Tissue-specific responses to the LRPPRC founder mutation in French Canadian Leigh Syndrome

Florin Sasarman†, Tamiko Nishimura†, Hana Antonicka, Woranontee Weraarpachai and Eric A. Shoubridge*, LSFC Consortium

Montreal Neurological Institute and Department of Human Genetics, McGill University, Montreal, QC H3A 2B4, Canada

Received June 30, 2014; Revised August 1, 2014; Accepted September 9, 2014

French Canadian Leigh Syndrome (LSFC) is an early-onset, progressive neurodegenerative disorder with a distinct pattern of tissue involvement. Most cases are caused by a founder missense mutation in LRPPRC. LRPPRC forms a ribonucleoprotein complex with SLIRP, another RNA-binding protein, and this stabilizes polyadenylated mitochondrial mRNAs. LSFC fibroblasts have reduced levels of LRPPRC and a specific complex IV assembly defect; however, further depletion of mutant LRPPRC results in a complete failure to assemble a functional oxidative phosphorylation system, suggesting that LRPPRC levels determine the nature of the biochemical phenotype. We tested this hypothesis in cultured muscle cells and tissues from LSFC patients. LRPPRC levels were reduced in LSFC muscle cells, resulting in combined complex I and IV deficiencies. A similar combined deficiency was observed in skeletal muscle. Complex IV was only moderately reduced in LSFC heart, but was almost undetectable in liver. Both of these tissues showed elevated levels of complexes I and III. Despite the marked biochemical differences, the steady-state levels of LRPPRC and mitochondrial mRNAs were extremely low, LRPPRC was largely detergent-insoluble, and SLIRP was undetectable in all LSFC tissues. The level of the LRPPRC/SLIRP complex appeared much reduced in control tissues by the first dimension blue-native polyacrylamide gel electrophoresis (BN-PAGE) analysis compared with fibroblasts, and even by second dimension analysis it was virtually undetectable in control heart. These results point to tissue-specific pathways for the post-transcriptional handling of mitochondrial mRNAs and suggest that the biochemical defects in LSFC reflect the differential ability of tissues to adapt to the mutation.

INTRODUCTION

Deficiencies in oxidative phosphorylation (OXPHOS) are associated with a diverse array of multisystem disorders that are often referred to as mitochondrial encephalomyopathies because of the prominent involvement of the nervous system and striated muscle (1–3). The molecular basis for the extraordinarily broad clinical spectrum observed in OXPHOS disorders remains an enduring mystery. Mutations in mtDNA are the most frequent cause of mitochondrial disease in adults and more than 100 such pathogenic mutations have been identified. In the pediatric population, the majority of OXPHOS disorders (~80%) are transmitted as autosomal recessive traits, usually with a severe phenotype and a fatal outcome.

Leigh syndrome is one of the most common presentations in this population. First described by Denis Leigh in a single case (4), the so-called classical form of the disease is characterized by early-onset neurodegeneration, particularly in the brain stem and basal ganglia. The genetic underpinnings of this disorder are found in structural subunits of the OXPHOS complexes, associated assembly factors or in pyruvate dehydrogenase (5,6). A variant known as Leigh Syndrome French Canadian (LSFC) has a characteristic pattern of tissue involvement and a different clinical evolution, with acute acidotic crises usually leading to very early fatality (7–9). This disorder is caused by a founder missense mutation in LRPPRC, a gene coding for a pentatricopeptide repeat (PPR) family protein (10). PPR proteins, which belong to the alpha-solenoid protein family, have undergone a huge radiation in land plants where they number several hundred (11). Virtually all of them localize to mitochondria or chloroplasts where they mediate a large variety of RNA transactions including splicing, editing, stability and translation.
initiation. A combinatorial code that specifies the recognition of specific RNA substrates has been determined for the chloroplast PPR protein PPR10 (12), a result that was substantiated by the solution of the crystal structure of the protein bound to its mRNA substrate (13). Humans have only seven identified PPR proteins, all of which are mitochondrial (14).

