**Yeast models of mutations in the mitochondrial *ATP6* gene found in human cancer cells**

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**Abstract**

Since the discovery of somatic mtDNA mutations in tumor cells, multiple studies have focused on establishing a causal relationship between those changes and alterations in energy metabolism, a hallmark of cancer cells. Yet the consequences of these mutations on mitochondrial function remain largely unknown. In this study, *Saccharomyces cerevisiae* has been used as a model to investigate the functional consequences of four cancer-associated missense mutations (8914C>A, 8932C>T, 8953A>G, 9131T>C) found in the mitochondrial MT-ATP6 gene. This gene encodes the *a*-subunit of F1FO-ATP synthase, which catalyzes the last steps of ATP production in mitochondria. Although the four studied mutations affected well-conserved residues of the *a*-subunit, only one of them (8932C>T) had a significant impact on mitochondrial function, due to a less efficient incorporation of the *a*-subunit into ATP synthase. Our findings indicate that these *ATP6* genetic variants found in human tumors are neutral mitochondrial genome substitutions with a limited, if any, impact on the energetic function of mitochondria.

**Keywords**: ATP synthase, MT-ATP6, mitochondria, bioenergetics, cancer, mtDNA mutation.

1. **Introduction**

Cancer cells actively metabolize glucose producing excessive lactic acid and, at the same time, consume oxygen via oxidative phosphorylation (OXPHOS), a phenomenon called ‘aerobic glycolysis’ or the ‘Warburg effect’ ([Warburg, 1956a](#_ENREF_97" \o "Warburg, 1956 #1), [b](#_ENREF_98" \o "Warburg, 1956 #2)). Warburg interpreted this as a consequence of mitochondrial dysfunction. In support to this hypothesis, a number of studies reported that tumor mitochondria are structurally and functionally abnormal and incapable of generating normal levels of energy ([Chen et al., 2009](#_ENREF_17" \o "Chen, 2009 #4); [Galluzzi et al., 2010](#_ENREF_33" \o "Galluzzi, 2010 #7); [John, 2001](#_ENREF_40" \o "John, 2001 #6); [Ramanathan et al., 2005](#_ENREF_73" \o "Ramanathan, 2005 #5)). Furthermore, nuclear mutations in key mitochondrial metabolic enzymes, such as succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase 1 and 2 (IDH1/2) have been linked to uterine leiomyomas and paragangliomas ([Bardella et al., 2011](#_ENREF_7" \o "Bardella, 2011 #113); [Berrada et al., 2013](#_ENREF_8" \o "Berrada, 2013 #118); [Gupta et al., 2012](#_ENREF_38" \o "Gupta, 2012 #117); [Ward et al., 2010](#_ENREF_99" \o "Ward, 2010 #116); [Xiao et al., 2012](#_ENREF_102" \o "Xiao, 2012 #115)). Altered mitochondrial metabolism can have wide spread effects, such as an increase production of reactive oxygen species (ROS) and reduced Ca2+ uptake, which can in turn modulate the activities of several transcription factors, such as HIF1α (hypoxia-inducible factor 1α), induce apoptosis and activate mitochondrial-nucleus retrograde signaling pathways ([Wallace, 2012](#_ENREF_96" \o "Wallace, 2012 #8)). Based on these findings, it has been proposed that cancer is primarily a metabolic disease ([Seyfried and Shelton, 2010](#_ENREF_80" \o "Seyfried, 2010 #15)).

Almost two thousands of mitochondrial DNA (mtDNA) mutations have been found in various tumors, in the D-loop region and in RNA and protein-encoding genes ([Lu et al., 2009](#_ENREF_55" \o "Lu, 2009 #17)). However, for most of them, their impact on mitochondrial function has not been characterized and a role for these mutations in tumorigenesis remains elusive. Most data about a possible link between mtDNA mutations and cancer come from studies using cell lines bearing detrimental mtDNA mutations from patients with mitochondrial cytopathies. For instance, cybrids containing a common HeLa nucleus and mtDNA with mutations of the MT-ATP6 gene (8993T>G and 9176T>G) leading to NARP (neuropathy ataxia retinitis pigmentosa) or LS (Leigh) syndromes proved to confer an advantage in the early stage of tumor growth in a nude mice assay ([Shidara et al., 2005](#_ENREF_82" \o "Shidara, 2005 #20)). In another study, PC3 prostate cancer cell lines in which the 8993T>G mutation was introduced, generated much larger tumors than cells carrying wild type mtDNA ([Petros et al., 2005](#_ENREF_69" \o "Petros, 2005 #35)). It has been argued that the 8993T>G and 9176T>G mutations promote tumorigenesis by preventing apoptosis ([Shidara et al., 2005](#_ENREF_82" \o "Shidara, 2005 #20)). As mutations in mtDNA affecting the efficiency of the OXPHOS system result in an increased ROS production, this mechanism may be linked to ROS signaling ([Gupta et al., 2012](#_ENREF_38" \o "Gupta, 2012 #117)). Indeed, ROS levels were shown to be elevated in 8993T>C cybrids ([Carrozzo et al., 2004b](#_ENREF_15" \o "Carrozzo, 2004 #45); [Mattiazzi et al., 2004](#_ENREF_56" \o "Mattiazzi, 2004 #53)). Whereas a strong increase in ROS levels should induce apoptosis, a moderate increase in ROS may lead to the up-regulation of antioxidant enzymes and, therefore, stimulate tumor growth. Such mechanism was postulated in a colorectal cell line, where a heteroplasmic, but not homoplasmic, MT-ND5 mutation promoted tumorigenesis by an alteration of ROS production ([Park et al., 2009](#_ENREF_68" \o "Park, 2009 #27)).

