5 mM EDTA, and 1% Triton-X100) 20 minutes on ice. Enriched mitochondrial membranes were harvested by centrifugation at 10,000g for 10 minutes at 4°C and finally boiled in sample buffer with reducing agent.

**Western Blotting**

Lysis of myoblasts and fibroblasts was performed as previously described.19 Primary antibodies anti-LC3-B (clone 4E12, 1:1,000; MBL International, Woburn, MA) and anti-β-actin (sc-81178; Santa Cruz Biotechnology, Dallas, TX) were detected with horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescence detection. Lipoic acid (Abcam ab18724 1:800) and SDH-A (Abcam ab14715 1:2,000) were used after mitochondrial enrichment as described above.

**FGF-21 Measurements**

Plasma from 4 healthy controls and FDX1L and ISCU-deficient individuals was used to perform ELISA measurements (Quantikine ELISA, Human FGF-21; R&D Systems, Bio-Techne, Minneapolis, MN) as previously described.19

**Data Availability**

Cells and raw data sets are available on reasonable request.
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They obtained their general bachelor's degree. They have recurrent RM episodes precipitated by febrile illnesses, fasting, exercise, cold, or fatigue, sometimes without trigger, associated with palpitations and dyspnea related to lactic acidosis. The first episode occurred at age 6 years in P1 and 14 years in P2, associated with lactic acidosis. The presenting symptoms were generalized weakness, inability to walk, myalgia, polypnea, and dark urine. The reflexes were usually preserved. The CNS was typically spared during the episodes. In P1, the number of RMs was 2–3 per year until age 12 years; then, the frequency has gradually decreased, without recurrence since age 18 years. Renal failure was frequently associated. Cardiac arrest occurred in 1 episode. P2 presented with 9 episodes of RM since age 14 years. At disease onset, a facial edema with transient malar erythema was associated with P2 RM. Both patients displayed 1 episode of pulmonary embolism at age 18 years. During acute RM, plasma creatine kinase (CK) levels were markedly elevated (maximum peak levels 105,000 U/L in P1, 200,000 U/L in P2, N <200 U/L) with overt myoglobinuria. Lactate dehydrogenase and aspartate aminotransferase were concomitantly elevated (data not shown) as well as lactate concentrations in blood (4–23 mmol/L; N <2.2 mmol/L) and acidosis (i.e., pH 6.80). During the episodes, treatment with IV fluids and alkalinization was proposed, associated in a few cases with dialysis.

Between episodes, the individuals presented generalized weakness, more pronounced in P1 who had muscle pain worsened by mild effort, predominantly in the lower limbs, an amyotrophy, and a mild muscle deficit. The walking perimeter was 100–150 m without assistance for P1, and longer for P2, who was able to go to school by herself. CK levels were normal or subnormal in both individuals. Sensory and motor nerve conductions were normal, and needle EMG showed a myogenic pattern in both patients. The P1 leg muscle MRI showed fatty infiltration at age 12 years. Echocardiogram was normal in both patients. Cardiopulmonary exercise tests were performed in P1 and P2 at age 18 years. Both tests were maximal, as suggested by the ventilatory ratio >1.1 and the effort intensity score (on the modified Borg scale graduated from 0 to 10) >8. P1 reached a peak oxygen uptake equal to 5.8 mL/kg/min (18% of theoretical value), whereas P2 reached an oxygen uptake equal to 12.7 mL/kg/min (40% of theoretical value), indicating a much more impaired adaptation of peripheral muscle in P1.

Amino acid analysis in P1 plasma showed constant elevation of alanine and mild decrease of branched chain amino acids, whereas it was normal in P2 plasma. In urine, lactate and alpha-ketoglutarate were detected at higher levels in P1 than in P2. Normal results were obtained for total and free carnitine and blood acylcarnitine profile.

In P1, the administration of coenzyme Q10 and vitamins including riboflavin and thiamine did not prevent RM. In P2, administration of steroids for 4 months and methotrexate for an initial clinical suspicion of dermatomyositis did not improve the patient. Her antibodies were negative (RaCh, MuSK, and MSA).

