RESEARCH ARTICLE

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A Novel Mutation in *YARS2* Causes Myopathy with Lactic Acidosis and Sideroblastic Anemia

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ABSTRACT: Mutations in the mitochondrial aminoacyl-tRNA synthetases (ARSs) are associated with a strikingly broad range of clinical phenotypes, the molecular basis for which remains obscure. Here, we report a novel missense mutation (c.137G>A, p.Gly46Asp) in the catalytic domain of *YARS2*, which codes for the mitochondrial tyrosyl-tRNA synthetase, in a subject with myopathy, lactic acidosis, and sideroblastic anemia (MLASA). YARS2 was undetectable by immunoblot analysis in subject myoblasts, resulting in a generalized mitochondrial translation defect. Retroviral expression of a wild-type *YARS2* complementary DNA completely rescued the translation defect. We previously demonstrated that the respiratory chain defect in this subject was only present in fully differentiated muscle, and we show here that this likely reflects an increased requirement for YARS2 as muscle cells differentiate. An additional, heterozygous mutation was detected in *TRMU/MTU1*, a gene encoding the mitochondrial 2-thiouridylase. Although subject myoblasts and myotubes contained half the normal levels of TRMU, thiolation of mitochondrial tRNAs was normal. YARS2 eluted as part of high-molecular-weight complexes of ~250 kDa and 1 MDa by gel filtration. This study confirms mutations in *YARS2* as a cause of MLASA and shows that, like some of the cytoplasmic ARSs, mitochondrial ARSs occur in high-molecular-weight complexes.

KEY WORDS: mitochondrial aminoacyl-tRNA synthetase; YARS2; MLASA; TRMU; MTU1; mitochondrial translation

Introduction

Mitochondrial disorders are frequently associated with defects of mitochondrial translation caused by mutations in either the mitochondrial [Taylor and Turnbull, 2005; Tuppen et al., 2010] or nuclear genome [Kemp et al., 2011]. Although most of these mutations have been detected in the transfer RNAs (tRNAs) or ribosomal RNAs (rRNAs) encoded by the mitochondrial genome, several nuclear mutations have been described over the past few years. The first nuclear, pathogenic mutations resulting in defects of mitochondrial translation were identified in 2004 in a mitochondrial ribosomal protein gene, *MRPS16* (MIM# 609204) [Miller et al., 2004], the mitochondrial translation elongation factor, *EFG1* (MIM# 606639) [Coenen et al., 2004], and a gene coding for a tRNA-modifying enzyme, *PUS1* (MIM# 608109) [Bykhovskaya et al., 2004]. Since then, mutations have been uncovered in several nuclear genes encoding two other mitochondrial ribosomal proteins, *MRPS22* (MIM# 605810) [Saada et al., 2007] and *MRPL3* (MIM# 607118) (Galmiche et al., 2011); four additional translation factors or activators, *TUFM* (MIM# 602389) [Valente et al., 2007], TSFM (MIM# 604723) [Smeitink et al., 2006], *c12orf65* (MIM# 613541) [Antonicka et al., 2010], and *TACO1* (MIM# 612958) [Weraarpachai et al., 2009]; another tRNA-modifying enzyme, *TRMU* (MIM# 610230) [Zeharia et al., 2009]; and seven mitochondrial aminoacyl-tRNA synthetases (ARSs), *DARS2* (MIM# 610956) [Scheper et al., 2007], *RARS2* (MIM# 611524) [Edvardson et al., 2007], *YARS2* (MIM# 610957) [Riley et al., 2010], *SARS2* (MIM# 612804) [Belostotsky et al., 2011], *HARS2* (MIM# 600783) [Pierce et al., 2011], *AARS2* (MIM# 612035) [Gotz et al., 2011], and EARS2 (MIM#612799) [Steenweg et al., 2012].