Given the high mutational rate of the mitochondrial genome and the presence of numerous family or population-specific polymorphisms, it is difficult to distinguish between a neutral mtDNA variant and a disease-causing mutation. Multiple studies have also determined that the effects of deleterious mtDNA mutations may be exacerbated by concomitant mtDNA nucleotide changes that are not pathogenic *per se* and by unknown factors in the nuclear genetic background, i.e. so-called modifier genes ([Cai et al., 2008](#_ENREF_12" \o "Cai, 2008 #170); [Swalwell et al., 2008](#_ENREF_88" \o "Swalwell, 2008 #171)). Owing to the absence of mutagenesis methods for the mammalian mitochondrial genomes, *S. cerevisiae* has been utilized as an alternative model to investigate mtDNA mutations found in patients. Mitochondrial genetic transformation can be achieved in *S. cerevisiae* in a highly controlled fashion, by the biolistic delivery into mitochondria of *in*-*vitro*-made mutated mtDNA fragments, followed by their integration into wild type mtDNA by homologous DNA recombination ([Bonnefoy and Fox, 2001](#_ENREF_10" \o "Bonnefoy, 2001 #48)). Unable to stably maintain heteroplasmic mtDNA ([Okamoto et al., 1998](#_ENREF_66" \o "Okamoto, 1998 #121)), it is relatively easy to obtain yeast homoplasmic populations where all mtDNA molecules carry a mutation of interest. Several groups have exploited these attributes for the study of various pathogenic mtDNA mutations, for example in the genes encoding subunits of complexes III (MT-CYB), IV (MT-CO1, MT-CO3) ([Meunier, 2001](#_ENREF_57" \o "Meunier, 2001 #173); [Meunier et al., 2013](#_ENREF_58" \o "Meunier, 2013 #174)) and V (MT-ATP6) ([Kucharczyk et al., 2010](#_ENREF_46" \o "Kucharczyk, 2010 #39); [Kucharczyk et al., 2013](#_ENREF_47" \o "Kucharczyk, 2013 #37); [Kucharczyk et al., 2009a](#_ENREF_48" \o "Kucharczyk, 2009 #42); [Kucharczyk et al., 2009b](#_ENREF_49" \o "Kucharczyk, 2009 #41); [Rak et al., 2007a](#_ENREF_71" \o "Rak, 2007 #1); [Vindrieux et al., 2013](#_ENREF_94" \o "Vindrieux, 2013 #86)), and in MT-tRNA genes ([Feuermann et al., 2003](#_ENREF_31" \o "Feuermann, 2003 #278); [Montanari et al., 2008](#_ENREF_61" \o "Montanari, 2008 #265)), which have helped to better define the functional consequences of these mutations. Importantly, the consequences of these mutations in yeast corresponded to the reported severity of these mutations in humans in most cases, likely reflecting a high level of evolutionary conservation within the regions of the affected proteins and RNAs.

In this study, yeast has been used as a model to investigate the functional consequences of four missense mutations (8914C>A, 8932C>T, 8953A>G and 9131T>C) of the MT-ATP6 gene found in thyroid, parathyroid, prostate and breast cancer cells ([Abu-Amero et al., 2006](#_ENREF_1" \o "Abu-Amero, 2006 #33); [Costa-Guda et al., 2007](#_ENREF_21" \o "Costa-Guda, 2007 #63); [Petros et al., 2005](#_ENREF_69" \o "Petros, 2005 #35); [Tan et al., 2002](#_ENREF_89" \o "Tan, 2002 #36)). The MT-ATP6 gene encodes an essential component (*a*-subunit) of F1FO-ATP synthase (or complex V) that enables this enzyme to produce ATP by utilizing the energy from the electrochemical proton gradient across the mitochondrial inner membrane during electron transfer to oxygen by respiratory chain. Among four studied mutations, only one affected the function of ATP synthase, which argues against a role, at least for the three other mutations, in carcinogenesis by compromising the energetic activity of mitochondria.

1. **Materials and Methods**
   1. *Yeast strains and growth media.* The sources and genotypes of the strains used in this study are listed in Table 1. Yeast cells were grown on standard yeast rich media (1% Bacto yeast extract, 2% Bacto peptone) with 2% of glucose (YPGA), 2% of galactose (YPGalA), 2% of glycerol (YPGlyA), supplemented with 40 mg/L of adenine and 2% of Bacto agar for solid media ([Sherman, 1991](#_ENREF_81" \o "Sherman, 1991 #122)).

# Table 1. Genotypes and sources of yeast strains. 0 refers to the complete absence of mtDNA. + designates a complete mitochondrial genome. - cells contain only a fragment of the mitochondrial genome. The four + yeast cancer models investigated in this study (RKY60, RKY61, AKY1, and AKY2) are isogenic to the MR6 wild type yeast strain, except for the mtDNA *atp6* mutation.

|  |  |  |  |
| --- | --- | --- | --- |
| Strain | Nuclear genotype | mtDNA | Ref. |
| DFS160 | *MAT****a*** *leu2 ura3-52 ade2-101 arg8::URA3 kar1-1* | ρo | [Steele et al., 1996](#_ENREF_85) |
| NB40-3C | *MAT****a*** *lys2 leu2-3,112 ura3-52 his3HinDIII arg8::hisG* | ρ+ *cox2-62* | [Steele et al., 1996](#_ENREF_85) |
| MR6 | *MAT****a*** *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ+ *ATP6* | [Rak et al., 2007b](#_ENREF_72) |
| MR10 | *MAT****a*** *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ+ *atp6::ARG8m* | [Rak et al., 2007b](#_ENREF_72) |
| RKY57 | *MAT leu2Δ ura3-52 ade2-101 arg8URA3 kar1-1* | ρ*- atp6-P157T* | This study |
| RKY58 | *MAT leu2Δ ura3-52 ade2-101 arg8URA3 kar1-1* | ρ*- atp6-P163S* | This study |
| AKY8 | *MAT leu2Δ ura3-52 ade2-101 arg8URA3 kar1-1* | ρ*- atp6-I170V* | This study |
| AKY9 | *MAT leu2Δ ura3-52 ade2-101 arg8URA3 kar1-1* | ρ*- atp6-L232P* | This study |
| RKY60 | *MAT****a*** *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ+ *atp6 P157T* | This study |
| RKY61 | *MAT****a*** *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ*+atp6-P163S* | This study |
| AKY1 | *MAT****a*** *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ*+atp6-I170V* | This study |
| AKY2 | *MAT****a*** *ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 CAN1 arg8::hisG* | ρ*+atp6-L232P* | This study |

