BRIEF REPORT

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A Homozygous Mutation in *LYRM7/MZM1L* Associated with Early Onset Encephalopathy, Lactic Acidosis, and Severe Reduction of Mitochondrial Complex III Activity

Federica Invernizzi,[1†] Marco Tigano,[2†] Cristina Dallabona,[2] Claudia Donnini,[2] Ileana Ferrero,[2] Maurizio Cremonte,[3] Daniele Ghezzi,[1] Costanza Lamperti,[1] and Massimo Zeviani[1,4*]

[1] *Unit of Molecular Neurogenetics, Fondazione IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) Istituto Neurologico "Carlo Besta", Milan, Italy;* [2] *Department of Life Sciences, University of Parma, Parma, Italy;* [3] *Department of Child Neuropsychiatry, C. Arrigo Hospital, Alessandria, Italy;* [4] *MRC Mitochondrial Biology Unit, Cambridge, UK*

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ABSTRACT: **Mutations in nuclear genes associated with defective complex III (cIII) of the mitochondrial respiratory chain are rare, having been found in only two cIII assembly factors and, as private changes in single families, three cIII structural subunits. Recently, human *LYRM7/MZM1L*, the ortholog of yeast *MZM1*, has been identified as a new assembly factor for cIII. In a baby patient with early onset, severe encephalopathy, lactic acidosis and profound, isolated cIII deficiency in skeletal muscle, we identified a disease-segregating homozygous mutation (c.73G>A) in *LYRM7/MZM1L*, predicting a drastic change in a highly conserved amino-acid residue (p.Asp25Asn). In a *mzm1Δ* yeast strain, the expression of a $mzm1^{D25N}$ mutant allele caused temperature-sensitive respiratory growth defect, decreased oxygen consumption, impaired maturation/stabilization of the Rieske Fe–S protein, and reduced complex III activity and amount. LYRM7/MZM1L is a novel disease gene, causing cIII-defective, early onset, severe mitochondrial encephalopathy.**

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Mitochondrial complex III (cIII; ubiquinol–cytochrome *c* reductase, bc1 complex, E.C.1.10.2.2) catalyzes the transfer of electrons from coenzyme Q to cytochrome *c* (cyt *c*). The energy liberated by this reaction sustains the translocation of four protons across the inner mitochondrial membrane (IMM), which contributes to the formation of the electrochemical potential (ΔP).

Complex III is composed of 11 subunits. Cytochrome *b* (cyt *b*) is the only cIII subunit encoded by a mtDNA gene, whereas the remaining 10 are encoded by nuclear DNA genes. Several other nuclear genes are required for the proper formation and function of cIII [Crivellone et al., 1988]. The assembly of cIII in mammalian cells is a still poorly understood process, including the very mechanisms leading to the module-based, stepwise formation of the mature enzyme, the exact subunit composition of the assembly intermediates, and the complete set of specific cIII assembly factors.

We report here the identification of the first *LYRM7/MZM1L* pathogenic mutation in a patient with early onset, severe encephalopathy and lactic acidosis. *LYRM7* (LYR motif containing 7) gene product is the human ortholog of the *Saccharomyces cerevisiae* protein MZM1 (Mitochondrial-Zinc-Maintenance 1); in both organisms, this protein is a cIII assembly factors that participates in the incorporation of the Rieske Fe–S protein into nascent cIII [Atkinson et al., 2011; Sánchez et al., 2013].