We previously characterized an adult subject of Lebanese descent who presented with myopathy, lactic acidosis and sideroblastic anemia (MLASA), associated with a severe combined respiratory chain deficiency in skeletal muscle, as shown by enzymatic assays and immunoblot analysis [Sasarman et al., 2002]. We demonstrated that the causal gene was nuclear through the generation of cybrid cells. Muscle cells cultured from the subject showed a mitochondrial translation defect, which did not, however, result in a measurable enzyme deficiency in the respiratory chain. This suggested a mutation in a developmentally regulated translation factor. Overexpression of several different mitochondrial translation factors in cultured myoblasts of the subject did not rescue the translation defect, and analysis of *PUS1*, which has also been associated with MLASA, did not reveal any pathogenic mutations.

Here, we report, in this subject, the identification of a novel, homozygous mutation in *YARS2* encoding the mitochondrial tyrosyl-tRNA synthetase, which was recently associated with MLASA in two independent Lebanese families [Riley et al., 2010].

Materials and Methods

Mutation Detection

Total RNA was isolated from myoblasts using the RNeasy kit (Qiagen, Toronto, ON., Canada). *YARS2* (NM_001040436.2) and *TRMU* (NM_018006.4) complementary DNAs (cDNAs) were amplified with specific primers by using the One-Step Reverse

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Transcriptase-Polymerase Chain Reaction (RT-PCR) kit (Qiagen) and the gel-purified fragments were directly sequenced (Institut de Recherche en Immunologie et Cancérologie, Montréal, Canada). Total genomic DNA was extracted from cultured cells using the DNeasy kit (Qiagen). Primers specific for exon 1 of *YARS2* and for exon 9 of *TRMU* were used to amplify genomic DNA (gDNA), which was then digested with either BgIII, or NciI for Restriction Fragment Length Polymorphism (RFLP) analysis. Genomic DNA from a total of 100 controls was used for RFLP analysis and confirmed the presence of the YARS2 homozygous mutation and the TRMU heterozygous mutation exclusively in the subject. All variants identified in this study were named according to the journal guidelines (with +1 as the A of the initiation codon in the reference sequence; www.hgvs.org) and were submitted to locus-specific databases (http://www.LOVD.nl/YARS2 and http://www.LOVD.nl/TRMU).

Cell Culture

Myoblast cultures were established from muscle biopsies, then sorted, immortalized, propagated, and fused as described previously [Sasarman et al., 2002].

Pulse Labeling of Mitochondrial Translation Products

[^{35}S]-labeling of mitochondrial translation products in cultured muscle cells was performed with 200 μCi/ml of [^{35}S]-methionine/cysteine mix (PerkinElmer, Waltham, MA) in Dulbecco's Modified Eagle Medium (DMEM) lacking methionine and cysteine, and containing 100 μg/ml of cytoplasmic translation inhibitor (emetine), for 60 min, followed by chasing of the label for 10 min, as described in detail elsewhere [Sasarman and Shoubridge, 2012].

Antibody Production and Immunoblot Analysis

Rabbits were injected with the synthetic peptide CESPSDSPED conjugated to Imject® Maleimide Activated mcKLH (Pierce, Rockford, Il, as specified by the manufacturer), and then boosted three times at intervals of 3 to 4 weeks, bled periodically, and screened for antibody response by immunoblot analysis. Anti-TRMU polyclonal antibodies were subsequently affinity purified from serum of immunized animals with the SulfoLink® Kit (Pierce, as instructed by the manufacturer), and used for Western blotting at a dilution of 1:1000.

For immunoblotting, whole cells protein extracts were prepared in 1.5% *n*-dodecyl maltoside in phosphate-buffered saline, then 20–30 μg of protein per sample was used for Tris–glycine Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and used for detection of TRMU, YARS2 (with a purified rabbit poyclonal antibody from Abgent), the Co-II-70kDa subunit of complex II of the respiratory chain (with a monoclonal antibody from Molecular Probes, Burlington, ON, Canada), or prohibitin (with a rabbit polyclonal antibody from Abcam, Cambridge, MA).