LRPPRC forms a ribonucleoprotein (RNP) complex of about 250 kDa with the stem-loop RNA-binding protein SLIRP, and this complex appears necessary to stabilize the majority of polyadenylated mitochondrial mRNAs (15–17), protecting them against 3’ exoribonuclease digestion (16). In vitro studies with purified LRPPRC also show a stimulation of polyA addition to RNA substrates, suggesting that the complex might play a dual role in the synthesis and maintenance of the 3’ ends of mitochondrial mRNAs (16). The protein carrying the A354V substitution subunits (15). These data suggest that tissue-specific differences assembly defect in all OXPHOS complexes with mtDNA-encoded al; however, further siRNA-mediated depletion results in an as-

LSFC cultured muscle cells have a combined OXPHOS deficiency

To investigate the nature of the biochemical phenotype in another cell type, we cultured myoblasts obtained from two LSFC cases, differentiated them into myotubes, and immunoblotted for LRPPRC and SLIRP. Similar to LSFC fibroblasts, the level of mutant LRPPRC was reduced to 20–30%, and the levels of SLIRP, to 10–20% of control in LSFC myoblasts and myotubes (Figs 2A and 3A). The synthesis of the COX subunits was particularly affected in LSFC muscle cells, and an anomalous translation product appeared in post-natal LSFC myoblasts and myotubes (Figs 2B and 3B). However, unlike LSFC fibroblasts, all of the cultured muscle cells from LSFC subjects (fetal and post-natal myoblasts and myotubes) showed a combined OXPHOS defect, with reduced levels of assembled complex I and complex IV as shown by blue-native polyacrylamide gel electrophoresis (BN-PAGE) analysis (Figs 2C and 3C).

To determine whether a further reduction in the steady-state level of LRPPRC would lead to a more severe OXPHOS deficiency in cultured muscle cells (as it does in fibroblasts), we used siRNA to deplete LRPPRC below 10% of control levels in control and LSFC myoblasts and myotubes. This resulted in a generalized OXPHOS defect in which all complexes with mtDNA-encoded subunits were affected, as demonstrated by immunoblot (Fig. 3A), mitochondrial translation (Fig. 3B) and BN-PAGE analyses (Fig. 3C). The smaller molecular weight complex V species in Figure 3C represent F1-containing subcomplexes.

The reduction in steady-state levels of mitochondrial mRNAs does not correlate with the biochemical phenotype in LSFC tissues

Given the role of LRPPRC in binding mature mitochondrial mRNAs, we started our analysis of LSFC tissues by northern blot analysis of the steady-state levels of mitochondrial mRNAs in the skeletal muscle, heart and liver tissues from an LSFC patient (Fig. 4A). In all three tissues, there was a generalized decrease in the levels of all analyzed mitochondrial mRNAs, the most severe reduction in mRNA levels being observed in skeletal muscle. Additionally, an increase in the levels of the 12S mitochondrial ribosomal RNA was observed in the three tested LSFC tissues, and an increase in the 16S mitochondrial ribosomal RNA, in the LSFC heart and liver, presumably indicating a compensatory response to the decrease in mitochondrial mRNA levels. The levels of mitochondrial tRNAs were indistinguishable from control in all three LSFC tissues (Fig. 4B).

