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Functional investigation of an universally conserved leucine residue in subunit *a* of ATP synthase targeted by the pathogenic m.9176 T > G mutation

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A B S T R A C T

Protons are transported from the mitochondrial matrix to the intermembrane space of mitochondria during the transfer of electrons to oxygen and shuttled back to the matrix by the *a* subunit and a ring of identical *c* subunits across the membrane domain (F_O) of ATP synthase, which is coupled to ATP synthesis. A mutation (m.9176 T > G) of the mitochondrial *ATP6* gene that replaces an universally conserved leucine residue into arginine at amino acid position 217 of human subunit *a* ($aL_{217}R$) has been associated to NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) and MILS (Maternally Inherited Leigh's Syndrome) diseases. We previously showed that an equivalent thereof in *Saccharomyces cerevisiae* ($aL_{237}R$) severely impairs subunit *a* assembly/stability and decreases by > 90% the rate of mitochondrial ATP synthesis. Herein we identified three spontaneous first-site intragenic suppressors ($aR_{237}M$, $aR_{237}T$ and $aR_{237}S$) that fully restore ATP synthase assembly. However, mitochondrial ATP synthesis rate was only partially recovered (40–50% *vs* wild type yeast). In light of recently described high-resolution yeast ATP synthase structures, the detrimental consequences of the $aL_{237}R$ change can be explained by steric and electrostatic hindrance with the universally conserved subunit *a* arginine residue (aR_{176}) that is essential to F_O activity. aL_{237} together with three other nearby hydrophobic residues have been proposed to prevent ion shortage between two physically separated hydrophilic pockets within the F_O. Our results suggest that aL_{237} favors subunit *c*-ring rotation by optimizing electrostatic interaction between aR_{176} and an acidic residue in subunit *c* (cE_{59}) known to be essential also to the activity of F_O.

1. Introduction

In the inner mitochondrial membrane, an F-type ATP synthase catalyzes the last step of oxidative phosphorylation, which provides aerobic eukaryotes with ATP [1]. In this process, electrons are shuttled to oxygen along four respiratory chain (RC) complexes (I–IV) and protons are concomitantly transported from the mitochondrial matrix into the space between the outer and inner membranes of the organelle (IMS). Protons are returned to the matrix by the ATP synthase, which is coupled to ATP synthesis from ATP from ADP and inorganic phosphate [2]. High-resolution structures of mitochondrial ATP synthase from various organisms have been described recently [3–5]. This enzyme organizes into a matrix-localized F_1 catalytic sector where ATP is synthesized and a membrane-embedded F_O domain that shuttles protons from IMS to the matrix [3,6,7]. Protons are moved through the membrane by the subunit *a* and a ring of identical *c* subunits (8 in mammals, 10 in yeast), which leads to rotation of the subunit *c*-ring and conformational changes in the F_1 that favor the production of ATP and its release into the mitochondrial matrix.

Devastating human neuromuscular disorders have been associated to the m.9176 T > G mutation in the mitochondrial *ATP6* gene that encodes the subunit *a* of ATP synthase [8–10]. It changes an universally conserved leucine residue into arginine at amino acid position 217 of subunit *a* ($aL_{217}R$) [11]. In reported cases, the m.9176 T > G mutation was always found co-existing at varying degrees with wild type mtDNA (heteroplasmy) and showed different distributions in cells and tissues, which likely contributes to the high clinical variability of affected individuals. To better understand how it impacts ATP synthase, we investigated the functional consequences of this mutation in *Saccharomyces cerevisiae* [12]. Mitochondrial genetic transformation is well controlled in this organism [13], and owing to its inability to stably maintain heteroplasmy [14] and its strong fermenting capacity, it is possible to isolate and propagate at will homoplasmic populations carrying a defined mutation in mtDNA even if it totally inactivates oxidative phosphorylation. Using this system we found that an equivalent of the $aL_{217}R$ change in yeast subunit *a* ($aL_{237}R$) severely compromises the assembly/stability of ATP synthase [12]. As a consequence, yeast can no longer grow on non-fermentable substrates (*e.g.*

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glycerol, lactate, ethanol), conditions under which the presence of a functional ATP synthase is absolutely essential. This finding indicates that the aL_{217} residue might be important for a tight packing of subunit *a* or its interactions with protein partners within the ATP synthase. However, its immediate proximity to two residues known for a long time to be essential to the activity of F_O (aR_{176} and cE_{59}) (see below) suggests that it may additionally, or instead, help the functioning of ATP synthase.

In this study we further investigated the function of the aL_{237} residue through the isolation of genetic suppressors of the $aL_{237}R$ mutation. In this way, it is possible to identify novel amino acid residues at position 237 of subunit *a* that are compatible with ATP assembly and/or function, as well as second-site suppressors that can make the primary mutation no longer or less detrimental. Applying this approach to another pathogenic mutation of subunit *a* (m.8969G > A, $aS_{148}N$, [15]) revealed that the inactivation of F_O induced by this mutation is possibly caused by the establishment of hydrogen bonds with a glutamate residue of subunit *a* (aE_{162} in yeast) supposed to be important for the exit of protons from the *c*-ring and their transport towards the mitochondrial matrix [16]. Herein we identified three spontaneous first-site intragenic suppressors ($aR_{237}M$, $aR_{237}T$ and $aR_{237}S$) in the yeast model of the m.9176 T > G mutation. The results lead us to propose that aL_{237} optimizes the functioning of F_O by favoring electrostatic interactions between aR_{176} and cE_{59}.

2. Materials and methods

2.1. Growth media and genotypes

Yeast strains were grown on the following media: YPGA (1% Bacto yeast extract, 1% Bacto Peptone, 2% or 10% glucose, 40 mg/L adenine), YPGalA (1% Bacto yeast extract, 1% Bacto Peptone, 2% galactose, 40 mg/L adenine), YPEGA (1% Bacto yeast extract, 1% Bacto Peptone, 3% ethanol, 2% glycerol, 40 mg/L adenine), W0 (2% glucose, 0.67% Nitrogen base with ammonium sulfate from Difco). Solid media were obtained by adding 2% Bacto Agar (Difco, Becton Dickinson). The genotypes of the used strains are given in Table 1.

2.2. Selection of revertants from strain RKY25 ($aL_{237}R$)

The strain RKY25 that carries an equivalent of the m.9176 T > G mutation was subcloned on rich 2% glucose plates. Forty subclones were picked up and individually grown for three days in 10% glucose. 10^8 cells from each culture were spread on rich glycerol/ethanol (YPEGA) plates and incubated at 28 °C for at least 16 days. Maximum two revertants per plate were retained for further analyses. They were purified by subcloning on glucose plates and their *ATP6* gene was PCR-amplified and entirely sequenced with primers 5′TAATATACGGGGGT GGGTCCCTCAC and 5′GGGCCGAACTCCGAAGGAGTAAG.