[(N-Acryloylamino)Phenyl]Mercuric Chloride (APM)–Northern Blotting Analysis

Total RNA was extracted from muscle tissue with Trizol reagent (Invitrogen, Burlington, ON, Canada) and 1.5 μg was run on a 10% polyacrylamide gel containing 7 M urea and 1 μg/ml APM (a kind gift from T. Suzuki, University of Tokyo), followed by transfer to Hybond N+ membrane (GE Healthcare, Mississauga, ON, Canada). For prehybridization and hybridization, the EXPRESSHyb solution (Clontech, Mountain View, CA) was used according to the manufacturer's instructions. The oligonucleotides used for the generation of the [^{32}P] end-labeled probes were 24 nucleotides in length and had sequences complementary to the 3′ end of the tRNA of interest; for example, the probe for the mitochondrial tRNA^{Lys} had the following sequence: 5′tggtcactgtaaagaggtgttggt3′. Quantification of the radioactive signal was performed with the ImageQuant software (Molecular Dynamics/GE Healthcare, Bridgewater, NJ).

Size-Exclusion Chromatography

Soluble proteins from mitochondrial extracts were fractionated on a Tricorn Superdex 200 10/30 HR column (GE Healthcare) as described previously [Kaufman et al., 2007], and the elution profiles of YARS2 and glycyl-tRNA synthetase (GARS) were revealed by immunoblot analysis of the fractions with purified rabbit poyclonal antibodies from Abgent (YARS2) or Abcam (GARS).

Results

Mutation Analysis in the Index Subject

On the basis of similarities of the clinical phenotype and molecular findings between the index subject (E in [Sasarman et al., 2002]) and recently described individuals with mutations in *YARS2* [Riley et al., 2010], we amplified and sequenced the cDNA for this gene from subject myoblasts. We identified a novel homozygous c.137G>A mutation, which changes the highly conserved Gly residue at position 46 to Asp (Fig. 1A and C). The presence of the mutation in the gDNA isolated from subject myoblasts was confirmed by restriction enzyme analysis (Fig. 1B). The mutation was absent in 50 ethnically matched controls and in 50 additional controls from the general population.

Previous mutational analysis in this patient had also uncovered a heterozygous c.967C>T mutation in *TRMU*, coding for the mitochondrial 2-thiouridylase, the enzyme responsible for the 2-thiolation of the wobble uridine in mitochondrial tRNA^{Lys}, tRNA^{Glu}, and tRNA^{Gln}. The presence of this mutation in the gDNA of the index subject was confirmed by restriction enzyme analysis (data not shown). The mutation predicts a p.Arg323Trp substitution, is absent in 100 controls, and is not a reported Single Nucleotide Polymorphism (SNP).

Retroviral Expression of YARS2 Rescues the Translation Defect in Subject Muscle Cells

To test whether the mutation in YARS2 was responsible for the translation defect, myoblasts were transduced with a retroviral vector expressing the wild-type *YARS2* cDNA. The expression of the protein was confirmed by immunoblotting (Fig. 2B). The mitochondrial translation defect observed in cultured myoblasts and myotubes of the subject was completely rescued by the overexpression of YARS2 (shown in Fig. 2A for myoblasts). Retroviral expression of TRMU on the other hand had no effect (data not shown).

Regulation of YARS2 Expression During Differentiation of Cultured Muscle Cells

We next assessed the steady-state levels of the YARS2 and TRMU proteins in myoblasts and myotubes from the index subject