* 1. *ATP6 mutagenesis*. A schematic description of the method used to create the yeast *ATP6* cancer models is provided in Figure 1. Nucleotide and amino-acid changes induced by the mutations studied in the human *a*-subunit, and their equivalents in yeast are listed in Table 2. The QuikChange XL Site-directed Mutagenesis Kit of Stratagene and the following mutagenic oligonucleotides were used for the mutagenesis of the yeast *ATP6* gene (in bold is the modified codon): 5’GGTTGAGTATTCTTCTCATTATTCGTA**ACT**GCTGGTACACCATTACC (P157T); 5’TCATTATTCGTACCTGCTGGTACACCATTA**TCA**TTAGTACCTTTATTAGTTATTATTGAAACTTTATCTTATTTCGCTAGA (P163S); 5’CCATTAGTACCTTTATTAGTT**GTT**ATTGAAACTTTATCTTATTTCGCTAGAGC (I170V); and 5’GCTATGATCTTAGCCATTATGATG**CCA**GAATTCACTGGCCGTCGTTTTACAACGTCG (L232P).Mutagenesis was performed on a *Bam*HI-*Eco*RI fragment containing the 5’partof the *ATP6* gene cloned into pUC19 (plasmid pSDC8, ([Zeng et al., 2007a](#_ENREF_103" \o "Zeng, 2007 #44))). The mutated *ATP6* fragments were liberated and ligated at the same sites with pJM2 ([Steele et al., 1996](#_ENREF_85" \o "Steele, 1996 #105)). The resulting plasmids are called (pRK29 (with the P157T change), pRK27 (P163S), pRK30 (I170V), and pRK36 (L232P). The 3’ part of the wild type *ATP6* locus, excised from pSDC9 ([Rak et al., 2007a](#_ENREF_71" \o "Rak, 2007 #1)) with *Sap*I + *Eco*RI, was introduced in these plasmids, resulting in the final plasmids used for yeast transformation: pRK33, pRK31, pAK4-6, and pAK5-1, respectively. These plasmids were introduced by co-transformation with the nuclear selectable *LEU2* gene contained in the Yep351 plasmid, into the DFS160 strain devoid of mtDNA (ρo), by microprojectile bombardment using a biolistic PDS-1000/He particle delivery system (Bio-Rad) as described ([Bonnefoy and Fox, 2001](#_ENREF_10" \o "Bonnefoy, 2001 #48)). The DFS160 strain carries a mutation (*kar1-1*) that delays nuclear karyogamy ([Conde and Fink, 1976](#_ENREF_18" \o "Conde, 1976 #133)). Mitochondrial transformants were identified among the Leu+ nuclear transformants by their ability to produce respiring clones when mated to the non-respiring NB40-3C strain, bearing a partial deletion in the mitochondrial *COX2* gene. The resulting synthetic ρ*-* mtDNA clones were called RKY57 (ρ*-* P157T change), RKY58 (ρ*-* P163S), AKY8 (ρ*-* I170V), and AKY9 (ρ*-* L232P) (Table 1). These clones were crossed to MR10 strain ([Rak et al., 2007b](#_ENREF_72" \o "Rak, 2007 #47)) with a *arg8* nucleus and in which the coding sequence of *ATP6* gene was replaced by *ARG8m* (*atp6::ARG8m*), a mitochondrial version of the nuclear *ARG8* gene encoding a mitochondrial protein involved in arginine biosynthesis ([Steele et al., 1996](#_ENREF_85" \o "Steele, 1996 #105)). In these crosses the mutated *atp6* genes can replace the *ARG8m* marker by mtDNA recombination, resulting in strains with a complete (+) mtDNA carrying one of the *atp6* mutations: RKY60 (+ P157T), RKY61 (+ P163S), AKY1 (+ I170V) and AKY2 (+ L232P) (Table 1). These clones were identified by arginine auxotrophy and verified by DNA sequencing.