**Muscle Pathology**

In P1, a first muscle biopsy realized at age 6 years was considered as normal in terms of pathologic findings (data not shown). A second deltoid biopsy performed at age 18 years (Figure 1) revealed slight mitochondrial (fuchsinophile) subsarcolemma overload (A), an important lipid accumulation (B), a predominance of fibers type 1 (C), an absence of...
cytochrome c oxidase coloration in a few fibers (D), an almost complete absence of SDH enzyme activity in muscle fibers (E), and no iron deposit in Perls coloration (F). Ultrastructural study showed the presence of abnormal mitochondria containing palisade crystals, proliferation of mitochondrial crests with osmiophilic inclusions in some mitochondria, and numerous lipid droplets (G–I).

In P2, deltoid muscle biopsy performed at age 18 years (Figure 2) showed mild myopathic changes with centronucleated fibers and perifascicular basophilic fiber (A).

Molecular Investigations
We first excluded fatty acid oxidation defects by biochemical investigation in both individuals (data not shown). Subsequently,
The lipoylation levels of the E2 subunit of PDHc and α-KGDHc were not altered in fibroblasts of both individuals (Figure 3A, right panel) in agreement with a previous report on P1 fibroblasts.18 No difference was observed in both individuals, deficient for either FDX1L or ISCU proteins. Importantly, the lipoylation normality was not due to the choice of the tissue as it was also normal in FDX2-deficient myoblasts (Figure 3A, left panel).

Oxygen consumption rates decreased in FDX2-deficient myoblasts, even in basal conditions (Figure 3B). These results correlated with the decreased oxygen uptake by peripheral muscle during the effort test that was worsened in P1 than in P2 and with the result of FGF-21 measured in basal condition that was very elevated in plasma of P1 and moderately increased in P2 (Figure 3C). These observations strongly suggest a greater respiratory capacity failure downstream of mutated FDX2 than mutated ISCU.

**FDX2 and ISCU Variants Cause Mitochondrial Dysfunction**

Considering the key role of both genes in mitochondrial homeostasis, we suspected that key mitochondrial functions might be perturbed in the absence of functional FDX2 and ISCU protein. Therefore, we investigated and compared when possible different mitochondrial tests including respiratory chain enzymes by spectrophotometry, mitochondrial lipoylated proteins by immunoblotting, oxygen consumption by the Seahorse technology in patient myoblasts, and FGF-21.29

The enzymatic activities of the mitochondrial respiratory chain complexes were realized in fibroblasts of both patients (P1 with FDX2 variants and P2 with ISCU variants). The results showed a moderated decrease of complex I activity and lipoylated PDHc in fibroblasts of P1, whereas complex II, complex III, α-KGDHc, and aconitase activities were normal.18 In fibroblasts of P2, results were normal for all mitochondrial complexes and for α-KGDHc activity (data not shown).

The lipoylation levels of the E2 subunit of PDHc and α-KGDHc were not altered in fibroblasts of both individuals (Figure 3A, right panel) in agreement with a previous report on P1 fibroblasts.18 No difference was observed in both individuals, deficient for either FDX1L or ISCU proteins. Importantly, the lipoylation normality was not due to the choice of the tissue as it was also normal in FDX2-deficient myoblasts (Figure 3A, left panel).

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**Variants in FDX2 Individuals Do Not Cause Defective Mitophagic Clearance**

We speculated that FDX2 and ISCU diseases might perturb mitophagy, as adenosine triphosphate (ATP) is required for initiation and progression of macroautophagy30 and for degradation of soluble cytosolic proteins in lysosomes.31 Thus, we examined the consequence of a loss of FDX2 in the activity of autophagy, by determining the regulation of the autophagosomal marker LC3 level in basal conditions, starvation (Earle’s balanced salt solution [EBSS]), and refeeding. Immunoblot revealed the presence of LC3 II staining in fasting condition (EBSS) in control and patient myoblasts deficient for FDX2, suggesting correct induction of autophagy (Figure 3, D and E). Moreover, the accumulation of LC3-II was similar after blocking autophagosome degradation by bafilomycin, and LC3 II level normalized in individuals’ myoblasts after refeeding. Thus, autophagic flux was not altered by the absence of FDX2.