2.3. Miscellaneous procedures

Mitochondria were prepared by the enzymatic method [17] from cells strains grown until middle exponential phase (3–4 × 10^7 cells/mL) in rich galactose medium. Oxygen consumption and ATP synthesis rates were measured as previously described [18]. Mitochondrial ATPase activity was assayed as in [19]. Variations in transmembrane potential ($\Delta\Psi$) were evaluated using Rhodamine 123 with a SAFAS Monaco fluorescence spectrophotometer as described [20]. SDS- BN-PAGE analyses were performed according to [21]. Polyclonal antibodies against Atp6 (subunit *a*), Atp9 (subunit *c*) and Atp1 (α-F_1 subunit) were used after 1:10.000 dilution, and those against cytochrome *b* (a gift of T. Langer) and Cox2 (from Molecular Probes) were diluted 1:2.000 and 1:5.000 respectively. 1:10.000 diluted peroxidase-labeled antibodies and the ECL reagent of Amersham International were used to reveal the probed proteins. Cytochrome spectra analysis was performed at liquid nitrogen temperature from whole cells grown for 2 days in rich galactose with a Cary 128 spectrophotometer after reduction of the cytochromes with dithionite as described [22].

Table 1
Intragenic suppressors of $aL_{237}R$.

Codon change	Amino acid change	Number
Original mutant		
$TTA_{247}AGA$	$aL_{237}R$	–
Intragenic suppressors		
$AGA_{247}ATA$	$aR_{237}M$	39
$TAGA_{247}ACA$	$aR_{237}T$	3
$TAGA_{247}AGT$	$aR_{237}S$	2

2.4. Amino-acid alignments and topology of subunit a mutations

Subunits *a* of various origins were aligned using Clustal Omega [23]. The topology of the mutations is based on the atomic structures of yeast F_O [4,24,25]. The shown figures were built using PyMOL molecular graphic system [26].

2.5. Statistical analysis

At least three biological and three technical replicates were performed for all experiments. The *t*-test was used for all data sets. Significance and confidence level was set at 0.05.

3. Results

3.1. Isolation of revertants from the mutant $aL_{237}R$

Yeast subunit *a* (also referred to as subunit *6* or Atp6) is synthesized as a pre-protein of which the first ten N-terminal residues are removed in the mitochondrial intermembrane space by a protease called Atp23 [27–29]. The leucine residue at position 217 of human subunit *a* that is changed into arginine by the m.9176 T > G mutation ($aL_{217}R$) corresponds to aL_{237} in the mature yeast protein (aL_{247} in the non-processed form) (Fig. 4A). As we have shown [12], changing the corresponding leucine codon into an arginine one ($TTA_{247}AGA$; $aL_{237}R$) very severely compromises the growth of yeast on non-fermentable carbon sources like glycerol or ethanol (see also Fig. 1A, B). Cells from the $aL_{237}R$ mutant having recovered the ability to grow in respiratory conditions were isolated using a procedure that avoids over-representation of specific suppressor alleles through multiple mitotic divisions of a same genetically rescued cell (see details in Materials and Methods). To this end, the $aL_{237}R$ mutant was subcloned on glucose plates and forty subclones were grown in liquid 10% glucose until stationary phase. 10^8 cells from each of these cultures were spread separately on solid glycerol medium. This ensures that revertants appearing on different selection plates are genetically independent (*i.e.*, not 'brothers' or 'sisters'). Revertants appeared at a 10^{-7} frequency (on average 10 clones per selection plate). Forty-four clones were retained. The *ATP6* gene of these clones was entirely sequenced. None had recovered the wild type leucine codon 247. This is not surprising because a double nucleotide change would have been required ($AGA_{247}TTA$), the occurrence of which is far below 10^{-10}, and there is no other leucine codon that can be derived by a single nucleotide substitution from the mutant codon AGA. All the analyzed revertants were issued from a single mono-substitution leading to a novel amino acid at codon 247: $AGA_{247}ATA$ ($aR_{237}M$) in thirty-nine clones, $AGA_{247}ACA$ ($aR_{237}T$) in three clones, and $AGA_{247}AGT$ ($aR_{237}S$) in two clones (Table 1). It is somewhat surprising that one of these suppressors was by far much more frequent than the two others. This was not due to a bias in the choice of revertants, since as described below they all grew very well on glycerol. Possibly, for some reason the mitochondrial DNA polymerase has a

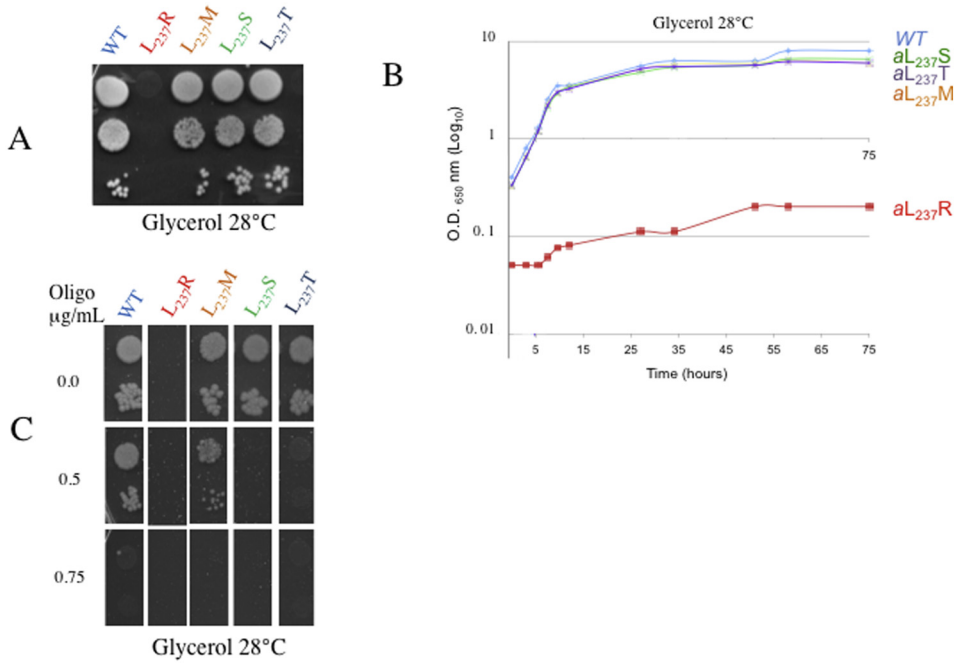

Fig. 1. *Growth phenotypes*. (A) Cells from the indicated strains grown in glucose were serially diluted and spread on rich glycerol plates and incubated at 28 °C for 7 days. (C) Growth tests on glycerol medium supplemented with oligomycin at with the indicated concentrations. (B) Growth curves in liquid glycerol medium.

Table 2
Mitochondrial respiration and ATP synthesis/hydrolysis.