The proband was one of four siblings born from Moroccan first-cousin parents; two of them are both alive and well, whereas one has isolated, static mental delay. The patient was born at the 39th week of gestation after uncomplicated pregnancy and delivery. Weight at birth was 2.8 kg (5–10 percentile). Her development was normal during the first 20 months of life, when she manifested rapidly progressive weakness with reduced spontaneous motor activity and alertness, and polypnea. The laboratory tests showed anemia (hemoglobin 7.3 g/dl, n.v. 13–16) associated with low plasma iron (15 mg/dl, n.v. > 50), treated by iron supplementation. At 21 months, during an infectious episode with fever, the patient developed acute, severe dyspnea and stupor, requiring ICU treatment. The clinical examination showed generalized hypotonia, a central breathing pattern, and a fluctuating comatose state with hardly any reaction to external stimuli. Laboratory tests showed severe metabolic acidosis (pH 6.78, pCO_2 16.5; O_2 saturation 96%), with increased plasma lactate (11, 2 mg/dl, n.v. < 2) and ammonia (279 μM, n.v. < 80), and severe anemia (Hb 6.8). A blood culture was positive for *Staphylococcus haemolyticus*. The profiles of urinary organic acids, plasma amino acids, and acylcarnitines were normal. Heart and liver were normal as well. A CT scan showed marked bilateral hypodensity of the centrum semiovale, later confirmed by brain MRI, indicating severe demyelinization and vacuolization of the white matter, and global atrophy with a thin corpus callosum (Fig. 1A and B). The patient was intubated for forced ventilation, and treated with blood transfusions and intravenous bicarbonates. In the next several days, the clinical features improved, with recovery

Additional Supporting Information may be found in the online version of this article.

[†]These authors contributed equally to this work.

*Correspondence to: Massimo Zeviani, MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building Hills Road, Cambridge CB2 0XY, UK. E-mail: mdz21@mrc-mbu.cam.ac.uk

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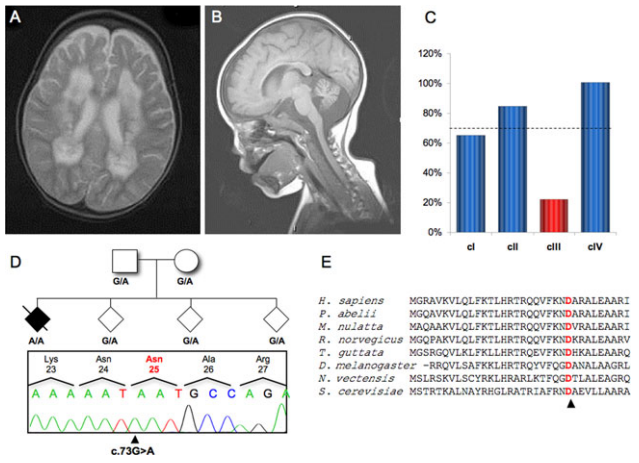

Figure 1. Radiological, biochemical, and genetic features. (**A**) and (**B**) Brain MRI: T2-weighed axial (**A**), and T1-weighed sagittal (**B**) MRI sections showing severe demyelinization and vacuolization of the white matter, and global atrophy with a thin corpus callosum. (**C**) Biochemical activities of mitochondrial respiratory chain complexes in muscle. All enzymatic activities are normalized for CS activity, and indicated as percentages relative to the mean control value. The dotted line represents the lower value of the control range. (**D**) Family tree and electropherogram of the mutant sequence. The proband is indicated by a black symbol. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. (**E**) Phylogenetic alignment of the LYRM7/MZM1L orthologs. The aspartic acid in position 25, mutated in our patient, is in bold red (arrowhead).

of spontaneous breathing, and oral alimentation was resumed. Nevertheless, severe psychomotor regression persisted with reduction of consciousness, no head control, inability to walk, and severe spastic tetraparesis with reduced spontaneous movements. The patient died at 28 months of respiratory failure. Informed consent, approved by the Ethical Committee of the Foundation IRCCS Istituto Neurologico "C.Besta," Milan, Italy, in agreement with the Declaration of Helsinki, was signed by the parents of the patient. Histochemical [Heckmatt and Dubowitz, 1984; Sciacco and Bonilla, 1996], biochemical [Bugiani et al., 2004], molecular, and yeast [Baruffini et al., 2010; Goffrini et al., 2009; Lodi and Ferrero, 1993; Schiestl and Gietz, 1989] studies were performed as previously reported. Complete and detailed methods are described in the Supporting Information, including the list of primers used in the experiments (Supp. Table S1).