**Iron Pathway**

The ISCU-deficient skeletal muscle (P2) showed a histology pattern of iron overload, whereas the FDX2-deficient muscle (P1) did not. Iron is transported into cells on its ferric form (Fe³⁺) after binding to the cytosolic transferrin receptor. Transcriptional regulation of iron-responsive proteins was investigated in skeletal muscle (P2) or myoblasts (P1) relative to controls, including IRP1 (aconitase 1 that regulates import of iron into cells by modulating transferrin), mitoferrin2 (a mitochondrial iron transporter that mediates iron transport across the mitochondrial inner membrane), and FXN (a mitochondrial protein involved in assembly of Fe-S clusters and in iron storage protein). In agreement with the iron overload in P2, mitoferrin2 and IRP1 gene expression was downregulated in P2’s skeletal muscle relative to 2 control muscles, at the same time that FXN gene expression was upregulated (Figure 3F). In the absence of P1 skeletal muscle, we performed similar experiments in myoblasts from P1 (Figure 3G), where we found no significant up- or down-regulation of any of the above genes.

**Discussion**

De novo synthesis of the 2Fe-2S cluster is accomplished on the scaffold protein ISCU with the help of FDX2 and FXN that form a dynamic complex with ISCU.36,37 In humans, ISCU deficiency has been widely reported, whereas FDX2 variants have been reported at least twice. Contrary to other diseases involving the ISC assembly machinery, the phenotype resulting from variants in either ISCU or FDX2 is mainly restricted to skeletal muscle.8,12 Here, we report 2 individuals with recessively inherited variants in either ISCU or FDX2 genes, with recurrent episodes of RM associated with muscle weakness and lactic acidosis. Except the onset of RM and a ptosis noted in P2, the clinical phenotype was similar in both individuals, although more severe in FDX2-deficient patient.
According to the clinical phenotype, the biochemical phenotype and the effort test were also more severe in FDX2 deficiency than in ISCU deficiency, with an increase of blood FGF-21 that was more pronounced in P1 than in P2 and a greater decrease of oxygen uptake by peripheral muscle during effort tests. We also found a decrease in mitochondrial respiratory chain complex I and PDHc activities in P1 fibroblasts. The mitochondrial respiratory chain complexes II and III were normal in fibroblasts from both individuals, although they were markedly decreased in previously reported patients with FDX2 or ISCU deficiency. In P1 myoblasts, basal respiration, spare respiratory capacity, and ATP production were severely reduced compared with healthy controls, strengthening the observations obtained previously in fibroblasts. On that report, we showed that unexpectedly, PDHc-E2 and α-KDHc-E2 were correctly lipoylated in the absence of FDX2, as opposed to several other Fe-S cluster-forming proteins. Here, we verified that finding, extended it to ISCU-deficient fibroblasts, and verified tissue independence by showing correct lipoylation in FDX2-deficient myoblasts. If lipoylation is considered as a proxy of ISC maturation, these data imply that both ISCU and FDX2 are redundant in Fe-S biogenesis. However, P1 has a severe phenotype, whereas her sister bearing the same variation already died. A possible explanation may rely on the second ferredoxin FDX1, which is also important for ISC biogenesis. Concerning ISCU protein, almost absent in muscle tissue from ISCU-deficient patients, it was present in other examined tissues. Therefore, the tissue specificity in ISCU myopathy can be attributed to several factors, including tissue and differentiation-specific abnormal mRNA splicing that our patient shares.

Alternatively, and beyond tissue specificity, both ISCU and FDX2 might have another function in addition to ISC biogenesis. Indeed, oxidative stress destabilizes the small amounts of normal ISCU protein generated in patient’s skeletal muscles. On that line, we suspected that mitophagy, a selective form of autophagy that specifically targets mitochondria through autophagolysosomal activity in response to the stresses, could also be affected in the absence of ISCU or FDX2. Indeed, mitophagy can be impaired as ATP is required for initiation and progression of macroautophagy or for degradation of soluble cytosolic proteins in lysosomes. Moreover, our ongoing investigations indicate that autophagy deficiency is linked with development of RM events in other genetic backgrounds. Unfortunately, we failed to see any alteration in autophagic flux of FDX2-deficient myoblasts, indicating that autophagy alteration is not a core molecular marker for RM events. In the absence of P2 myoblasts, we cannot extend the same statement to ISCU, although it is unlikely to have a major autophagic defect in a relatively milder disease scenario.