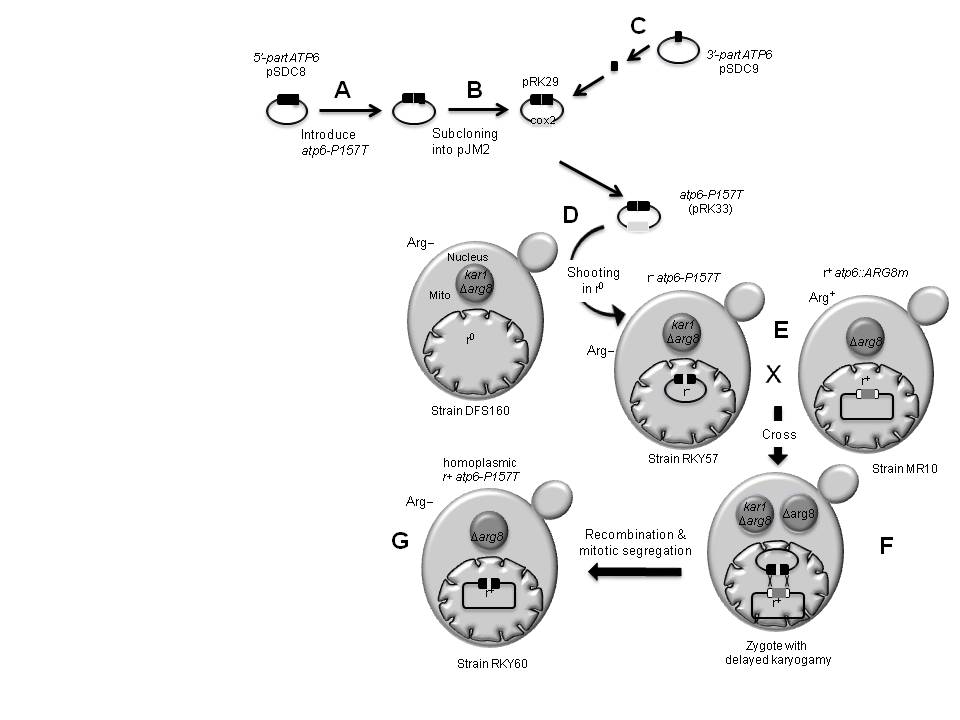


Figure 1. Construction of yeast model bearing a human mtDNA cancer mutation in the *ATP6* gene. Steps A, B, C describe the construction of the pRK33 plasmid bearing the yeast *ATP6* gene with the P157T mutation and the *COX2* gene as a marker for mitochondrial transformation. (D) pRK33 was introduced into the DFS160 strain mitochondria, devoid of mtDNA (ρ0), which has a null allele of the nuclear *ARG8* gene (*arg8Δ*) and a mutation (*kar1-1*) that strongly delays nuclear fusion, enabling mtDNA transfer from one haploid nuclear background to another ([Conde and Fink, 1976](#_ENREF_18" \o "Conde, 1976 #133)). (E) The resulting synthetic ρ− strain (RKY57), unable grow in the absence of external arginine (Arg−), was crossed with an arginine prototrophic (Arg+) strain that contains ρ+ mtDNA deleted for *ATP6* (*atp6Δ::ARG8m*). *ARG8m* is a mitochondrial version of a nuclear gene (*ARG8*) and encodes a yeast mitochondrial protein involved in arginine biosynthesis ([Steele et al., 1996](#_ENREF_85" \o "Steele, 1996 #105)). (F) Since the *ARG8m* clone used for the deletion of *ATP6* is flanked by ∼100 bp of the *ATP6* locus on each side, homologous recombination can mediate the replacement of *ARG8m* with the *atp6-P157T* gene. (G) The subsequent mitotic segregation then produces ρ+ cells with the *atp6-P157T* mutation, in a pure (homoplasmic) form that can be identified by virtue of their inability to grow in the absence of arginine.

* 1. *Measurement of mitochondrial respiration, ATP synthesis and mitochondrial membrane potential*. Mitochondria were prepared by the enzymatic method as described ([Guerin et al., 1979](#_ENREF_36)). For all assays, they were diluted to 0.15 mg/ml in 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 after consecutive addition of 4 mM NADH (state 4 respiration), 150 µM ADP (state 3) or 4 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (uncoupled respiration), as described previously ([Rigoulet and Guerin, 1979](#_ENREF_75)). The rates of ATP synthesis were determined as in ([Rak et al., 2007b](#_ENREF_72)), in the presence of 4 mM NADH and 750 µM ADP; aliquots were withdrawn from the oxygraph cuvette every 15 seconds and the reaction was stopped with 3.5% (w/v) perchloric acid, 12.5 mM EDTA. The samples were then neutralized to pH 6.5 by addition of KOH, 0.3 M MOPS. Synthesized ATP was quantified using a luciferin/luciferase assay (ATPLite kit from Perkin Elmer) on a LKB bioluminometer. Participation of the F1FO-ATP synthase to ATP production was assessed by measuring the sensitivity of ATP synthesis to oligomycin (3 μg/ml). Variations in mitochondrial membrane potential (ΔΨ) were evaluated as in ([Emaus et al., 1986](#_ENREF_29)) by monitoring the quenching of rhodamine 123 fluorescence (0.5 μM) using a λexc of 485 nm and a λem of 533 nm under constant stirring using a FLX Spectrofluorimeter (SAFAS, Monaco). The specific ATPase activity at pH 8.4 of non-osmotically protected mitochondria was measured as described in ([Somlo, 1968](#_ENREF_83)). Student’s t-test was used to assess significant differences with the respective control.
  2. *Determination of ROS levels in cells*. The cytosolic superoxide (O2–), hydroxyl (OH–) and peroxynitrite (ONOO–) anions accumulation were measured by flow cytometry using dihydroethidium (DHE, Sigma). Cells were grown in YPGA, YPGalA or YPGlyA media to OD=1-2. 3 OD of cells were then converted to protoplasts with zymolyase 20T (AsmBio) for 1 hour at 37°C in PBS pH 7.5/1 M sorbitol buffer. Protoplasts were washed twice and diluted to 1x107/ml density in the same buffer and 10 µM of DHE were added. The next day, the cells were washed with PBS pH 7.5/1 M sorbitol and suspended to 1x106/ml concentration for flow cytometry using BD FACS Calibur. The cells were sonicated and then subjected to FACS analysis; 10,000 cells were counted for each sample. Student’s t-test was used to assess significant differences with the respective control.
  3. *Miscellaneous procedures.* Scoring -/0 cells in yeast cultures, SDS–PAGE and BN–PAGE, Western blotting and pulse labeling of mtDNA encoded proteins were performed as described in ([Rak et al., 2007b](#_ENREF_72" \o "Rak, 2007 #47)), except that precast Novex 3-12% Bis-tris gels were used for BN-PAGE.
  4. *Multiple sequence* *alignment and homology modelling of yeast ac10 complex*. Multiple sequence alignment of ATP synthase *a*-subunits was performed using ClustalW ([Thompson et al., 1994](#_ENREF_91" \o "Thompson, 1994 #126)). TMHMM 2.0 and PSIPRED 3.3 servers were used to predict transmembrane segments and secondary structures, respectively ([Buchan et al., 2013](#_ENREF_11" \o "Buchan, 2013 #127); [Krogh et al., 2001](#_ENREF_45" \o "Krogh, 2001 #128)). Figure 2A was drawn using the ESPRIPT program ([Robert and Gouet, 2014](#_ENREF_76" \o "Robert, 2014 #129)). The homology model of yeast *ac10* complex is based on the atomic model built in the cryo-electron microscopy density map of the bovine ATP synthase (pdb:5arh; emdb:3166) ([Zhou et al., 2015](#_ENREF_105" \o "Zhou, 2015 #108)) and the yeast *c*10-ring crystal structure (pdb:2hld) ([Kabaleeswaran et al., 2006](#_ENREF_42" \o "Kabaleeswaran, 2006 #124)). It was built using Phire2 ([Kelley et al., 2015](#_ENREF_43" \o "Kelley, 2015 #110)), regularized using Coot ([Emsley et al., 2010](#_ENREF_30" \o "Emsley, 2010 #130); [Pettersen et al., 2004](#_ENREF_70" \o "Pettersen, 2004 #131)), and energy minimized using Phenix ([Adams et al., 2010](#_ENREF_2" \o "Adams, 2010 #132)). Figure 2B was drawn using Pymol (DeLano WL, DeLano Scientific, San Carlos California, USA, 2002).