Strain	Respiration (nmol O/min/mg)				ATP synthesis (nmol/min/mg)		P/O
	+NADH	+NADH +ADP	+NADH +CCCP	Asc/TMPD +CCCP	−oligo	+oligo	
WT[a]	279 ± 17	613 ± 12	1081 ± 107	2013 ± 290	637 ± 18	15 ± 8	1.03 ± 0.01
$L_{237}R$[a]	52 ± 1	44 ± 3	70 ± 1	89 ± 19	26 ± 1	21 ± 2	0.60 ± 0.07
$L_{237}M$	180 ± 4	324 ± 8	640 ± 60	805 ± 35	230 ± 11	9 ± 2	0.70 ± 0.02
$L_{237}S$	213 ± 23	414 ± 45	694 ± 58	1053 ± 84	369 ± 22	0 ± 0	0.96 ± 0.07
$L_{237}W$	169 ± 22	339 ± 30	578 ± 104	851 ± 154	308 ± 56	0 ± 0	0.9 ± 0.11

Strain	% ρ^+	ATPase (µmoles/min/mg)		
		−oligo	+oligo	% Inhib.
WT[a]	> 98	4.474 ± 0.222	0.665 ± 0.108	85
$L_{237}R$[a]	64	3.108 ± 0.020	2.797 ± 0.100	10
$L_{237}M$	> 98	3.494 ± 0.274	0.500 ± 0.021	86
$L_{237}S$	> 98	3.311 ± 0.204	0.520 ± 0.044	85
$L_{237}W$	> 98	3.340 ± 0.147	0.472 ± 0.021	86

Mitochondria were isolated from cells grown for 5–6 generations in rich galactose medium (YPGalA) at 28 °C. Reaction mixes for assays contained 0.15 mg/mL protein, 4 mM NADH, 150 µM ADP (for respiration assays), 750 µM ADP (for ATP synthesis assays), 12.5 mM ascorbate (Asc), 1.4 mM *N,N,N,N*-tetramethyl-*p*-phenylenediamine (TMPD), 4 µM carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), 3 µg/mL oligomycin (oligo). The values reported are averages of triplicate assays ± standard errors.

[a] The data of WT and $L_{237}R$ strains have been reported [12] and are here included for comparision with the revertants. Cultures of the $aL_{237}R$ strain contained 35% of *petite* ρ^-/ρ^o cells whereas those from the other strains contained < 3–5% of *petites*.

higher propensity to convert the mutant AGA codon into ATA rather than ACA or AGT.

It is to be noted that three other amino acid residues could have been derived by a single nucleotide change from the mutant AGA codon: glycine ($AGA_{247}GGA$), lysine ($AGA_{247}AAA$) and tryptophane ($AGA_{247}TGA$). Possibly these changes were not selected because they are not compatible with ATP synthase function. However because of the high frequency of the $AGA_{247}ATA$ suppressor mutation, such a conclusion would be hazardous. Henceforth, the three identified suppressor mutations will be designated as $aL_{237}M$ (instead of $aR_{237}M$), $aL_{237}T$ and $aL_{237}S$, to indicate the amino acid changes relative to the wild type subunit *a* sequence.

3.2. Respiratory growth and mtDNA stability of the revertants

The $aL_{237}M$, $aL_{237}T$ and $aL_{237}S$ strains grew on solid (Fig. 1A) and liquid (Fig. 1B) glycerol medium as wild type (*WT*) yeast at 28 °C (the optimal temperature for yeast respiration-dependent growth). This does not imply that despite their rescuing activity the suppressor mutations have no deleterious effects on ATP synthase. Indeed, decreasing ATP synthase activity by ~85% is required to obviously affect yeast proliferation on non-fermentable substrates [30,31]. However, with ATP

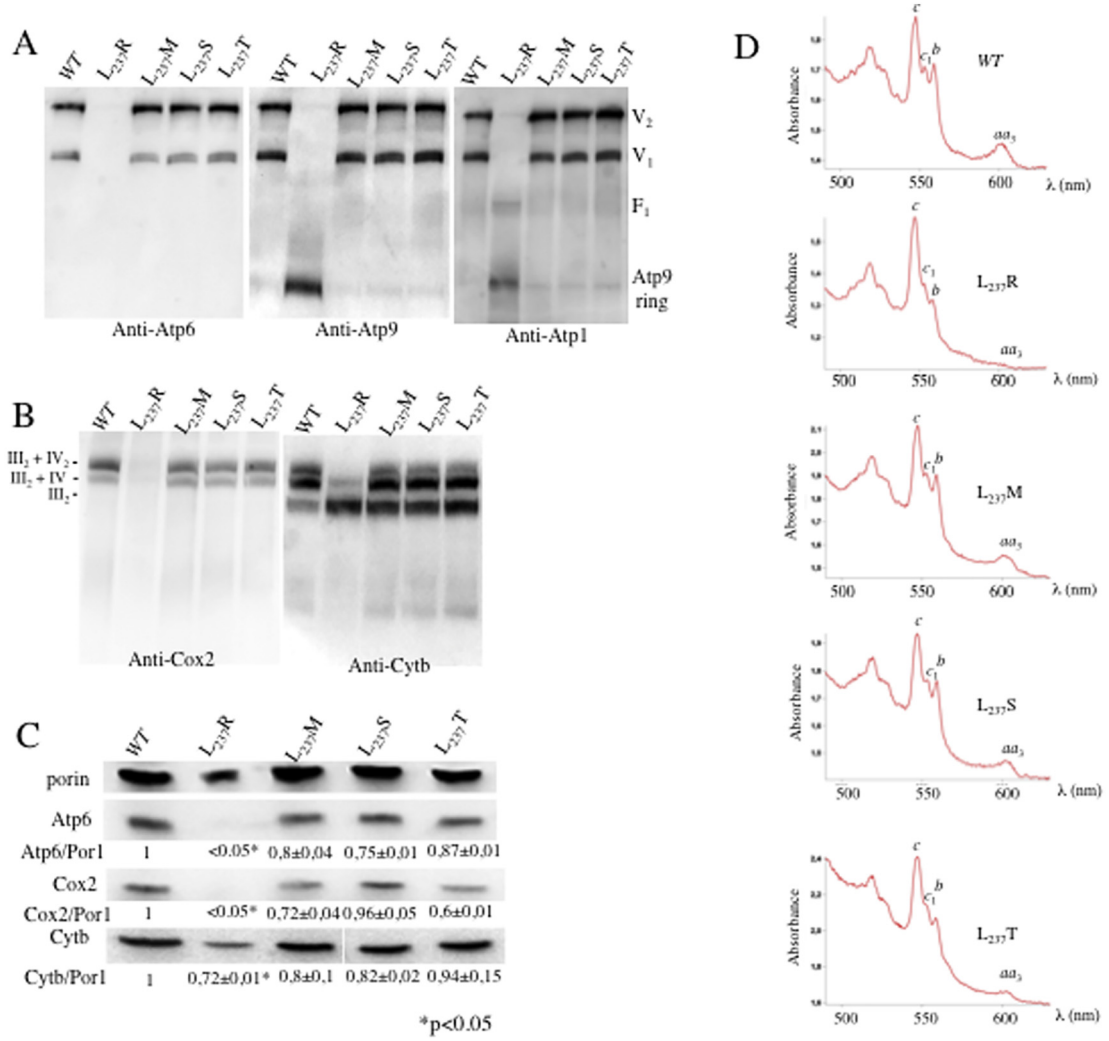

Fig. 2. *Assembly of ATP synthase and respiratory chain complexes.* (A, B) Mitochondria isolated from the indicated strains were solubilized with digitonin (2 g/g protein) and 200 μg of proteins were separated by BN-PAGE in gels containing a 3–10% polyacrylamide gradient. The proteins were transferred to a PVDF membrane and probed with antibodies against Atp6 (subunit *a*), Atp9 (subunit *c*) and Atp1 (α-F_1 subunit) (A), cytochrome *b* (subunit of complex III) or Cox2 (subunit of complex IV) (B). (C) Mitochondrial proteins (50 μg per lane) of proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane and probed with antibodies against the indicated proteins. (D) Mitochondrial cytochrome spectra recorded at liquid nitrogen temperatures established with whole cells of the indicated strains grown in rich galactose medium at 28 °C.