Loss of LRPPRC and SLIRP results in tissue-specific OXPHOS deficiencies

BN-PAGE analysis of the LSFC tissues revealed an almost complete lack of assembled complex IV in LSFC liver (not detectable even in overexposed blots), and ~50% of control levels of assembled complex IV in heart (Fig. 5A). Additionally, both of these tissues displayed an increase in the levels of assembled complexes I and III. The differences in intensity of the bands for complexes I, III, IV and V in the control samples reflect real
difference in the relative abundance of these complexes between heart and liver, as the same amount of mitochondrial protein was loaded in all lanes and the level of complex II is similar in all lanes. On the other hand, in skeletal muscle we observed a combined complex I and IV deficiency, similar to that seen in cultured muscle cells (Fig. 5A). This pattern of OHPHOS deficiency was confirmed by immunoblotting cleared mitochondrial extracts from LSFC and control tissues, which showed decreases in the steady-state levels of structural subunits for the same complexes that showed assembly defects by BN-PAGE analysis (Fig. 5B). Despite the tissue-specific OHPHOS defects, mutant LRPPRC and SLIRP were virtually undetectable in all three analyzed LSFC tissues. An immunoblot analysis of the endogenous levels of LRPPRC and SLIRP in cleared mitochondrial extracts from control tissues showed comparable levels, relative to proteins in the OXPHOS complexes, in all three analyzed LSFC tissues (Fig. 5C).

Detergent solubility of LRPPRC and a subset of proteins involved in mitochondrial translation is altered in LSFC liver

Since mutant LRPPRC appeared to be present at levels below immunodetection in LSFC tissues, yet with such tissue-specific biochemical consequences, we asked whether residual LRPPRC might be present in a detergent-insoluble fraction, that is a fraction that could not be extracted with the concentration of the non-ionic detergent dodecyl maltoside (DDM), that we routinely use for immunoblot analysis of proteins, in some or all of these tissues. To test this, mitochondria from LSFC and control tissues extracted in DDM were mixed with the loading buffer [containing sodium dodecyl sulfate (SDS)], electrophoresed by PAGE as usual, and subsequently immunoblotted. As shown in Figure 6A, while LRPPRC was still not detected in total extracts of LSFC muscle, residual mutant protein was present in LSFC heart and liver at ~10% of control levels. It is important to point out that mutant LRPPRC could easily be extracted from fibroblasts or myoblasts with DDM, so this is a tissue-specific and not a technical problem. While the above findings might explain a combined complex I and IV deficiency in LSFC muscle versus an isolated complex IV defect in LSFC heart and liver, it still does not clarify why complex IV deficiency is severe in liver and mild in heart. Hence, we further investigated the distribution of LRPPRC and other proteins involved in mitochondrial translation between the soluble and insoluble fractions following detergent extraction, in LSFC and control liver. Interestingly, while LRPPRC was found predominantly (80–90%) in the supernatant (soluble fraction) in control liver, the residual amount of mutant LRPPRC was detected exclusively in the pellet (insoluble fraction) in the LSFC liver (Fig. 6B). A similar reversal in distribution between supernatant and pellet and, in some cases, a decrease in total amounts was also observed for a subset of proteins involved in mitochondrial translation, including two translation factors (TACO1, EFG1), a mitochondrial ribosomal protein (MRPS27) and the mitochondrial formyltransferase, MTFMT (Fig. 6B). By contrast, another mitochondrial translation factor (CCDC56), the ribosomal protein MRPL44, the mitochondrial peptide deformylase (PDF) and two subunits of OXPHOS complexes (SDHA, ATP5A1) displayed a normal distribution between the soluble

---

**Figure 1.** Knockdown of SLIRP in control fibroblasts phenocopies the mitochondrial translation defect in LSFC fibroblasts. (A) Control fibroblasts were transiently transfected with two different siRNA constructs specific to SLIRP, or with a fluorescent control siRNA (Alexa). On the sixth day post-transfection, the mitochondrial translation products were pulse-labeled with a mix of [35S]-methionine and cysteine in the presence of emetine, an inhibitor of cytoplasmic translation, and analyzed by PAGE. The 13 mitochondrial translation products are indicated to the left of the figure: seven subunits of complex I (ND), one subunit of complex III, three subunits of complex IV (COX) and two subunits of complex V (ATP). The asterisk indicates an anomalous, unidentified translation product typically detected in LSFC fibroblasts. (B) The knockdown of SLIRP and the concomitant reduction in steady-state levels of LRPPRC were confirmed by immunoblotting with specific antibodies. The 70 kDa subunit of complex II was used as a loading control.
and insoluble fractions in the LSFC liver, demonstrating that this was not a general problem of protein aggregation. The mitochondrial apoptosis-inducing factor AIFM1 also showed a control pattern of distribution between fractions following detergent extraction of LSFC liver mitochondria, suggesting that the change in solubility of the subset of proteins in patient tissue is not linked to pro-apoptotic events. The different distribution of LRPPRC between fractions in LSFC liver does not appear to be the consequence of compromised mitochondrial membranes, as transmembrane proteins of the outer (VDAC1) and inner (ATP5A1) membranes were extracted the same way in LSFC and control. Strikingly, SLIRP, which appeared only in the soluble fraction in control tissues, was not detectable by immunoblot analysis in any LSFC tissue.