1. **Results**

The four MT-ATP6 mutations investigated in this study (8914C>A, 8932C>T, 8953A>G and 9131T>C) affect well conserved residues of the *a*/6 subunit from various origins (*Homo sapiens*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Alligator mississippiensis*, *Brachydanio rerio*, *Caenorhabditis elegans*, *Cionia intestalis*, *Drosophilia melanogaster*, *Neurospora crassa*, *Podospora anserina* and *Xenopus laevis* ([Kucharczyk et al., 2009c](#_ENREF_50" \o "Kucharczyk, 2009 #43)); see also Figure 2A). These mutations lead to the following amino acid changes in the human *a*-subunit: P130T, P136S, I143V and L202P, respectively (Figure 2A). The corresponding *ATP6* codons in yeast were modified to induce equivalent amino acid changes: P157T, P163S, I170V and L232P, respectively (Table 2, Figure 2A).

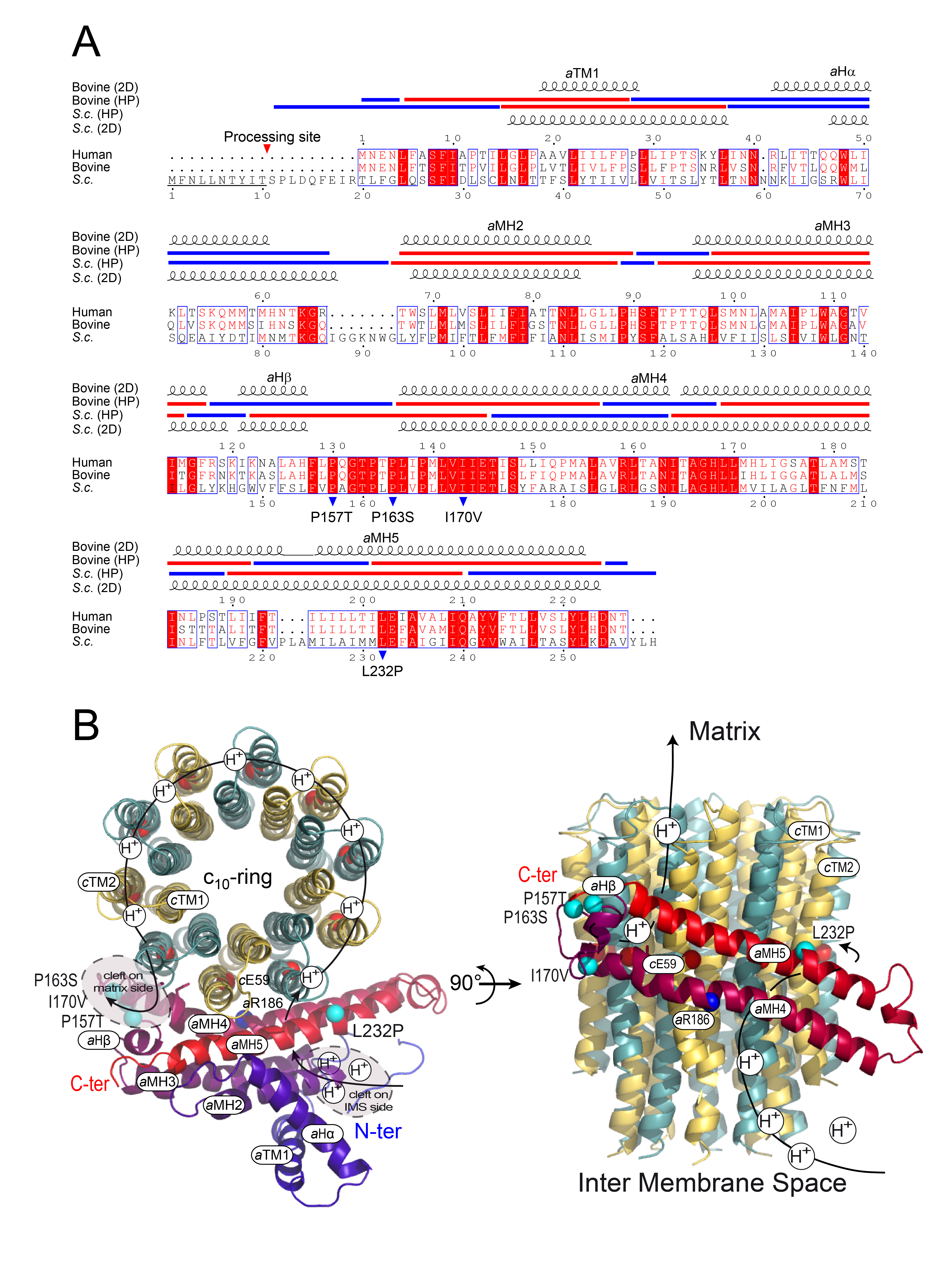
**Table 2.** Cancer-related mutations in human *ATP6* and their equivalents in yeast. \* according to MITOMAP (www.mitomap.org); n.d., not determined; A.a., amino acid; Ps, polymorphic site; Frequency, means occurrence in mtDNA from cancer samples.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| In human | | | | | | |  | In yeast | |
| Type of tumor | Nucleotide change | A.a.  change | Hetero-plasmy | Ps\* | Frequency | Ref. |  | Codon change | A.a. change |
| Thyroid | 8914C>A | P130T | Yes | No | 2 / 26 | [Abu-Amero et al., 2006](#_ENREF_1) |  | CCT>ACT | P157T |
| Prostate | 8932C>T | P136S | n.d. | Yes | n.d. | [Petros et al., 2005](#_ENREF_69) |  | CCA>TCA | P163S |
| Para-thyroid | 8953A>G | I143V | n.d. | Yes | 1 / 34 | [Costa-Guda et al., 2007](#_ENREF_21) |  | ATT>GTT | I170V |
| Breast | 9131T>C | L202P | No | No | 1 / 19 | [Tan et al., 2002](#_ENREF_89) |  | TTA>CCA | L232P |