production deficits < 85% respiratory growth becomes more sensitive to oligomycin - a chemical that inhibits the F_O - because less of this drug is then needed to reach the ATP synthase threshold activity [31]. As shown in Fig. 1C, the $aL_{237}T$ and $aL_{237}S$ strains ceased to grow on glycerol in the presence of an oligomycin concentration (0.5 μg/mL) that did not interfere significantly with the growth of the *WT*, and a significant impairment was observed also with $aL_{237}M$, indicating that the suppressors did not fully restored ATP synthase function.

The original $aL_{237}R$ mutant has a higher propensity to produce ρ^-/ρ^o cells issued from large deletions in mtDNA, 35% *vs* < 3% for the *WT* [12], which is a common feature of mutants where ATP synthase function is very severely compromised [32–36]. Consistent with their good growth on glycerol, the $aL_{237}M$, $aL_{237}T$ and $aL_{237}S$ strains showed an almost normal genetic stability with < 3–5% of *petites* (Table 2).

3.3. Oxidative phosphorylation and mitochondrial membrane potential in the revertants

As we reported [12], the $aL_{237}R$ mutant shows *vs* the *WT* a 95% drop in the rate of mitochondrial ATP synthesis because of major defects in the incorporation/stability of subunit *a* within ATP synthase. As was usually observed in yeast ATP synthase defective mutants [33,37–41], except in those with proton leaks through the F_O [44–46], mitochondria from the $aL_{237}R$ mutant have a diminished content in respiratory complexes, mostly the complex IV ([12], Fig. 2). Possibly, as previously discussed [33,42,43], complex IV biogenesis is in yeast modulated by the proton transport activity of F_O *via* the transmembrane electrical potential ($\Delta\Psi$) across the mitochondrial inner membrane as a means to balance electron transfer and ATP synthesis activities.

We biochemically evaluated the rescuing activity of the suppressors in mitochondria isolated from cells grown in a rich galactose medium, in exactly the same conditions previously used to characterize the consequences of the $aL_{237}R$ mutation [12]. The electron transfer to oxygen activity was measured using NADH, alone (state 4), after a further addition (75 μM) of ADP (state 3) and in the presence of the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (uncoupled, maximal respiration). Ascorbate/TMPD was used to measure complex IV's activity in isolation. ATP synthesis coupled to NADH oxidation was assayed in the presence of a large excess of external ADP (750 μM), conditions where ATP is produced only by ATP synthase using the proton-motive force generated by complexes III and IV (there is no complex I in *S. cerevisiae*). We additionally monitored changes in

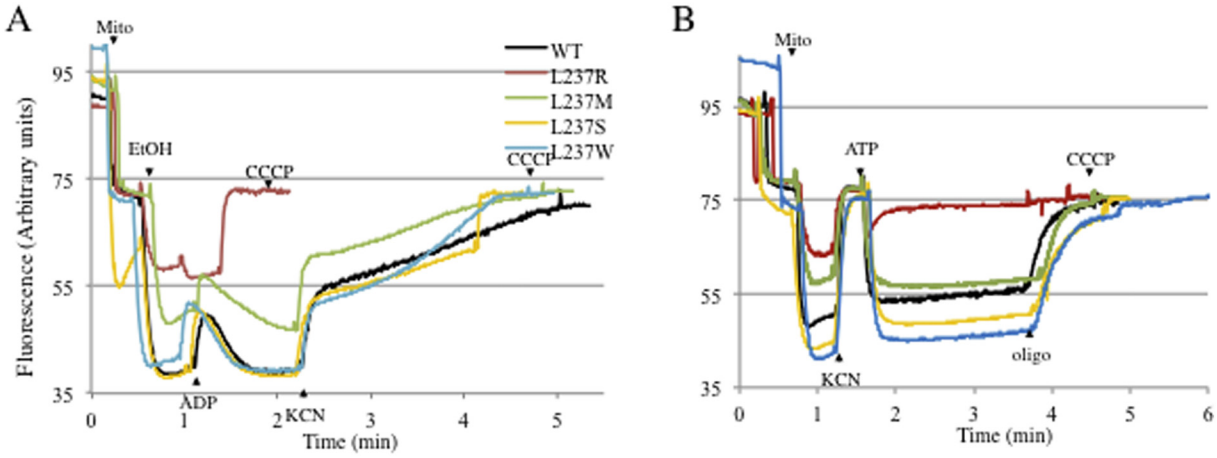

Fig. 3. *Mitochondrial membrane potential.* Variations in mitochondrial ΔΨ were monitored by fluorescence quenching of Rhodamine 123 in mitochondria isolated from the indicated strains. The tracings in panel A show how the mitochondria responded to externally added ADP, those in panel B reflect ATP-driven proton-pumping by ATP synthase. The additions were 75 µM ADP, 0.5 µg/mL Rhodamine 123, 75 µg/mL mitochondrial proteins (Mito), 10 µL ethanol (EtOH), 2 mM potassium cyanide (KCN), 4 µg/mL oligomycin (oligo), and 4 µM carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP). The shown tracings are representative of three experiments.

the transmembrane electrical potential induced by ethanol, ADP and ATP, and evaluated the amounts of complexes III, IV and V by BN- and SDS-PAGE, and cytochrome spectral analysis. The previously reported data relative to the aL_{237}R and *WT* strains [12] are included in Figs. 2 and 3, and Table 2 for comparison with the revertants.