Muscle morphology showed normal COX reaction with no ragged-red fibers. Biochemical assays showed severe, isolated decrease of cIII specific activity normalized to citrate synthase (CS) activity (22.2, n.v. 70–130). The other respiratory chain enzymes were normal, although the cI/CS ratio was at the lower limit of the control range (13.4, n.v. 13.6–27.7) (Fig. 1C). The CS-specific activity was also slightly below the lower control limit (74.7 nmol/min mg^{-1}, n.v. 80–210). Because of the profound cIII deficiency, we sequenced cIII-related genes, including those encoding cyt *b*, BCS1L, and TTC19, which were all normal. Contrariwise, analysis of the recently reported *LYRM7* gene revealed a homozygous c.73G>A transition (NM_181705.2), predicting a Asp25Asn amino-acid change in the corresponding protein (NP_859056.2) (Fig. 1D). The c.73G>A variant was absent in 100 alleles from subjects of North-Africa origin and is not reported in any public SNP databases, including dbSNP and the Exome Variant Server, which contains >10,000 alleles; this variant has been submitted to LSDB (http://www.lovd.nl/LYRM7). The Asp25 residue is highly conserved in the phylogenesis (Fig. 1E). The mutation was present in heterozygosis in both parents and in all three unaffected siblings, according to autosomal recessive inheritance of the disease trait. Neither fibroblast cells, nor other tissues of the proband were available. However, the *LYRM7* gene is the human ortholog of *MZM1* in *Saccharomyces cerevisiae* [Atkinson et al., 2011; Sánchez et al., 2013] and the Asp25 residue is conserved between the two species (Fig. 1E). We then introduced the change equivalent to the c.73G>A, p.Asp25Asn of human *LYRM7* into the yeast *MZM1* wild-type (wt) gene, cloned in a centromeric monocopy vector. The *MZM1* and $mzm1^{D25N}$ constructs were used to transform a *mzm1*Δ mutant, giving rise to *mzm1*Δ/*MZM1* and *mzm1*Δ/$mzm1^{D25N}$ yeast strains.

In order to reveal a possible respiratory growth defect, serial dilutions of the strains were incubated on minimum medium supplemented with either dextrose or glycerol, at 28°C and 37°C. A clear growth defect of the *mzm1*Δ/$mzm1^{D25N}$ was observed in glycerol-containing plates incubated at 37°C but not at 28°C, (Fig. 2A), similar to, albeit less pronounced than, the *mzm1*Δ mutant [Atkinson et al., 2010]. To further analyze the respiratory deficiency, we measured oxygen consumption, cIII activity and cyt spectra. The O_2 consumption rates of *mzm1*Δ/$mzm1^{D25N}$ and *mzm1*Δ were 36% and 54% less than that of *mzm1*Δ/*MZM1*, respectively (Fig. 2B). Likewise, the cIII activity was reduced in *mzm1*Δ/$mzm1^{D25N}$ (65% residual activity) and *mzm1*Δ (50%) mitochondria (Fig. 2C). Moreover, the *mzm1*Δ/$mzm1^{D25N}$ strain showed marked reduction of the peak at 570 nm, corresponding to respiratory cyt *b* (not shown). These results are consistent with the temperature-sensitive respiratory growth defect observed in the *mzm1*Δ/$mzm1^{D25N}$ serial dilution spot test.