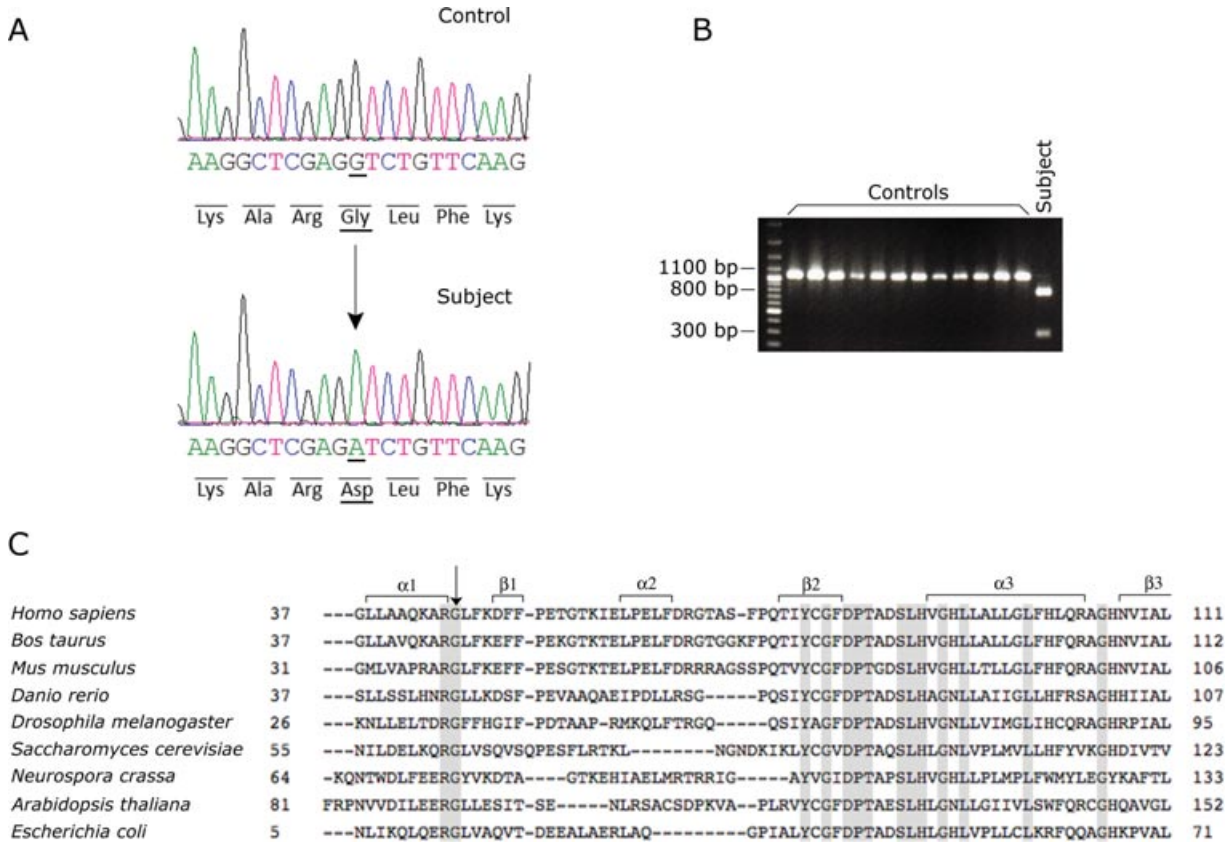

Figure 1. Mutation analysis of *YARS2* in the index subject. **A:** Sequencing analysis of the *YARS2* cDNA, indicating the position of the homozygous c.137G>A mutation and the corresponding p.G46D amino acid substitution in the subject. **B:** RFLP analysis of exon 1 of *YARS2* amplified from the genomic DNA of the subject and 12 controls and digested with BglII confirms the mutation. **C:**. Alignment of the YARS2 amino acid sequence shows the complete evolutionary conservation of the mutated residue (arrow). Conserved residues are shaded. Secondary structural elements identified from the crystal structure of the human YARS2 [Bonnefond et al., 2007] are indicated above the sequence.

and three controls. Immunoblot analysis showed that YARS2 was undetectable in the subject's myoblasts and myotubes, while the levels of TRMU were less than half of those present in control cells (Fig. 3). Additionally, the amount of YARS2 protein present in control myotubes was approximately twice that in control myoblasts, suggesting an increased requirement for YARS2 as muscle differentiation progresses.