* 1. *Topological positions of cancer related mutations in the a-subunit.*

The *a*-subunit and a ring of *c*-subunits (8 in humans ([Watt et al., 2010](#_ENREF_100" \o "Watt, 2010 #320)), 10 in yeast ([Stock et al., 1999](#_ENREF_87" \o "Stock, 1999 #275))) are responsible for proton transport across the membrane domain (FO) of ATP synthase ([Fillingame et al., 2003](#_ENREF_32" \o "Fillingame, 2003 #307); [Walker, 2013](#_ENREF_95" \o "Walker, 2013 #472)). This transport drives the rotation of the *c*-ring and leads to conformational changes in the catalytic domain (F1) of the enzyme ultimately promoting ATP synthesis. Cryo-electron microscopy analysis of ATP synthase from mammals, fungi and bacteria have revealed four horizontal membrane-intrinsic -helices (MH) in the *a*-subunit running along the *c*-ring ([Allegretti et al., 2015a](#_ENREF_3" \o "Allegretti, 2015 #180); [Morales-Rios et al., 2015](#_ENREF_63" \o "Morales-Rios, 2015 #111); [Zhou et al., 2015](#_ENREF_105" \o "Zhou, 2015 #108)). Due to the high conservation of *a*- and *c*-subunits during evolutin, a structural homology model of this 4-helix bundle was constructed for the yeast *a*-subunit (referred to as *a*MH2-5) in interaction with the *c*10-ring ([Watt et al., 2010](#_ENREF_100" \o "Watt, 2010 #320)) (Figure 2B). In the N-terminal domain of the *a*-subunit, the density maps of bovine ATP synthase ([Zhou et al., 2015](#_ENREF_105" \o "Zhou, 2015 #108)) revealed an additional hydrophilic helical segment (referred to as *a*H) located in the matrix and a helical segment that spans the membrane (referred to as *a*TM1). As a result, the N-ter of *a*TM1 is located in the IMS. Consistent with this, it has been shown that the yeast protein is synthesized as a precursor containing a stretch of 10 residues on the N-ter that is cleaved in the inter membrane space ([Michon et al., 1988](#_ENREF_59" \o "Michon, 1988 #188); [Osman et al., 2007](#_ENREF_67" \o "Osman, 2007 #148); [Zeng et al., 2007b](#_ENREF_104" \o "Zeng, 2007 #147)). *a*TM1 is located on the outer surface of the complex. Indeed, previous cross-linking experiments have provided strong indication that *a*TM1 belongs to the contact zone between two ATP synthase monomers ([Velours et al., 2011](#_ENREF_93" \o "Velours, 2011 #517)). Two electrically charged residues (*a*R186 and *c*E59) known to be involved in the transfer of protons between *a*-subunit and the *c*-ring, are located at the interface between *a*-subunit and the *c*-ring, near the middle of the membrane ([Fillingame et al., 2003](#_ENREF_32" \o "Fillingame, 2003 #307); [Walker, 2013](#_ENREF_95" \o "Walker, 2013 #472)). A hydrophilic cleft on the external side of the inner membrane is presumed to enable protons to reach *c*E59 from the IMS and, upon *c*-ring rotation, the protons are supposed to be released into a second hydrophilic cleft on the matrix side of the membrane ([Allegretti et al., 2015b](#_ENREF_4" \o "Allegretti, 2015 #514)) (Figure 2B).

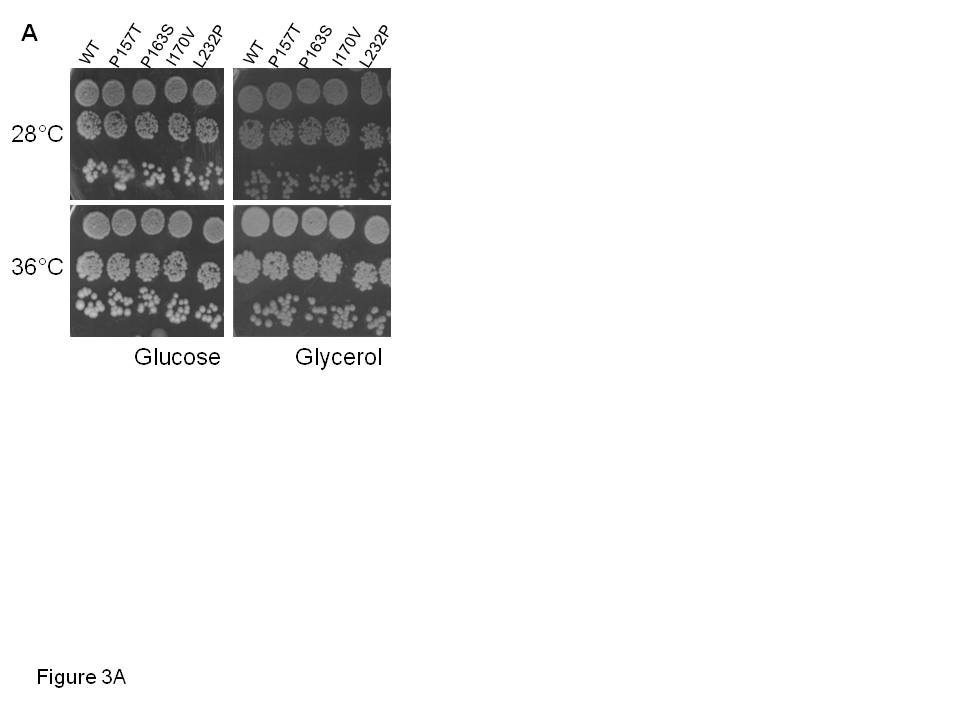
Three of the four studied Atp6p mutations are located on the matrix side of the membrane, in the loop connecting *a*MH3 and *a*MH4 (P157T, P163S), and in the beginning of *a*MH4 (I170V). The fourth one (L232P) is more embedded in the membrane in *a*MH5 (Figure 2B).