We confirmed the presence of LRPPRC in the insoluble fraction following DDM extraction of LSFC liver mitochondria by repeating the analysis with two additional controls (Fig. 6C). While a certain percentage of LRPPRC (between 20 and 50%) is associated with the pellet in control liver, in the LSFC liver, the residual, mutant LRPPRC is found exclusively in the

**Figure 2.** Mitochondrial translation defect and combined OXPHOS deficiency in LSFC cultured muscle cells. (A) Immunoblot analysis showing reduced levels of LRPPRC and SLIRP in extracts from fetal and post-natal cultured muscle cells from LSFC subjects. Prohibitin was used as loading control. The sample for LSFC fetal myotubes was slightly under loaded. (B) Mitochondrial translation products in fetal and post-natal myoblasts and myotubes from LSFC subjects were pulse-labeled with a mix of [35S]-methionine and cysteine in the presence of emetine, an inhibitor of cytoplasmic translation and analyzed by PAGE. The 13 mitochondrial translation products are indicated to the left of the figure: seven subunits of complex I (ND), one subunit of complex III, three subunits of complex IV (COX) and two subunits of complex V (ATP). The asterisk indicates a translation product with anomalous migration also detected in LSFC fibroblasts. (C) Blue-native PAGE analysis of the five OXPHOS complexes revealed reduced levels of assembled complex IV (COX) and complex I in cultured muscle cells from LSFC subjects. Individual complexes were detected with subunit-specific antibodies.
pellet. A similar analysis of heart mitochondria revealed that all tested proteins are found predominantly (≏80%) in the insoluble fraction following DDM extraction, in both control and LSFC tissues (data not shown).

**LRPPRC and SLIRP are partially associated with mitochondrial membranes**

The presence of LRPPRC in the insoluble fraction following DDM extraction, even in control mitochondria, prompted us to investigate whether LRPPRC and/or SLIRP are associated with the mitochondrial membranes. While neither of these two proteins is predicted to contain a transmembrane domain, alkaline carbonate extraction of isolated mitochondria revealed that up to 50% of LRPPRC and SLIRP are found in the membrane fraction in HEK293 cells (Fig. 7A) and in fibroblasts, whether control or LSFC (Fig. 7B).

**The levels of mitochondrial small ribosomal subunits and the monosome are decreased in LSFC liver**

The alteration of the detergent solubility of a subset of proteins involved in mitochondrial translation in the LSFC liver, including a protein of the small ribosomal subunit (mt-SSU), MRPS27 (Fig. 6B), might reflect a qualitative or quantitative change of the mitochondrial ribosomes in this tissue. To test this, we performed sucrose gradient centrifugation of cleared mitochondrial extracts from control and LSFC liver. Consistent with the decrease in levels and detergent solubility of MRPS27, the amounts of fully assembled small subunit (mt-SSU) and the monosome were decreased in the LSFC liver, while the levels of assembled large ribosomal subunits (mt-LSU) were normal (Fig. 8A). In contrast, in the LSFC heart mitochondria there was an increase in the steady-state levels of subunits of the small and large ribosomal subunits and of assembled small and large ribosomal subunits.
large ribosomal subunits (Fig. 8B and C), suggesting an adaptive
response to the decrease in the levels of mRNAs in this tissue.