Increased Levels and Normal 2-Thiolation of Mitochondrial tRNAs in the Index Subject

Since mutations in other mitochondrial ARSs were shown to result in decreased steady-state levels of the corresponding tRNA [Belostotsky et al., 2011; Edvardson et al., 2007], we performed Northern blotting analysis to assess the levels of the mitochondrial $tRNA^{Tyr}$ and of other tRNAs in the muscle of the subject and two controls (Fig. 4). Given the decrease in the amount of the TRMU protein observed in the subject muscle cells, we also assessed the extent of 2-thiouridylation of the mitochondrial tRNAs for Lys, Glu, and Gln in the subject's muscle by APM–Northern blotting analysis [Sasarman et al., 2011]. APM retards the migration of thio-modified tRNAs during electrophoresis by binding to the sulfur in the tRNA. The levels of all mitochondrial tRNAs tested were increased twofold to threefold (and those of mitochondrial $tRNA^{Gln}$, sixfold) in the subject muscle when compared with the controls, while the level of the cytoplasmic $tRNA^{Glu}$ was unchanged (Fig. 4). The fraction of the three mitochondrial tRNAs that are thiolated was not significantly different between the subject and controls.

YARS2 and GARS Elute in High-Molecular-Weight Complexes by Gel Filtration

Some of the cytoplasmic ARSs associate in a high–molecular-weight complex containing several different synthetases and three nonenzymatic factors [Hausmann and Ibba, 2008]. One study reported that the mitochondrial tyrosyl-tRNA synthetase from bovine liver is found exclusively in a noncomplexed form [Walker et al., 1983]. To verify this finding, we separated mitochondrial extracts from control fibroblasts by size-exclusion chromatography, followed by SDS-PAGE analysis of the resulting fractions. YARS2 was detected predominantly in a high-molecular-weight complex of ~250 kDa, roughly four times its monomeric weight of 53 kDa (Fig. 5). A small amount of YARS2 was also detected in the higher molecular weight fractions around 1 MDa. We then used an antibody against another mitochondrial ARS, the GARS, and detected a single peak at ~250 kDa (the GARS monomer is 83 kDa). These findings support the notion that human mitochondrial YARS2 and GARS function as part of high-molecular-weight complexes.