Another difference between both individuals with variants in either ISCU or FDX2 genes was the muscle biopsy findings. Both showed a specific decreased SDH coloration, but only ISCU myopathy was associated with an iron load. Under normal conditions, on increase in iron concentration, the mRNA synthesis of the transferrin receptor is downregulated and vice versa. IRP1 (aconitase 1) controls import of iron in cells by modulating transferrin, whereas mitoferrin2, a mitochondrial iron transporter, mediates iron transport across the mitochondrial inner membrane. The intracellular iron overload in ISCU (P2) skeletal muscle was associated with a decrease in IRP1, as already reported and a decrease of mitoferrin2 gene expression that is in contradiction with another study. These results are consistent with a muscle-specific alteration of iron homeostasis in this disease compared with FDX2 disease. Whether gene regulation is a cause or a consequence cannot be established from our experiments. However, we favor the hypothesis of an initial iron load coming from the extracellular milieu that cannot be passed through the ISC biogenesis chain; in turn, iron will accumulate inside the cell, thereby downregulating genes necessary for iron import. Compared with P2, we found that in P1 myoblasts, there was no modification of IRP1 or mitoferrin2, confirming the absence of iron accumulation in skeletal muscle of this patient.

Like in P1 myoblasts in which FXN tended to be upregulated, there is a clear upregulation of such gene in P2 muscle cells. FXN is a mitochondrial protein involved in assembly of Fe-S clusters through its interaction with the ISCU-NFS1-ISD11 complex, but also in iron storage or as an iron chaperone. Its deficiency leads to ISC deficiency, mitochondrial dysfunction, and major mitochondrial iron accumulation. Because FXN binds ISCU and stimulates persulfide transfer to ISCU, it might act as a compensatory protein to fulfill the demand for Fe-S biogenesis, altered in case of ISCU deficiency. It might be interesting to know whether in Friedreich ataxia (FRDA), where FXN expression is insufficient, ISCU might play a compensatory effect. In the case of FDX2, FXN might also mildly compensate for its absence, although from our experiments, this is not totally evident despite a trend. However, since FDX2 intervenes only after FXN in the ISC machinery, it could be expected that FXN does not compensate FDX2 deficiency. Of interest, the increased iron uptake in FRDA is related to marked transferrin receptor 1 and mitoferrin2 upregulations that facilitate mitochondrial iron influx and a decreased expression of the iron exporter, ferroportin 1, leading to decreased cellular iron flux. Thus, we can conclude by our findings that ISCU deficiency and FRDA differ by the expression of IRP1.

In conclusion, variants in FDX2 and ISCU genes lead to a similar phenotype associating RM, muscle weakness, and lactic acidosis, which is more severe in the FDX2 individual. The phenotype is partially related as a common energy deficiency, with P1 showing more mitochondrial-derived inflammation (FGF-21) but no iron accumulation and functional autophagy, whereas P2 shows iron accumulation followed by a regulation of iron regulatory genes, and mild FGF-21 production. Our data confirm that FDX2 is not
involved in mitochondrial iron influx contrary to FXN. In ISCU deficiency, IRP1 downregulation and FXN upregulation also suggest that ISCU is not involved in mitochondrial iron influx. Further studies should focus on the potential effects of iron chelators to modulate ISCU absence.

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Disclosure

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Appendix

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<td>Analysis or interpretation of data and OXPHOS studies</td>
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Appendix

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<tr>
<td>Sebastian Montealegre, PhD</td>
<td>Inserm U1151, Institut Necker Enfants-Malades, Paris, France</td>
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</tr>
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<tr>
<td>François Jérôme Authier, MD, PhD</td>
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References


FDX2 and ISCU Gene Variations Lead to Rhabdomyolysis With Distinct Severity and Iron Regulation
Sebastian Montealegre, Elise Lebigot, Hugo Debruge, et al.

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