The LRPPRC/SLIRP complex is not detectable in heart
tissue

We next asked whether the LRPPRC/SLIRP complex is prefer-
entially stabilized in the LSFC heart. First dimension blue-native
PAGE analysis showed that $\approx 250$ kDa complex of LRPPRC
and SLIRP was undetectable with antibodies against LRPPRC
in the LSFC liver as compared with control (Fig. 9A), whereas
in the heart, it was undetectable in both LSFC and control mito-
chondria (data not shown). This led us to hypothesize that the
reason why heart is spared in LSFC might be a quantitative or
qualitative difference of the LRPPRC/SLIRP complex in this
tissue, as opposed to liver or muscle. To test this, we analyzed
mitochondria from heart, liver and muscle of two controls, and
from two control fibroblast lines, by the first and second dimen-
sion BN-PAGE. Indeed, the LRPPRC–SLIRP complex was
undetectable in the heart of both controls in the first dimension
and was greatly reduced in muscle or liver as compared with
fibroblasts (Fig. 9B). This contrasts with the levels of assembled
complex IV, which are highest in heart and muscle, and lowest
in liver and fibroblasts (Fig. 9B and (19)). Additionally, the
LRPPRC–SLIRP complex in muscle migrates at a slightly
higher molecular mass than in the other analyzed tissues and
cells, suggesting it might have different properties or composition.
Heart was the only tissue in which LRPPRC and SLIRP were
equally undetectable in the second dimension (Fig. 9C). These
observations suggest that heart has alternative pathways to the
LRPPRC/SLIRP complex for post-transcriptional handling of
mitochondria-encoded proteins.

DISCUSSION

The results presented in this study demonstrate striking differences
in the post-transcriptional handling of mitochondrial mRNAs both
among tissues and between cultured cells and fully differentiated
tissues. In fibroblasts, myoblasts and differentiated myotubes,
we show that the LRPPRC–SLIRP complex is essential for mito-
chondrial translation and, as a consequence, assembly of all
OXPHOS complexes containing mtDNA-encoded structural sub-
units. Reducing the level of LRPPRC to levels undetectable by
immunoblot analysis abolishes translation and completely abro-
gates assembly of a functional respiratory chain. Depletion of
SLIRP phenocopies the translation defect, emphasizing the inter-
dependence of the two proteins. What was unexpected, and
remains to be explained, is that despite very similar translation
defects, LSFC fibroblasts and muscle cells have quite distinct bio-
chemical phenotypes, the latter showing a marked reduction in
complex I assembly, similar to that observed in patients with iso-
lated complex I deficiencies, in addition to a complex IV assembly
defect. This suggests that the basis for the different biochemical
defects lies downstream of mitochondrial translation.

What is most extraordinary, however, is the fact that in the
three LSFC tissues that we were able to study, the steady-state
levels of the mutant form of LRPPRC were extremely low, the
protein was largely detergent-insoluble and SLIRP was un-
detectable (Note that we have no evidence that tissue-specific
isoforms or paralogues exist for either protein). Despite this
rather severe and relatively homogeneous phenotype, it seems
evident that many of the mitochondrial mRNAs are translated
to a degree that underwrites normal (or increased as for com-
plexes I and III in heart and liver) assembly of some of the
OXPHOS complexes, but that marked tissue-specific deficien-
cies persist. This occurs despite the fact that the steady-state
levels of the mRNAs were severely reduced in all three tissues, as might have been predicted from the results we obtained in fibroblasts showing the interdependence of LRPPRC, SLIRP and mRNA (15). The only overt phenotype reported in a recent study of a mouse germ line deletion of SLIRP was a small decrease in male fertility, although the study did not include any biochemical analyses of OXPHOS complex assembly or function (20). It thus appears that SLIRP may not be an essential component of the molecular machinery involved in the processing and handling of mitochondrial mRNAs, or that some component(s) of the translation can compensate for its absence. In any case, we were not able to convincingly demonstrate the presence of the LRPPRC/SLIRP complex in control heart tissue by either first or second dimension BN-PAGE analysis.