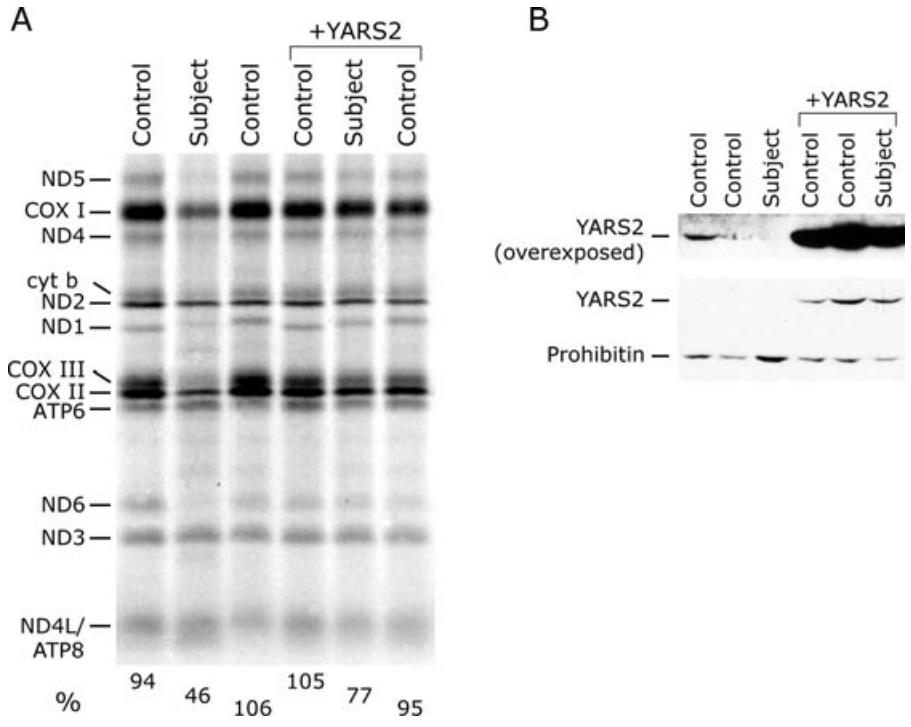

Figure 2. Overexpression of YARS2 rescues the mitochondrial translation defect in cultured muscle cells of the subject. **A:** Mitochondrial translation products in myoblasts from the subject and two controls were pulse labeled with [^{35}S]-(methionine and cysteine) in the presence of emetine, after which 50 μg of total protein per sample was run on a 15–20% polyacrylamide gradient gel. The seven subunits of complex I (ND), one subunit of complex III (cyt b), three subunits of complex IV (COX), and two subunits of complex V (ATP) are indicated at the left of the gel. Total mitochondrial translation levels expressed as percentages of the average of the two controls are indicated at the bottom of the gel. **B:**. Immunoblot analysis of the steady-state levels of YARS2 in myoblasts from the subject and two controls before and after transduction with the *YARS2* cDNA. Prohibitin was used as a loading control.

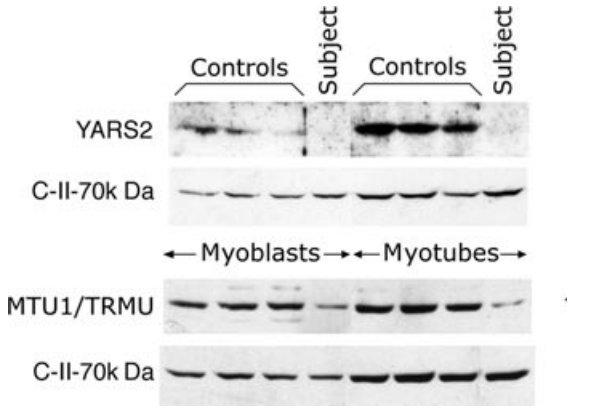

Figure 3. Analysis of the steady-state levels of YARS2 and TRMU in cultured muscle cells of the subject. Immunoblotting was carried out for YARS2 and TRMU in whole cells extracts of myoblasts and myotubes from the subject and three controls. The 70 kDa subunit of complex II was used as a loading control.

Discussion

We have identified a novel, homozygous mutation in *YARS2*, resulting in MLASA in a patient of Lebanese origin. This is the second mutation reported in the *YARS2* gene. The first mutation, reported in subjects from two apparently unrelated consanguineous families, likely represents a founder mutation in the Lebanese population [Riley et al., 2010]. The two mutations, which are only six amino acid residues apart (amino acid residues 46 and 52), are located in the catalytic domain of the enzyme in the vicinity of the catalytic center where tRNA^{Tyr} is tyrosylated [Bonnefond et al., 2007]. The glycine residue mutated in the subject in this report is completely conserved evolutionarily as far back as bacteria. The pathogenicity of the p.Gly46Asp mutation was confirmed by complementation of the mitochondrial translation defect by retroviral expression of the YARS2 cDNA.

The steady-state levels of YARS2 protein increase as differentiation of cultured muscle cells proceeds, from myoblasts to myotubes (Fig. 3), suggesting an increased requirement for YARS2 in fully differentiated muscle. This observation could explain why the translation defect is more easily detectable in myotubes versus myoblasts or fibroblasts of *YARS2* subjects [Riley et al., 2010; Sasarman et al., 2002], and why these individuals present with a very severe clinical myopathy.