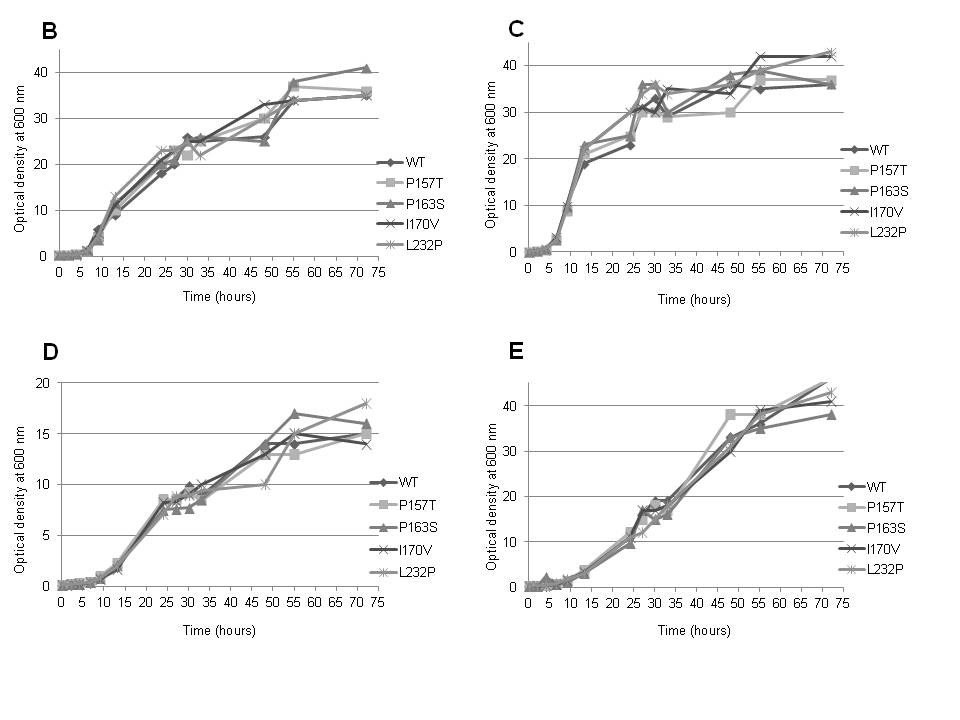


**Fig. 2.** *Sequence alignments of a-subunits from human, bovine and yeast S. cerevisiae* and *3D topology of cancer-related mutations in the yeast ac10 model*. (A) Human (top) and yeast (bottom) amino-acid sequences are numbered. The yeast protein contains a cleavable 10 residues stretch at the N-ter, as indicated. Blue arrowheads indicate the cancer-related mutations investigated in this study, and the mutated residues are numbered according to the yeast *a*-subunit. Above the alignment, bovine and yeast proteins are predicted by PSIRED (2D, secondary structure) and TMHMM (HP, hydropathy plot). Hydrophilic and hydrophobic regions are indicated by blue and red lines, respectively. (B)The figure shows a structural homologymodel of the yeast *a-*subunit in complex with the *c*10-ring (PDB id: 2hld) viewed from the matrix (left) and from the membrane plane (right). The *a*R186 and *c*E59 residues and the cancer-related mutations (P157T, P163S, I170V and L232P) are shown as blue, red and cyan spheres, respectively. The arrowed line indicates the proton pathway during ATP synthesis. Protons enter and exit the *c*-ring/*a*-subunit through hydrophilic clefts within the membrane, represented by grey areas surrounded by dashed lines ([Allegretti et al., 2015b](#_ENREF_4" \o "Allegretti, 2015 #514)). For clarity, the predicted *a*TM1, *a*Hαand *a*HM2-3 helices are not represented in the right panel. The *a*-subunit is coloured using a linear ramp from blue (N-ter) to red (C-ter); adjacent *c*-subunits are in different colours (gold and green).

* 1. *Respiratory growth and stability of the mitochondrial genome*

The optimal growth temperature for yeast is 28°C. At 37°C, mitochondria display some partial damage, apparently due to heat sensitivity of the yeast mitochondrial translation system ([Dibrov et al., 1998](#_ENREF_24" \o "Dibrov, 1998 #347); [Lefebvre-Legendre et al., 2001](#_ENREF_51" \o "Lefebvre-Legendre, 2001 #195)). Since our study aimed to model human mtDNA mutations in yeast their consequences were investigated in yeast cells grown at 28°C and 36°C (not at 37°C, in order to limit the detrimental heat effect). Neither of the studied Atp6p mutations presented an obvious effect on yeast growth on fermentable substrates (glucose) or carbon sources, where the presence of a functional ATP synthase is required (like glycerol), both at 28°C and 36°C (Figure 3A,B). These results did not necessarily mean that the activity of ATP synthase was unaffected in the mutants. Indeed, a decrease in its activity by at least 80% is necessary for the affectation of the respiratory growth in yeast ([Kucharczyk et al., 2010](#_ENREF_46" \o "Kucharczyk, 2010 #39); [Mukhopadhyay et al., 1994](#_ENREF_65" \o "Mukhopadhyay, 1994 #116)). Mutations in *ATP6* often increased, for unknown reason, the production of -/0 *petite* cells issued from large deletions in the mtDNA ([Bietenhader et al., 2012](#_ENREF_9" \o "Bietenhader, 2012 #58); [Contamine and Picard, 2000](#_ENREF_19" \o "Contamine, 2000 #59); Rak et al., 2007a; Kucharczyk et al., 2009). Interestingly, one of the mutants analyzed in this study (P163S) had a significantly higher tendency to lose mtDNA when grown at 36°C (55% of *petites* *versus* 34% in the MR6 wild type strain), indicating that this mutation is possibly detrimental to ATP synthase at elevated temperature.

