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Research paper

# Defining the impact on yeast ATP synthase of two pathogenic human mitochondrial DNA mutations, T9185C and T9191C

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ABSTRACT

Mutations in the human mitochondrial ATP6 gene encoding ATP synthase subunit *a*/6 (referred to as Atp6p in yeast) are at the base of neurodegenerative disorders like Neurogenic Ataxia and Retinitis Pigmentosa (NARP), Leigh syndrome (LS), Charcot–Marie–Tooth (CMT), and ataxia telangiectasia. In previous studies, using the yeast *Saccharomyces cerevisiae* as a model we were able to better define how several of these mutations impact the ATP synthase. Here we report the construction of yeast models of two other ATP6 pathogenic mutations, T9185C and T9191C. The first one was reported as conferring a mild, sometimes reversible, CMT clinical phenotype; the second one has been described in a patient presenting with severe LS. We found that an equivalent of the T9185C mutation partially impaired the functioning of yeast ATP synthase, with only a 30% deficit in mitochondrial ATP production. An equivalent of the mutation T9191C had much more severe effects, with a nearly complete block in yeast Atp6p assembly and an >95% drop in the rate of ATP synthesis. These findings provide a molecular basis for the relative severities of the diseases induced by T9185C and T9191C.



## 1. Introduction

A quite large number of point mutations (sixteen) has been found in the mitochondrial *ATP6* gene in patients presenting with various neurodegenerative disorders, Neurogenic Ataxia and Retinitis Pigmentosa (NARP), Leigh syndrome (LS), Leber's Hereditary Optic Neuropathy (LHON), Charcot–Marie–Tooth (CMT) or ataxia telangiectasia [1–8]. The *ATP6* gene encodes ATP synthase subunit *a*, which is referred to as Atp6p in yeast. The ATP synthase (also called complex V) synthesizes ATP from ADP and inorganic phosphate using the energy of the electrochemical proton gradient established by the mitochondrial electron transport chain (complexes I–IV) [9]. Atp6p is a key subunit of the $F_O$ proton-translocating domain of the ATP synthase. Proton movements mediated by Atp6p lead to the rotation of a transmembrane ring of Atp9p subunits (referred to as subunit *c* in humans) which ends up in conformational changes at the level of the catalytic sites in the $F_1$ extra-membrane domain of the enzyme that favor the synthesis ATP and its release into the mitochondrial matrix [10,11].

We previously constructed yeast models of the pathogenic ATP6 mutations T8993G [12], T8993C [13], T9176G [14], T9176C [15] and T8851C [16]. The effects of these mutations on yeast ATP synthase correlated well with those observed in humans, which reflects the high level of evolutionary conservation within the regions of Atp6p affected by these mutations.

Two other pathogenic mutations at the focus of the present study were described at positions 9185 (T9185C) and 9191 (T9191C) of ATP6 [17]. The first one changes a leucine into proline at position 220 near the carboxyl terminus of the protein. It was found in thirty-four patients from eight independent families suffering from LS, NARP, CMT or spinocerebellar ataxia syndromes [3,17–21]. In all cases the disease was maternally inherited, with a relatively mild, sometimes reversible, clinical phenotype and occurred at a minimum of 85% heteroplasmy. Mitochondria from patients's cells (muscle or skin fibroblasts) showed normal complexes I–IV activities [3,21] and only a slightly reduced ATPase activity [18,20]. The second mutation, T9191C, was found in a patient presenting with very severe LS [17]. It changes a leucine to proline at position 222 of the human homolog of yeast Atp6p. This mutation causes a substantial (50%) reduction in mitochondrial ATPase activity and a lower respiration rate (60% *vs.* control) [17]. We report here yeast models of

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the mutations T9185C and T9191C that help to better define how they impact the ATP synthase.

## 2. Materials and methods

### 2.1. Construction of yeast atp6-S250P and atp6-L252P mutants

The strains used in the study are listed in Table 1. Using the QuikChange XL Site-directed Mutagenesis Kit of Stratagene, we changed the serine TCA codon at position 250 in the yeast *ATP6* gene into proline CCA codon, with primers 5′ GTCTGGGCTATTT-TAACAGCA**CCA**TATTTAAAAGATGCAGTATACTTACAT and 5′ ATGTAA GTATACTGCATCTTTTAAATA**TGG**TGCTGTTAAAATAGCCCAGAC and the leucine TTA codon at position 252 into proline CCA codon, with primers 5′ GTCTGGGCTATTTTAACAGCATCATAT**CCA**AAAGATGCAGT ATACTTACAT and 5′ ATGTAAGTATACTGCATCTTT**TGG**ATATGATGC TGTTAAAATAGCCCAGAC (in bold the mutator codon). The mutagenesis was performed on an EcoRI–BamHI fragment containing the last 38 codons of *ATP6* cloned in pUC19 (plasmid pSDC9) [12]. The mutated fragment was liberated by restriction with EcoRI and SapI and ligated with pSDC14 [12] cut with the same enzymes to reconstruct a whole *ATP6* gene with the S250P or L252P mutations. The resulting plasmids (pRK37 and pRK38, respectively) also contain the yeast mitochondrial *COX2* gene as a marker for mitochondrial transformation. The plasmids were introduced by co-transformation with the nuclear selectable *LEU2* plasmid Yep351 into the rho$^0$ strain DFS160 by microprojectile bombardment using a biolistic PDS-1000/He particle delivery system (Bio-Rad) as described [22]. Mitochondrial transformants (synthetic AKY13 and AKY14 respectively) were identified among the Leu + nuclear transformants by their ability to produce respiring clones when mated to the nonrespiring NB40-3C strain bearing a deletion in the mitochondrial *COX2* gene. One AKY13 and AKY14 clone was crossed to the *atp6::ARG8m* deletion strain MR10 [23] for the production of clones (called AKY5 and RKY66) harboring the MR10 nucleus and where the *ARG8m* ORF [24] had been replaced by recombination with the mutated *atp6-S250P* or *atp6-L252P* genes. The AKY5 clone was identified by its inability to grow in the absence of an external source of arginine and the ability to grow on respiratory medium. The RKY66 clone was identified by its inability to grow in the absence of an external source of arginine and the ability to grow on respiratory medium when crossed with the SDC30 strain bearing in the mitochondrial DNA the wild type copy of *ATP6* gene. Sequencing of the mutated *atp6* locus in AKY5 and RKY66 revealed no other changes than S250P or L252P, respectively.