3.3.1. Accumulation of complexes III, IV and V

Complete F_1F_O complexes showed normal accumulation levels in the revertants and the ATP synthase assembly subcomplexes detected in the aL_{237}R mutant (F_1, subunit *c*-ring, and low molecular weight complexes containing subunit α) were much less abundant if not totally absent (Fig. 2A). Furthermore, the steady-state levels of subunit *a* were the same in the revertants and the *WT* (Fig. 2C). Mitochondria from the revertants had also a much better content in oligomers of complexes III and IV (III_2–IV_2 and III_2–IV_1) compared to the aL_{237}R mutant (Fig. 2B), as revealed with Cox2 and cytochrome *b* antibodies. The cytochrome *b* antibodies additionally produced an immunological signal corresponding to free complex III dimers (III_2). This signal was stronger in the mutant and the revertants compared to the *WT*, indicating that the large (90%) deficit in complex IV induced by the aL_{237}R mutation was not totally compensated by the genetic suppressors. Consistently, mitochondria from the revertants showed a 50–60% deficit in complex IV activity *vs* the WT (Table 2). Considering the good genetic stability of the revertants with < 3–5% of ρ^-/ρ^o petites, it can be inferred that their reduced content in complex IV was not due to a lack in mtDNA. The BN gels probed with cytochrome *b* antibodies falsely give the impression that complex III was in higher amounts in samples from the revertants *vs* the *WT*. This is because the complex III reacts much more efficiently with these antibodies when it is not associated with complex IV, which is apparent when comparing the III_2–IV_2 to III_2-IV_1 ratios in *WT* samples: with Cox2 antibodies the III_2–IV_2 immunological signal is stronger than the one produced by III_2-IV_1 whereas the opposite is observed with the cytochrome *b* antibodies. The much better accumulation of complex IV in the revertants *vs* the original aL_{237}R mutant was manifested also in the cytochrome spectra shown in Fig. 2D.

3.3.2. Respiration and ATP synthesis

Although they were substantially improved *vs* the aL_{237}R mutant, the rates of oxygen consumption and ATP synthesis were still reduced by 40–50% in the revertants in comparison to the *WT* (Table 2). As state 4 respiration was not stimulated, it can be inferred that the genetic suppressors did not affect the passive permeability to protons of the mitochondrial inner membrane. The yield in ATP per electron transferred to oxygen in mitochondria from the aL_{237}S and aL_{237}T strains was almost the same as the one measured in *WT* mitochondria. Thus if it efficiently assembled with a serine or threonine residue at position 237 of subunit *a* the functioning of ATP synthase was slow down. The yield in ATP per electron transferred was diminished by about 30% with the aL_{237}M mutation, indicating that in addition to slowing down the functioning of ATP synthase proton movements through the F_O were less efficiently coupled to ATP production.

3.3.3. Mitochondrial membrane potential

We further evaluated the influence of the suppressor mutations on oxidative phosphorylation by monitoring changes in the mitochondrial electrical transmembrane potential (ΔΨ) *via* fluorescence quenching of the dye Rhodamine 123. In a first series of experiments (Fig. 3A), we tested the capacity of ADP to induce ΔΨ in mitochondria fed with electrons from ethanol due to proton reentry through the F_OF_1-ATP synthase, followed by a total collapse of the membrane potential with further additions of KCN and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). As previously shown [12], mitochondria from the aL_{237}R strain responded poorly to ethanol and ADP, consistent with their strongly reduced capacity to respire and produce ATP. The ΔΨ profiles obtained with the mitochondria from the revertants confirmed that respiration and ATP synthesis were substantially restored by the suppressor mutations.

We next investigated the influence of the suppressors on the functionality of ATP synthase when working in the reverse mode (Fig. 3B). To release from F_1 its natural IF1 inhibitor [47], ethanol was added to the mitochondria and the resulting ΔΨ was collapsed with KCN. ATP was then rapidly added, which induced in *WT* mitochondria a large and stable ΔΨ that was fully reversed by oligomycin, whereas as reported [12], the mitochondria from the aL_{237}R mutant were mostly insensitive to ATP. A large F_OF_1-dependent fluorescence quenching was observed in the mitochondria from the revertants, which further illustrates their capacity to assemble an active F_O.

3.3.4. Mitochondrial ATP hydrolysis

In the F_1F_O-mediated proton pumping assays described above (Fig. 3B), only a small fraction of the ATP hydrolytic capacity of ATP synthase is used because the enzyme is working against a proton gradient. Thus we evaluated F_1-mediated ATP hydrolysis on non-osmotically protected mitochondria at pH 8.5, conditions under which this activity is maximal. When F_1 and F_O are properly connected, the ATP hydrolytic activity of ATP synthase is inhibited by blocking subunit *c*-