The subject in this study had an additional, heterozygous mutation in *TRMU*. Although TRMU levels are less than half of control in the subject muscle cells, 2-thiouridylation of three mitochondrial tRNAs was normal in subject muscle, and retroviral expression of TRMU did not suppress the translation phenotype, suggesting that this mutation does not contribute to the translation defect. The levels of all six mitochondrial tRNAs tested, including tRNA^{Tyr}, but not those of the cytoplasmic tRNA^{Glu}, are increased several fold in the subject's muscle. This is in agreement with the elevated steady-state levels of messenger RNAs and rRNA revealed previously by *in situ* hybridization and reflects the mitochondrial proliferation observed in the subject's muscle [Sasarman et al., 2002].

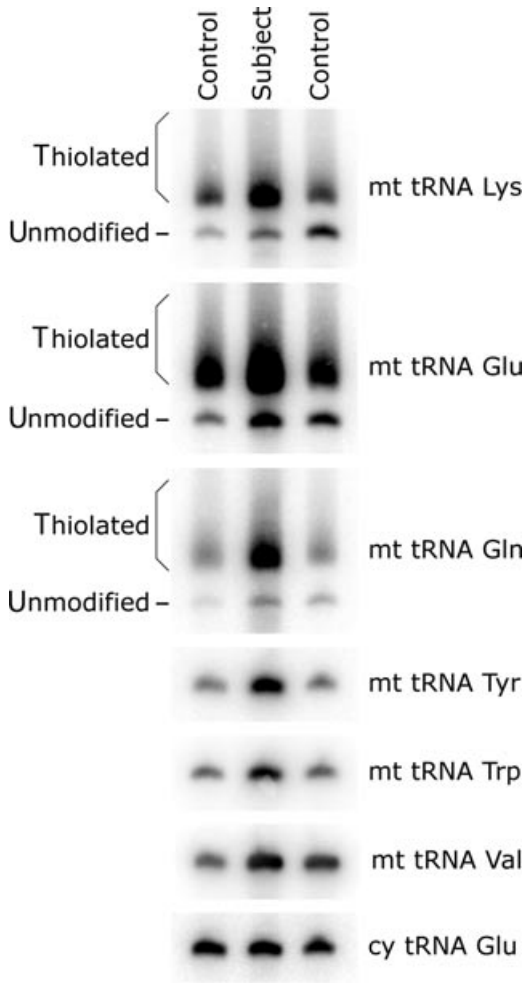

Figure 4. Normal thiolation and increased levels of mitochondrial tRNAs in the muscle of the subject. Total RNA was extracted from muscle tissue of the subject and two controls, after which 1.5 μg of total RNA/sample was run on a 10% polyacrylamide gel containing 7 M urea, in the absence or presence of 1 μg/ml APM, an organomercuric compound which specifically binds and retards the migration of sulfur-containing tRNAs ("thiolated" versus "unmodified" fractions, as indicated at the left of the figure). After transfer to membrane, hybridization was performed with oligonucleotide probes complementary to the mitochondrial tRNAs for Lys, Glu, Gln, Tyr, Trp, Val, or the cytoplasmic $tRNA^{Glu}$, as indicated.

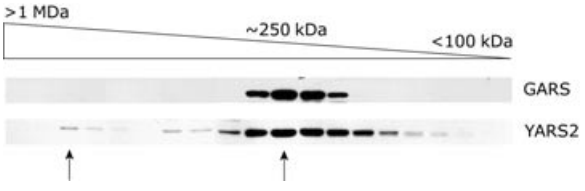

Figure 5. YARS2 and GARS are part of higher molecular weight complexes. Mitochondrial extracts from control fibroblasts were separated by size-exclusion chromatography and the resulting fractions were analyzed by SDS-PAGE and immunoblotting with antibodies against YARS2 and GARS. The elution profile of a set of standards was used to calculate the molecular weights of the individual fractions.