Figure 5. Tissue-specific OXPHOS deficiencies in LSFC tissues. Mitochondrial extracts from muscle, heart and liver of one LSFC subject, several controls and four disease controls were analyzed by BN-PAGE (A) and by western blotting (B) with antibodies against subunits of the OXPHOS complexes or against LRPPRC and SLIRP, as indicated. In (A), ‘Co I deficiency’ refers to two subjects with assembly defects of complex I, in (B), ‘COX15’ and ‘EFG1’ refer to two subjects with mutations in an assembly factor of COX and a mitochondrial translation factor, respectively. (C) Cleared mitochondrial extracts from control liver, heart and muscle were analyzed by immunoblotting with antibodies against LRPPRC, SLIRP or subunits of the OXPHOS complexes.

Figure 6. Altered detergent solubility of LRPPRC and of other mitochondrial translation proteins in LSFC liver. (A) Mitochondria from muscle, heart and liver samples of the LSFC subject and controls were extracted in dodecyl maltoside, then mixed with loading buffer containing SDS without extract clearing by centrifugation, followed by immunoblot analysis with antibodies against LRPPRC or the 70 kDa subunit of complex II. (B, C) Mitochondria isolated from liver samples of one LSFC subject and controls were extracted in dodecyl maltoside or taurodeoxycholate (for TACO1 detection), then loading buffer containing SDS was added to the extracts without extract clearing by centrifugation (‘Input’). Duplicate samples were centrifuged following extraction in dodecyl maltoside or taurodeoxycholate, and each soluble (‘Supernatant’) and insoluble (‘Pellet’) fraction was then mixed with loading buffer containing SDS. All samples were analyzed by immunoblotting with antibodies against LRPPRC, SLIRP, mitochondrial ribosomal proteins (MRPS27, MRPL44), factors or enzymes involved in mitochondrial translation (EFG1, TACO1, MTFMT, PDF, CCDC56) and other mitochondrial proteins, as detailed in the text.
These data lead us to two main conclusions. First, our data show that the way in which mitochondrial mRNAs are handled is tissue-specific. Although LRPPRC does not contain a predicted transmembrane domain, a significant fraction of it stays in the pellet after alkaline carbonate extraction in HEK cells and fibroblasts, and most of it remains DDM-insoluble in heart, but less so in liver. This suggests that LRPPRC may shuttle between a soluble matrix fraction and an inner membrane fraction that might depend on its association with SLIRP, and that this equilibrium shifts in some tissues in the presence of the mutant protein. The fact that the LRPPRC/SLIRP complex was more difficult to detect in the first versus second dimension BN-PAGE gels in muscle and liver, suggests that the epitope recognized by the LRPPRC antibody is less accessible in the native complex in tissues, perhaps because of a post-translational modification, or the presence of another protein in the complex, but this remains to be investigated.

Second, it seems clear that some tissues have the ability to adapt to the LRPPRC mutation better than others. The increase in the total number of monosomes in the heart is presumably a response to increase the efficiency of translation at lower steady-state levels of mRNAs. This signaling does not appear to function in the liver, as there were fewer monosomes in LSFC liver, due to a decrease in the assembled mt-SSU. We previously reported a re-organization of the translation elongation machinery in the heart of a patient with mutations in the translation elongation factor EFG1 (19). In this case, the ratio of EFTu:EFTs (the proteins involved in bringing the charged tRNAs to the ribosome) was completely reversed in the patient heart compared with control, driven by transcriptional reprogramming. These data suggest that there is a feedback mechanism that can adjust the functioning of the translation apparatus in some tissues, in this case in the face of a crippled polypeptide elongation factor EFG1. Why such adaptation does not occur in all tissues, to minimize the impact of the genetic mutation, remains unknown.