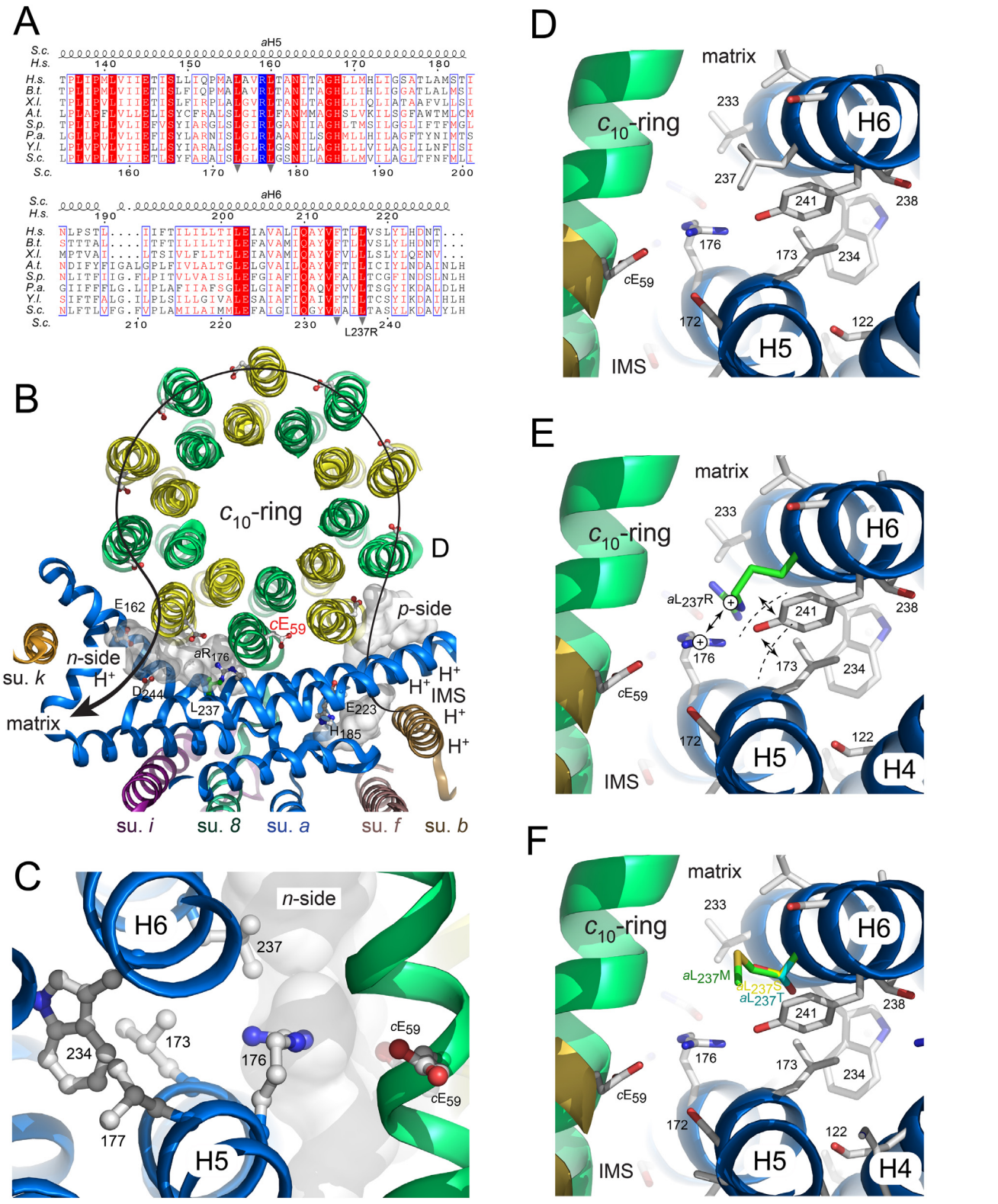

Fig. 4. *Evolutionary conservation and topology of the mutations.* (A) Amino-acid alignments of *a*-subunits from various sources. The aligned amino acid sequences are from *Homo sapiens* (*H.s.*), *Bos taurus* (*B.t.*), *Xenopus laevis* (*X.l.*), *Arabidopsis thaliana* (*A.t.*), *Schizosaccharomyces pombe* (*S.p.*), *Podospora anser*ina (*P.a.*), *Yarrowia lipolytica* (*Y.l.*) and *Saccharomyces cerevisiae* (*S.c.*). At the top and bottom, are numbered the residues of the *H.s.* protein and mature *S.c.* protein (the first 10 residues of the yeast protein are cleaved during assembly of the protein [27]), respectively. Strictly conserved residues are in white characters on a red background while similar residues are in red on a white background with blue frames. α-helices (*a*H5 and *a*H6) in the *S.c.* protein marked above the amino-acid alignments are according to [4]. The essential arginine (aR_{159} in *H.s.*, aR_{176} in *S.c.*) is on a blue background. The yeast residues aL_{237} (aL_{217} in *H.s.*) targeted by the 9176 T > G mutation, *a*L173, *a*L177 and *a*W234 that surround *a*R176 are indicated by a gray arrow. (B) View from the matrix of the entire *c*-ring and subunit *a* and the pathway along which protons are transported from the intermembrane space to the mitochondrial matrix. The side chains of the two residues essential to this transfer (aR_{176} and cE_{59}) are drawn as ball and stick with their carbon atoms in white. The *p*-side and *n*-side clefts are shown as gray surfaces. (C) Close view of cE_{59} and aR_{176} surrounded by aL_{173}, aL_{177}, aW_{234} and aL_{237}. (D-F) Side view from the *n*-side cleft of the *WT* protein (D), the $aL_{273}R$ mutation (E), and the three suppressor amino acids (F). The mutated side chains are in the lower energy conformation. Repulsive electrostatic interactions and steric hindrance are indicated by a double-headed arrow.

ring rotation with oligomycin, because then the central stalk cannot rotate and induce conformational changes in the ATP processing sites. If the F_1 is no longer connected to F_O, as in the $aL_{237}R$ where the assembly F_O is severely compromised, its ATP hydrolytic activity is preserved but becomes mostly insensitive to oligomycin (Table 2). Mitochondria from the $aL_{237}M$, $aL_{237}T$ and $aL_{237}S$ revertant strains showed a good ATP hydrolytic activity, only slightly diminished *vs* the WT, and this activity was quite efficiently inhibited by oligomycin (Table 2). These results further illustrate the beneficial effects of the suppressor mutations on ATP synthase.

3.4. Topology of the subunit a mutations

Two universally conserved residues are essential to the activity of F_O, an acidic amino acid in subunit *c* (Glu or Asp, cE_{59} in yeast) and an arginine residue in subunit *a* (aR_{176} in yeast). They face each other near the center of the membrane ([3,6,7], see Fig. 4B). The aR_{176} residue is located on the membrane embedded helix 5 of subunit *a* (*a*H5). Being kinked this helix follows the curvature of the subunit *c*-ring and seals two hydrophilic clefts that connect the *a*/*c*-ring interface to the intermembrane and matrix spaces [4,48–50]. The essential arginine points into the *n*-side cleft. This cleft has a funnel shape 15 Å long (from aR_{176} to aD_{244} or aE_{162}), 8 Å wide (from aD_{244} to aE_{162}) and 16 Å deep (from aS_{165} to the C-terminus of the protein) (Fig. 4B). The acidic residues aE_{162} and aD_{244} have been proposed to be important for moving protons out of the cleft towards the mitochondrial matrix [4]. The carboxyl group of cE_{59} is assumed to be protonated in all of the *c*-subunits except in those located at the *a*/*c*-ring interface where proton exchange between subunits *a* and *c* occurs. In the proposed series of events that occur during proton translocation [3–7,24,25], the carboxylate group inside the *p*-side channel is neutralized with a proton that originates from the IMS. The newly protonated *c*-subunit moves to the lipid phase and the adjacent monomer comes into the *p*-side channel, and in the *n*-side channel the incoming cE_{59} carboxyl group is deprotonated and the released proton is transported towards the mitochondrial matrix [3–7,24,25].

The leucine residue targeted by the m.9176 T > G mutation is in the immediate proximity of aR_{176} (Fig. 4D). As a result, the $aL_{237}R$ change leads to both steric hindrance and electrostatic repulsion with aR_{176} and secondary clashes with the neighboring aY_{241} and aL_{173} residues (Fig. 4E). This explains the dramatic consequences of the $aL_{237}R$ change on the assembly/stability or folding of subunit *a*. Consistently, replacement of the mutant arginine 237 with polar uncharged (serine, threonine) or hydrophobic (methionine) residues, all of which occupy a smaller space than arginine and not larger than leucine (Fig. 4F), efficiently restores ATP synthase assembly as described above (Fig. 2A).

4. Conclusion

The search for genetic suppressors in a yeast model of the m.9176 T > G mutation provides a molecular explanation for its pathogenicity and its detrimental consequences on the assembly/stability of ATP synthase. Indeed, the leucine-to-arginine change induced by this mutation in yeast subunit *a* ($aL_{237}R$) leads to both steric and electrostatic hindrance with the essential arginine residue of subunit *a* (aR_{176}), and this prevents a tight packing of the two membrane-embedded helical domains (*a*H5 and *a*H6) to which belong positions 173 and 237 respectively. Consistently, genetic reversions leading to replacement of the mutant arginine with the less bulkier and uncharged methionine, serine or threonine residues fully restored the assembly of subunit *a*.

None of the forty-four genetically independent revertants that were analyzed was issued from a second-site suppressor mutation. This indicates that it is unlikely that the structure of subunit *a* can be adapted so as to preserve ATP synthase function with an arginine residue at position 237. In a previous study, we showed that another pathogenic mutation of subunit *a* (m.8969G > A, $aS_{165}N$) can be efficiently suppressed by second-site suppressors some of which are distantly located (by 20–30 Å) from the original mutation, which holds promise for developing therapeutic molecules that can render the mutant asparagine residue less detrimental [16]. The present study reveals that there is little hope for the emergence of such pharmacological treatments in the case of the m.9176 T > G mutation.