Some of the cytoplasmic ARSs associate in a multiple synthetase complex containing at least nine different ARSs and three auxiliary factors [Lee et al., 2004]. Three ARSs are shared between the cytoplasmic and the mitochondrial translation systems: GARS, lysyl tRNA synthetase (KARS), and glutaminyl tRNA synthetase (QARS) [Antonellis et al., 2003]. The organization of the mitochondrial ARSs is largely unknown, but a single study of bovine liver mitochondria reported complexes of mitochondrial ARSs that were similar, although not identical, to those in the cytoplasm [Walker et al., 1983]. In that study, YARS2 was present entirely in a noncomplexed state, whereas GARS was largely noncomplexed [Walker et al., 1983]. By using size-exclusion chromatography, we demonstrate that neither YARS2 nor GARS could be detected immunologically in the fractions corresponding to their dimeric weights (both are active as dimers), but rather in a complex of ~250 kDa. There is a perfect overlap between the fractions in which these two ARSs are detected, suggesting that they might function together as part of the same complex. Low amounts of YARS2 were also detected in the high-molecular-weight fractions around 1 MDa, similar to the molecular weight of the multiple synthetase complex found in the cytoplasm.

To date, mutations in two cytoplasmic (*YARS* [Jordanova et al., 2006], *AARS* [Latour et al., 2011]), seven mitochondrial (*DARS2* [Scheper et al., 2007], *RARS2* [Edvardson et al., 2007], *YARS2* [Riley et al., 2010], *SARS2* [Belostotsky et al., 2011], *HARS2* [Pierce et al., 2011], and *AARS2* [Gotz et al., 2011]), EARS2 [Steenweg et al., 2012] and one shared (*GARS* [Antonellis et al., 2003]) ARS have been associated with disease. Initially, it appeared that the nervous system might be particularly sensitive to the disruption of normal ARS function, as mutations in the cytoplasmic, the shared, and the first two reported mitochondrial ARSs described were all associated with central or peripheral neuropathies. This led to the conclusion that ARSs might be particularly important in the development and function of neurons, and to the speculation that most (or all) ARS genes may play central roles in inherited neurological disease [Antonellis et al., 2003]. However, this speculation is no longer tenable as mutations in four additional mitochondrial ARSs have now been associated with distinct phenotypes: myopathy and sideroblastic anemia (*YARS2*), pulmonary hypertension and renal failure (*SARS2*), ovarian dysgenesis and sensorineural hearing loss (*HARS2*), and hypertrophic cardiomyopathy (*AARS2*). It is puzzling that mutations in enzymes that perform the same function of tRNA aminoacylation, and that are crucial for the same step of protein synthesis, should result in such diverse phenotypes. Several cytoplasmic ARSs have, however, secondary functions in, for instance, angiogenesis, immune responses, and inflammation, which are apparently completely unrelated to their role in protein translation [Antonellis et al., 2003; Park et al., 2008]. As the tRNA synthetases arose early in evolution, it has been suggested that they may have been among the earliest cell signaling molecules [Park et al., 2008]. It is possible that some mitochondrial ARSs similarly adopted secondary roles that might be related to the diverse phenotypes associated with mutation in these genes, but such functions have not yet been described or investigated. In the case of mutations in *YARS2*, mitochondrial translation is clearly impaired in cells from the affected tissue, making it the most likely pathogenic mechanism, at least in muscle.

It is also curious that the clinical phenotypes produced by mutations in the mitochondrial AARs are by and large not mirrored in patients with mutations in the cognate tRNAs (which are all encoded in the mitochondrial genome). Although mutations in the mitochondrial $tRNA^{Tyr}$ have been associated with muscle phenotypes, mostly chronic progressive external ophthalmoplegia and exercise intolerance [Pulkes et al., 2000; Raffelsberger et al., 2001;

Sahashi et al., 2001], one caused glomerulosclerosis and dilated cardiomyopathy [Scaglia et al., 2003], and none of the reported cases had sideroblastic anemia. Further characterization of the molecular interactions and additional functions of the mitochondrial ARSs should give clues about the pathogenic mechanism of this growing group of inherited diseases.

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