Recently, it has become clear that the polycistronic transcripts transcribed from mtDNA are processed in discrete granules that have been defined by the presence of the RNA-binding protein GRSF1 (21,22). Newly synthesized RNAs, visualized by pulse labeling with the nucleotide analog BrU, co-localize with GRSF1 (21). These foci also contain Suv3 and PNPase, the enzymes that are involved in mRNA degradation (23). Mitochondrial transcripts are matured by the activities of RNaseP and Z, which process the 5’ and 3’ ends of tRNAs, respectively (24,25). As tRNAs punctuate most of the open reading frames in mtDNA (26), this results in the release of mature mRNAs. It is not known how LRPPRC and SLIRP then associate with these processed RNAs, but it does seem clear that this association is necessary for mRNA stability. Immunofluorescence experiments with antibodies directed against either protein do not localize them to foci, but rather label the entire mitochondrial matrix. It is possible that either or both proteins shuttle between the foci and a free pool in order to deliver the mRNAs to ribosomes, which are thought to be tightly associated to the inner mitochondrial membrane where the mitochondrial polypeptides are co-translationally inserted into the inner membrane. This could explain why LRPPRC appears to exist both in a soluble and membrane-associated form.

The nature of the biochemical defects in LSFC patients that we report here shows that it is not a pure complex IV defect as had been previously thought. The study raises many questions that will require transcriptomic and proteomic analyses to resolve. We have recently developed a knock-in mouse model, in which we have replaced the wild-type allele with the murine homolog of the LSFC founder mutation, and this should allow us to address the molecular basis of tissue specificity in a definitive fashion.

MATERIALS AND METHODS

Cell culture and RNA interference experiments

Myoblasts were cultured from muscle biopsies of two LSFC patients according to an established procedure (27). Primary myoblasts were FACS-sorted and then immortalized by transduction with two retroviral vectors, one expressing the HPV-16 E6 and E7 genes, and the other expressing the catalytic
component of human telomerase (28). Myoblasts were grown in Ham’s F10-based, supplemented growth medium (29). Myotubes were generated by allowing confluent myoblasts to fuse for 2–4 days in dulbecco’s modified eagle medium (DMEM) medium supplemented with 2% horse serum and 0.4 μg/ml dexamethasone. Fibroblasts and HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum.

For knockdown of SLIRP, two Stealth RNA interference duplexes (Invitrogen) were used: CGG CGU CGU GUC AGC UGA AAG AAC A and ACA GAG GUU UGG GUU GGG UUC AGU U. LRPPRC was knocked down with the Stealth RNA interference duplex Stealth1173 (15). Stealth RNAi duplexes were transiently transfected at a concentration of 25 nM according to manufacturer’s instructions for reverse transfection. Transfection was repeated on day 3, and on day 6 cells were either harvested and analyzed (fibroblasts and myoblasts), or transferred to fusion medium and allowed to fuse for 2–4 days (myotubes) prior to analysis.

Radioactive labeling of mitochondrial translation products
Mitochondrial translation products in cultured muscle cells or fibroblasts were pulse-labeled for 1 h with 200 μCi/ml of a mixture of [35S]methionine and cysteine (Perkin Elmer) in DMEM lacking methionine and cysteine and containing 100 μl/ml emetine, an inhibitor of cytoplasmic translation, as detailed elsewhere (30).
Denaturing, native and second dimension PAGE

Denaturing SDS-PAGE was used for the electrophoretic separation of whole-cell extracts, isolated mitochondrial extracts from tissues or fractions from either alkaline carbonate extraction or sucrose gradient sedimentation. Whole cells or isolated mitochondria were extracted on ice with either 1.5–1.75% dodecyl maltoside/phosphate buffered saline (PBS) or 1% taurodeoxycholate/PBS, centrifuged, after which the cleared extract (‘supernatant’ fraction) and pellet (‘pellet’ fraction) were mixed with Laemmli sample buffer. Alternatively, the detergent extract was mixed with sample buffer without prior centrifugation (‘input’ fraction). Twenty to forty micrograms of protein were then electrophoresed, transferred to a nitrocellulose membrane and analyzed by immunoblotting with the antibodies described in (21), as well as with anti-TACO1 (in-house), anti-MTFMT (Abcam), anti-PDF (Abcam), anti-HSP60 and 75 (Stressgen), anti-SCO1 (in-house) and anti CCDC56 (a kind gift from Peter Rehling, Göttingen).