Mzm1p plays a role in the last steps of cIII assembly, being involved in the maturation of the Rieske iron–sulfur protein (Rip1p in yeast), an essential catalytic subunit of cIII [Atkinson et al., 2011; Cui et al., 2012]. Before incorporation into nascent cIII, the Rip1p precursor undergoes two proteolytic cleavages, which remove a 30-amino-acid import signal at the N-terminus. The mitochondrial matrix peptidase removes the first 22 N-terminal residues, giving rise to a partially processed, immature form of Rip1 (i-ISP). Next, eight additional N-terminal residues are removed by Oct1p, to generate the mature form (m-ISP), which is eventually incorporated in the nascent cIII [Graham et al., 1994; Nett and Trumpower, 1999]. *MZM1* deletion impairs Rip1p maturation and causes marked decrease in its steady state level. In order to evaluate the effects of the $mzm1^{D25N}$ mutation on Rip1p biogenesis and cIII function, we studied mitochondria purified from *mzm1*Δ/*MZM1*, *mzm1*Δ/$mzm1^{D25N}$, and *mzm1*Δ strains, cultured at 37°C in YNB medium. Mitochondrial proteins were resolved by SDS-page and both i-ISP and m-ISP were immunovisualized by Western-blot analysis. As shown in Figure 2D, m-ISP and i-ISP levels were strongly reduced in the $mzm1^{D25N}$ compared with the *MZM1* parental strain, resembling the *mzm1*Δ strain defects [Cui et al. 2012]. The reduced level of Rip1p was associated with a clear reduction of cIII holocomplex (Fig. 2E), with no trace of stable subassembly intermediates (Fig. 2F). These data indicate that the $Mzm1^{D25N}$ mutant protein impairs the stabilization/maturation of ISP and, as a consequence, the assembly of the cIII holoenzyme, thus supporting the pathogenic role of its human ortholog.

Whilst numerous mutations of the gene encoding cyt *b*, a mtDNA-encoded cIII subunit, have been associated with slowly progressive, usually adult onset, mitochondrial myopathy, mutations in nuclear genes associated with cIII deficiency are much rarer, and characterized by severe clinical course. In fact, only a handful of nucleus-encoded proteins related to cIII biogenesis have been linked