While ATP synthase assembled efficiently with methionine, serine or threonine at position 237, the rate of mitochondrial ATP synthesis was substantially decreased *vs* the wild type enzyme. This indicates that aL_{237} affords some structural specificity that optimizes the functioning of F_O, consistent with the strict evolutionary conservation of this residue. It has been proposed that this leucine residue could be important to avoid ion short-circuiting between the two pathways that enable the protons to reach the subunit *c*–ring from the intermembrane space and be released on the other side of the membrane after an almost complete rotation of the ring [25]. Our results lead us to propose that the aL_{237} residue may additionally contribute to constrain the guanidinium group of aR_{176} in a position that optimizes its interaction with the incoming acidic residue of subunit *c* after it has released its proton into the *n*-side channel.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contributions

R.K and F.G isolated, sequenced and investigated the biochemical properties of the revertants. A.D. performed the structural modeling analyses. All authors analyzed the data and contributed to the writing of the manuscript. J.-P.dR designed the research.

References

[1] M. Saraste, Oxidative phosphorylation at the fin de siècle, Science 283 (1999) 1488–1493.
[2] P.D. Boyer, The ATP synthase–a splendid molecular machine, Annu. Rev. Biochem. 66 (1997) 717–749.
[3] A. Zhou, A. Rohou, D.G. Schep, J.V. Bason, M.G. Montgomery, J.E. Walker, N. Grigorieff, J.L. Rubinstein, Structure and conformational states of the bovine mitochondrial ATP synthase by cryo-EM, elife 4 (2015).
[4] H. Guo, S.A. Bueler, J.L. Rubinstein, Atomic model for the dimeric FO region of mitochondrial ATP synthase, Science 358 (2017) 936–940.
[5] A. Hahn, K. Parey, M. Bublitz, D.J. Mills, V. Zickermann, J. Vonck, W. Kuhlbrandt, T. Meier, Structure of a complete ATP synthase dimer reveals the molecular basis of inner mitochondrial membrane morphology, Mol. Cell 63 (2016) 445–456.
[6] E. Morales-Rios, M.G. Montgomery, A.G. Leslie, J.E. Walker, Structure of ATP synthase from *Paracoccus denitrificans* determined by X-ray crystallography at 4.0 A resolution, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 13231–13236.
[7] M. Allegretti, N. Klusch, D.J. Mills, J. Vonck, W. Kuhlbrandt, K.M. Davies, Horizontal membrane-intrinsic alpha-helices in the stator a-subunit of an F-type ATP synthase, Nature 521 (2015) 237–240.
[8] A. Dautant, T. Meier, A. Hahn, D. Tribouillard-Tanvier, J.-P. di Rago, R. Kucharczyk, ATP synthase diseases of mitochondrial genetic origin, Front. Physiol. 9 (2018) 329.
[9] J. Houstek, A. Pickova, A. Vojtiskova, T. Mracek, P. Pecina, P. Jesina, Mitochondrial diseases and genetic defects of ATP synthase, Biochim. Biophys. Acta 1757 (2006) 1400–1405.
[10] E.A. Schon, S. Santra, F. Pallotti, M.E. Girvin, Pathogenesis of primary defects in mitochondrial ATP synthesis, Semin. Cell Dev. Biol. 12 (2001) 441–448.
[11] R. Carrozzo, A. Tessa, M.E. Vazquez-Memije, F. Piemonte, C. Patrono, A. Malandrini, C. Dionisi-Vici, L. Vilarinho, M. Villanova, H. Schagger, A. Federico,