### 2.2. Measurement of respiration and ATP synthesis/hydrolysis activities in whole mitochondria

For these assays, mitochondria were prepared by the enzymatic method of Ref. [25]. The rates of ATP synthesis were determined as described in Ref. [23]. For respiration ATP synthesis and transmembrane potential ($\Delta\Psi$) measurements, freshly prepared mitochondria were diluted to 0.15 mg/ml in the reaction medium thermostated at 28 °C and containing 10 mM Tris-maleate (pH 6.8), 0.65 M sorbitol, 0.3 mM EGTA, and 3 mM potassium phosphate. Oxygen consumption rates were measured using a Clarke electrode and an OXM204 oxymeter from Heito (France) as described [26]. The different respiration states were measured after consecutive additions of 4 mM NADH for State 2, 150 μM ADP for State 3 and State 4, 4 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) for uncoupled respiration and finally 12.5 mM ascorbate (Asc), 1.4 mM N,N,N,N,-tetramethyl-p-phenylenediamine (*TMPD*) for Complex IV respiration activity. The rates of ATP synthesis were determined in the same condition using 750 μM ADP. Aliquots were withdrawn from the oxygraph cuvette every 15 s and reaction was stopped by 3.5% (w/v) perchloric acid, 12.5 mM EDTA. Samples were then neutralized to pH 6.5 by addition of KOH, 0.3 M MOPS. ATP was quantified by luciferin/luciferase assay (ATPLite kit from Perkin Elmer) on an LKB bioluminometer. Participation of the $F_1F_O$-ATP synthase to ATP production was assessed by oligomycin addition (3 μg/ml). Variations in transmembrane potential ($\Delta\Psi$) were evaluated as in Ref. [27] by monitoring the quenching of rhodamine 123 fluorescence (0.5 μM) using a $\lambda_{exc}$ of 485 nm and a $\lambda_{em}$ of 533 nm using an FLX Spectrofluorimeter (SAFAS, Monaco) under constant stirring. Transmembrane potential was generated by addition of ethanol [1% (v/v) final concentration]. ATP synthesis (state 3 of respiration) was initiated by addition of 50 μM ADP. When State 4 was reached, respiratory was inhibited by adding 0.3 mM KCN in order to measure the $\Delta\Psi$ produced by the hydrolysis of the synthetized ATP. $\Delta\Psi$ was collapsed by adding 4 μM CCCP. The specific ATPase activity at pH 8.4 of non-osmotically protected mitochondria was measured as described in Ref. [28].

### 2.3. Miscellaneous procedures

Determination of $\rho^-/\rho^0$ cells in yeast cultures, SDS-PAGE and BN-PAGE, western blotting, Coomassie brilliant blue staining, pulse labeling of mtDNA encoded proteins were performed as described in Ref. [23].

## 3. Results

### 3.1. Respiratory growth and genetic stability of yeast mutants atp6-S250P and atp6-L252P

The leucine residues 220 and 222 of the human homolog of yeast Atp6p that are modified by the T9185C and T9191C mutations correspond respectively to serine 250 and leucine 252 of Atp6p [29]. The TCA and TTA codons specifying these residues were converted into proline CCA codon (see Materials and methods). Yeast *atp6*-S250P clones grew well on non-fermentable carbon

**Table 1**
Genotypes and sources of yeast strains.

| Strain | Nuclear genotype | mtDNA | Source |
|---|---|---|---|
| DFS160 | *MAT**a** leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1* | ρo | [24] |
| NB40-3C | *MAT**a** lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG* | ρ$^+$ *cox2-62* | [24] |
| MR6 | *MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ$^+$ | [23] |
| MR10 | *MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ$^+$ *atp6::ARG8$^m$* | [23] |
| SDC30 | *MAT**a** leu2Δ ura3-52 ade2-101 arg8: URA3 kar1-1* | ρ$^-$ *ATP6* | [23] |
| AKY13 | *MATα leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1* | ρ$^-$ *atp6-S250P* | This study |
| AKY14 | *MATα leu2Δ ura3-52 ade2-101 arg8::URA3 kar1-1* | ρ$^-$ *atp6-L252P* | This study |
| AKY5 | *MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ$^+$ *atp6 S250P* | This study This study |
| RKY66 | *MAT**a** ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ$^-$ *atp6-L252P* | This study<br>This study |