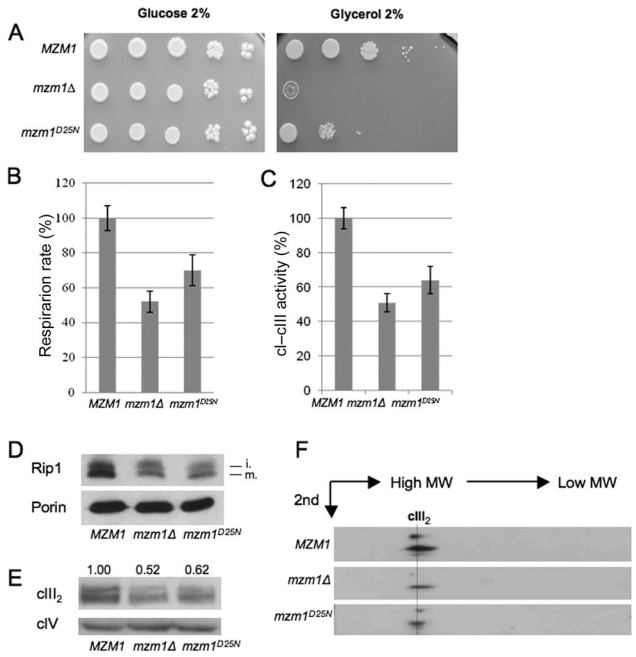

Figure 2. Phenotypes of the yeast model (**A**) oxidative growth. *Mzm1*Δ strains harboring a wild-type *MZM1* allele, the *mzm1*^{D25N} allele or the empty vector were serially diluted from 10^7 to 10^3 cells/mL. Five microliters of each dilution was spotted on YNB minimum agar plates minus uracil with dextrose or glycerol. Plates were incubated at 37°C for 4 days. (**B**) Oxygen consumption rate was recorded on cells grown at 37°C in YNB medium without uracil, supplemented with 0.6% dextrose. Values were normalized to wild-type rate of oxygen consumption (35 nmol O_2 min^{-1} mg^{-1}) and represented as the mean of at least three values ± SD. One-tail, unpaired Student's *t*-test was applied for statistical significance ($P < 0.05$). (**C**) Complex I–III specific activity was recorded on purified mitochondria from cells grown at 37°C as in B. Values were normalized to that of *MZM1* parental strain (413.5 units per mg of mitochondrial proteins) and represented as the mean of at least two independent experiments (**D**) ISP immunoblot analysis. Fifteen micrograms of total purified mitochondrial proteins was separated by a 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane; the ISP protein was immunovisualized by a specific antibody. (**E**) Western-blot immunovisualization of first dimension (1D) blue-native gel electrophoresis. An antibody against Core1 and 2 was used to detect complex III (cIII); an antibody against CoxII (cytochrome *c* oxidase, subunit II) was used for complex IV (cIV). Signal quantification and normalization were carried out using QuantityOne (Bio-Rad). The signal of the *mzm1*Δ/*MZM1* (wild-type) strain, taken as 1.00, was used to normalize those of the other samples. (**F**) Western-blot immunovisualization of second dimension (2D) blue-native gel electrophoresis. Antibodies against Core1 and 2 were used to detect complex III (cIII).

to disease in humans: three small structural subunits, Ubiquinol cyt *c*-reductase binding protein (UQCRB), Ubiquinol-cyt *c*-reductase subunit VII (UQCRQ), and Ubiquinol-cyt *c*-reductase core 2 protein (UQCRC2), and two cIII assembly factors, BC-synthesis-1 like (BCS1L) and tetra-trico-peptide repeat domain 19 (TTC19) [Miyake et al., 2013; Ghezzi and Zeviani, 2011]. Only one mutation in each of the cIII encoding subunits UQCRB, UQCRQ, and UQCRC2 has been reported, each in single families, and with clinical presentations of variable severity and features [Ghezzi and Zeviani, 2011]. However, several mutations in BCS1L are known, encompassing a clinical spectrum ranging from Biornstad syndrome (nerve deafness and *pili torti*) (MIM #209900), to isolated early onset encephalopathy (MIM #124000), to multisystem GRACILE syndrome (MIM #603358), linked to a founder mutation that is part of the Finnish disease heritage. TTC19-mutant patients are characterized by slowly progressive neurodegenerative disorder, usually starting in infancy or childhood, although a late-onset rapidly progressive neurological syndrome has also been described, resembling Jacob-Creutzfeldt Disease [Ghezzi et al., 2011]. LYRM7 is a novel human cIII assembly factor containing a LYR motif,. LYRM7 is involved in the maturation of the Rieske iron–sulfur protein, an essential catalytic subunit containing a (2Fe–2S) cluster, which is incorporated late during cIII assembly [Cui et al., 2012]. Interestingly, small LYR proteins are deemed to be involved in the biogenesis of iron–sulfur cluster containing structures, including respiratory chain complex I, where several LYR proteins are present as ancillary subunits, and complex II, for which a LYR-containing assembly factor, SDHAF1, was recently identified [Ghezzi and Zeviani, 2011]. The role of LYRM7 in maturation/incorporation of the Rieske protein in cIII explains the specific biochemical phenotype and the severe, early onset encephalopathy with lactic acidosis found in our patient, who carried a disease-segregating homozygous missense mutation in *LYRM7*, affecting a highly conserved amino-acid residue. In the yeast *Saccharomyces cerevisiae*, the ortholog of LYRM7 is MZM1, which plays the same role on cIII assembly. We took advantage of the phylogenetic conservation of both gene and mutant residue, to demonstrate the pathogenic role of the human variant in a yeast recombinant model carrying the equivalent mutant allele. We clearly showed that the mutant yeast strain has impaired oxidative growth, low cIII activity, reduced amount of cyt *b* and cIII holocomplex; the mature Rieske protein steady state level is also strongly decreased. Taken together, these results confirm the pathogenicity of the mutation, according to a recessive trait, and provide genetic evidence for a role of LYRM7 in a terminal step of cIII assembly, relevant to warrant OXPHOS proficiency in cells and tissues.

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