Mitoplasts prepared from fibroblasts, myoblasts or myotubes by treatment with 0.8, 1.2 or 0.4 mg digitonin/mg protein, respectively, or mitochondria isolated from tissues were run in the first dimension on non-denaturing, blue native gels (BN-PAGE) as detailed elsewhere (31). The native complexes were visualized with the following antibodies against the following subunits in all native gels: complex I, ND1 (a gift from Anne Lombes, Paris); complex II, SDHA (Abcam); complex III, core1 (Abcam); complex IV, COX IV (Abcam) and complex V, ATPalpha (Abcam). Second-dimension, denaturing PAGE was carried out as described previously (32).

Isolation of mitochondria and submitochondrial localization experiments

Mitochondria were isolated from cultured cells or from tissue specimens by differential centrifugation after homogenization in ice-cold SET buffer [250 mM sucrose/10 mM Tris–HCl/
1 mM ethylene diamine tetraacetic acid (EDTA)] containing protease inhibitor cocktail (Roche). EDTA was omitted from the buffer when mitochondria were isolated for sucrose gradient fractionation. For cells, 10 confluent 150 mm culture dishes were resuspended in 5–6 ml SET buffer and homogenized with 7–10 strokes in a pre-chilled, zero clearance homogenizer. Tissue samples were cut in small pieces and resuspended in SET buffer at a concentration of 10–15% (w/v) and homogenized with seven to ten strokes in a pre-chilled glass/teflon homogenizer. Homogenates were centrifuged twice at 4°C, 600 g for 10 min to generate post-nuclear supernatants, from which mitochondria were pelleted by centrifugation at 4°C, 8–10 000 g for 10–15 min. Mitochondrial pellets were washed once in cold SET buffer, then used immediately or stored at −80°C for later use. For sub mitochondrial localization experiments, 100 μg mitochondria were extracted in 100 μl sodium carbonate, pH 11.5, for 30 min on ice. Half of the extract volume was saved for immuno blot analysis (‘input’ fraction), the other half was centrifuged at 25 000 g for 1 h at 4°C. The resulting supernatant fraction (‘supernatant’ or ‘soluble’) and pellet fraction (‘pellet’ or ‘membrane’) were analyzed along with the ‘input’ fraction by immunoblotting as described.

Sucrose gradient sedimentation
Mitochondria isolated from liver or heart specimens were extracted in lysis buffer containing 260 mM sucrose, 100 mM KCl, 20 mM MgCl2, 10 mM Tris–HCl, pH 7.5, 1% Triton X-100, 5 mM β-mercaptoethanol and protease inhibitor cocktail without EDTA, for 20 min on ice, then the extract was cleared by centrifugation at 9400 g for 45 min at 4°C prior to loading on a 10–30% discontinuous sucrose gradient (21). After centrifugation, fractions were collected from the top and analyzed by immunoblotting.

RNA blotting analysis
Total RNA was isolated with Trizol reagent (Invitrogen) from muscle, heart or liver samples, and 1.5–10 μg were run on either MOPS/formaldehyde gels for detection of mitochondrial mRNAs (33), or on polyacrylamide/urea gels for detection of mitochondrial tRNAs with 24-mer oligonucleotide probes complementary to the 3′ end of mitochondrial and cytoplasmic tRNAs (15).

ACKNOWLEDGEMENTS
The LSFC Consortium is a group of scientists and clinicians dedicated to investigating the causes of and developing treatments for LSFC. The members of the consortium are (in alphabetical order) Bruce Allen, Yan Burelle, Guy Charron, Lise Coderre, Christine DesRosiers, Catherine Laprise, Charles Morin, John Rioux and Eric A. Shoubridge.

Conflict of Interest statement. None declared.

FUNDING
This research was supported by a CHIR emerging team grant (CGP 102168) to the LSFC consortium and by a CHIR operating grant (MT 15460) to E.A.S.

REFERENCES