E. Bertini, F.M. Santorelli, The T9176G mtDNA mutation severely affects ATP production and results in Leigh syndrome, Neurology 56 (2001) 687–690.
[12] R. Kucharczyk, B. Salin, J.P. di Rago, Introducing the human Leigh syndrome mutation T9176G into Saccharomyces cerevisiae mitochondrial DNA leads to severe defects in the incorporation of Atp6p into the ATP synthase and in the mitochondrial morphology, Hum. Mol. Genet. 18 (2009) 2889–2898.
[13] N. Bonnefoy, T.D. Fox, Genetic transformation of Saccharomyces cerevisiae mitochondria, Methods Cell Biol. 65 (2001) 381–396.
[14] K. Okamoto, P.S. Perlman, R.A. Butow, The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud, J. Cell Biol. 142 (1998) 613–623.
[15] S. Wen, K. Niedzwiecka, W. Zhao, S. Xu, S. Liang, X. Zhu, H. Xie, D. Tribouillard-Tanvier, M.F. Giraud, C. Zeng, A. Dautant, R. Kucharczyk, Z. Liu, J.P. di Rago, H. Chen, Identification of G8969 > A in mitochondrial ATP6 gene that severely compromises ATP synthase function in a patient with IgA nephropathy, Sci. Rep. 6 (2016) 36313.
[16] N. Skoczen, A. Dautant, K. Binko, F. Godard, M. Bouhier, X. Su, J.P. Lasserre, M.F. Giraud, D. Tribouillard-Tanvier, H. Chen, J.P. di Rago, R. Kucharczyk, Molecular basis of diseases caused by the mtDNA mutation m.8969G > A in the subunit a of ATP synthase, Biochim. Biophys. Acta 1859 (2018) 602–611.
[17] B. Guerin, P. Labbe, M. Somlo, Preparation of yeast mitochondria (*Saccharomyces cerevisiae*) with good P/O and respiratory control ratios, Methods Enzymol. 55 (1979) 149–159.
[18] M. Rigoulet, B. Guerin, Phosphate transport and ATP synthesis in yeast mitochondria: effect of a new inhibitor: the tribenzylphosphate, FEBS Lett. 102 (1979) 18–22.
[19] M. Somlo, Induction and repression of mitochondrial ATPase in yeast, Eur. J. Biochem. 5 (1968) 276–284.
[20] R.K. Emaus, R. Grunwald, J.J. Lemasters, Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties, Biochim. Biophys. Acta 850 (1986) 436–448.
[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[22] M.L. Claisse, P.F. Pajot, Presence of cytochrome c1 in cytoplasmic “petite” mutants of Saccharomyces cerevisiae, Eur. J. Biochem. 49 (1974) 49–59.
[23] F. Sievers, A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J.D. Thompson, D.G. Higgins, Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, Mol. Syst. Biol. 7 (2011) 539.
[24] A.P. Srivastava, M. Luo, W. Zhou, J. Symersky, D. Bai, M.G. Chambers, J.D. Faraldo-Gomez, M. Liao, D.M. Mueller, High-resolution cryo-EM analysis of the yeast ATP synthase in a lipid membrane, Science 360 (2018) 6389.
[25] A. Hahn, J. Vonck, D.J. Mills, T. Meier, W. Kuhlbrandt, Structure, mechanism, and regulation of the chloroplast ATP synthase, Science 360 (2018) 6389.
[26] W.L. DeLano, PyMOL Molecular Graphics System, (2002).
[27] T. Michon, M. Galante, J. Velours, NH2-terminal sequence of the isolated yeast ATP synthase subunit 6 reveals post-translational cleavage, Eur. J. Biochem. 172 (1988) 621–625.
[28] X. Zeng, W. Neupert, A. Tzagoloff, The metalloprotease encoded by ATP23 has a dual function in processing and assembly of subunit 6 of mitochondrial ATPase, Mol. Biol. Cell 18 (2007) 617–626.
[29] C. Osman, C. Wilmes, T. Tatsuta, T. Langer, Prohibitins interact genetically with Atp23, a novel processing peptidase and chaperone for the F1Fo-ATP synthase, Mol. Biol. Cell 18 (2007) 627–635.
[30] A. Mukhopadhyay, M. Uh, D.M. Mueller, Level of ATP synthase activity required for yeast Saccharomyces cerevisiae to grow on glycerol media, FEBS Lett. 343 (1994) 160–164.
[31] R. Kucharczyk, N. Ezkurdia, E. Couplan, V. Procaccio, S.H. Ackerman, M. Blondel, J.P. di Rago, Consequences of the pathogenic T9176C mutation of human mitochondrial DNA on yeast mitochondrial ATP synthase, Biochim. Biophys. Acta 1797 (2010) 1105–1112.
[32] V. Contamine, M. Picard, Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast, Microbiol. Mol. Biol. Rev. 64 (2000) 281–315.
[33] M. Rak, E. Tetaud, F. Godard, I. Sagot, B. Salin, S. Duvezin-Caubet, P.P. Slonimski, J. Rytka, J.P. di Rago, Yeast cells lacking the mitochondrial gene encoding the ATP synthase subunit 6 exhibit a selective loss of complex IV and unusual mitochondrial morphology, J. Biol. Chem. 282 (2007) 10853–10864.
[34] X. Zeng, M.H. Barros, T. Shulman, A. Tzagoloff, ATP25, a new nuclear gene of Saccharomyces cerevisiae required for expression and assembly of the Atp9p subunit of mitochondrial ATPase, Mol. Biol. Cell 19 (2008) 1366–1377.
[35] X. Zeng, A. Hourset, A. Tzagoloff, The Saccharomyces cerevisiae ATP22 gene codes for the mitochondrial ATPase subunit 6-specific translation factor, Genetics 175 (2007) 55–63.
[36] M. Bietenhader, A. Martos, E. Tetaud, R.S. Aiyar, C.H. Sellem, R. Kucharczyk, S. Clauder-Munster, M.F. Giraud, F. Godard, B. Salin, I. Sagot, J. Gagneur, M. Dequard-Chablat, V. Contamine, S. Hermann-Le Denmat, A. Sainsard-Chanet, L.M. Steinmetz, J.P. di Rago, Experimental relocation of the mitochondrial ATP9 gene to the nucleus reveals forces underlying mitochondrial genome evolution, PLoS Genet. 8 (2012) e1002876.
[37] R. Kucharczyk, M.F. Giraud, D. Brethes, M. Wysocka-Kapcinska, N. Ezkurdia, B. Salin, J. Velours, N. Camougrand, F. Haraux, J.P. di Rago, Defining the pathogenesis of human mtDNA mutations using a yeast model: the case of T8851C, Int. J. Biochem. Cell Biol. 45 (2013) 130–140.
[38] S. Marzuki, L.C. Watkins, W.M. Choo, H. Mitochondrial, +-ATPase in mutants of Saccharomyces cerevisiae with defective subunit 8 of the enzyme complex, Biochim. Biophys. Acta 975 (1989) 222–230.
[39] M.F. Paul, J. Velours, G. Arselin de Chateaubodeau, M. Aigle, B. Guerin, The role of subunit 4, a nuclear-encoded protein of the F0 sector of yeast mitochondrial ATP synthase, in the assembly of the whole complex, Eur. J. Biochem. 185 (1989) 163–171.
[40] M. Rak, E. Tetaud, S. Duvezin-Caubet, N. Ezkurdia, M. Bietenhader, J. Rytka, J.P. di Rago, A yeast model of the neurogenic ataxia retinitis pigmentosa (NARP) T8993G mutation in the mitochondrial ATP synthase-6 gene, J. Biol. Chem. 282 (2007) 34039–34047.
[41] M.J.B. Jean-Francois, H.B. Lukins, S. Marzuki, Post-transcriptional defects in the synthesis of the mitochondrial H +-ATPase subunit 6 in yeast mutants with lesions in the subunit 9 structural gene, Biochim. Biophys. Acta 868 (1986) 178–182.
[42] I.C. Soto, F. Fontanesi, M. Valledor, D. Horn, R. Singh, A. Barrientos, Synthesis of cytochrome c oxidase subunit 1 is translationally downregulated in the absence of functional F1F0-ATP synthase, Biochim. Biophys. Acta 1793 (2009) 1776–1786.
[43] C. Schwimmer, L. Lefebvre-Legendre, M. Rak, A. Devin, P.P. Slonimski, J.P. di Rago, M. Rigoulet, Increasing mitochondrial substrate-level phosphorylation can rescue respiratory growth of an ATP synthase-deficient yeast, J. Biol. Chem. 280 (2005) 30751–30759.
[44] Y. Xiao, M. Metzl, D.M. Mueller, Partial uncoupling of the mitochondrial membrane by a heterozygous null mutation in the gene encoding the gamma- or delta-subunit of the yeast mitochondrial ATPase, J. Biol. Chem. 275 (2000) 6963–6968.
[45] S. Duvezin-Caubet, M. Caron, M.F. Giraud, J. Velours, J.P. di Rago, The two rotor components of yeast mitochondrial ATP synthase are mechanically coupled by subunit delta, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13235–13240.
[46] E. Tetaud, F. Godard, M.F. Giraud, S.H. Ackerman, J.P. di Rago, The depletion of F (1) subunit epsilon in yeast leads to an uncoupled respiratory phenotype that is rescued by mutations in the proton-translocating subunits of F(0), Mol. Biol. Cell 25 (2014) 791–799.
[47] R. Venard, D. Brethes, M.F. Giraud, J. Vaillier, J. Velours, F. Haraux, Investigation of the role and mechanism of IF1 and STF1 proteins, twin inhibitory peptides which interact with the yeast mitochondrial ATP synthase, Biochemistry 42 (2003) 7626–7636.
[48] S.B. Vik, B.J. Antonio, A mechanism of proton translocation by F1F0 ATP synthases suggested by double mutants of the a subunit, J. Biol. Chem. 269 (1994) 30364–30369.
[49] W. Junge, H. Lill, S. Engelbrecht, ATP synthase: an electrochemical transducer with rotatory mechanics, Trends Biochem. Sci. 22 (1997) 420–423.
[50] D. Pogoryelov, A. Krah, J.D. Langer, O. Yildiz, J.D. Faraldo-Gomez, T. Meier, Microscopic rotary mechanism of ion translocation in the F(o) complex of ATP synthases, Nat. Chem. Biol. 6 (2010) 891–899.