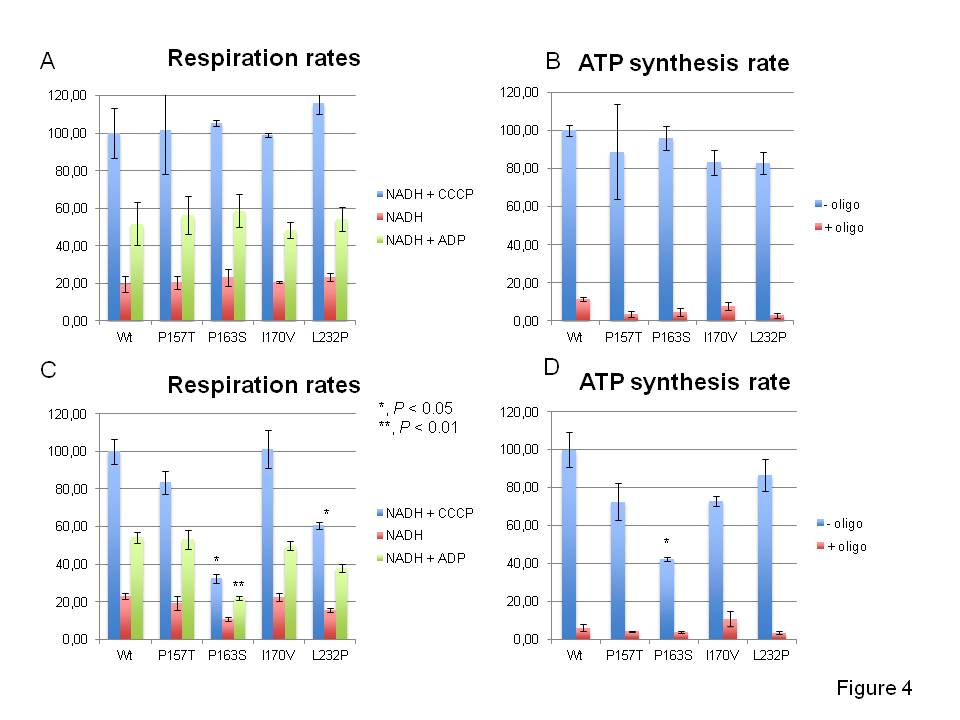


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**Fig. 3.** *Influence of cancer-related mutations in Atp6p on yeast respiratory growth*. (A) Fresh liquid glucose cultures of wild type yeast and the strains with cancer-related *atp6* mutations were serially diluted and spotted on rich glucose and rich glycerol plates. The plates were photographed after 4 days of incubation at the indicated temperatures. (B-D) Growth curves in liquid glucose (B, C) or glycerol (D, E) at 28°C (B, D) or 36°C (C, E). The growth tests are representative of at least two replicates.

* 1. *Mitochondrial respiration and ATP synthesis*

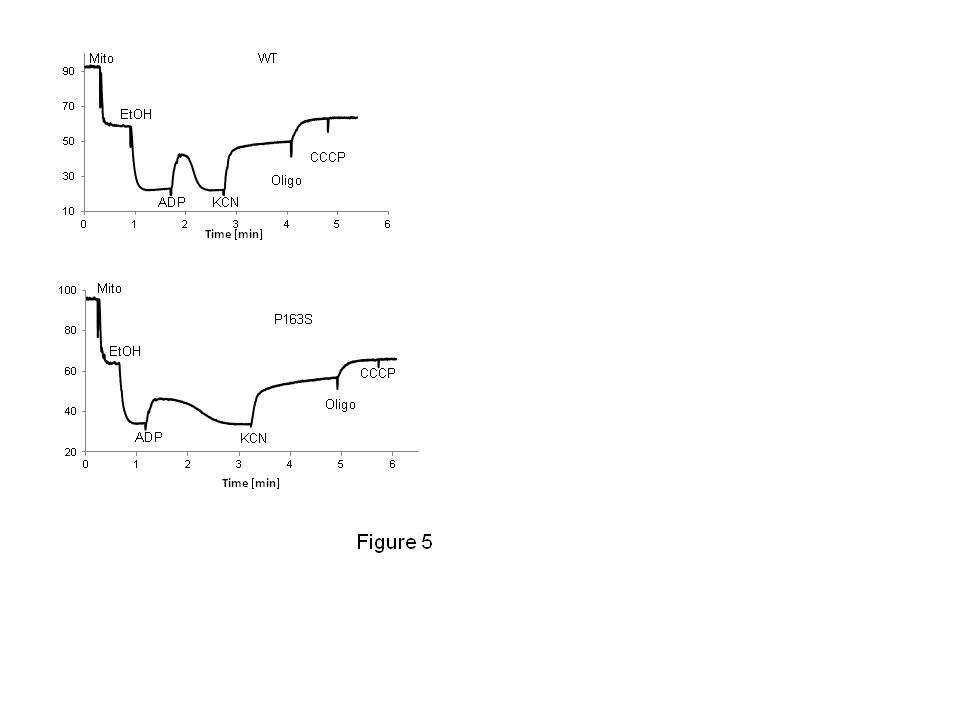
The impact of the cancer related *atp6* mutations on oxidative phosphorylation was evaluated by measuring the rates of oxygen consumption and ATP synthesis in isolated, osmotically-protected, mitochondria. The mutant mitochondria isolated from cells grown at 28°C respired and produced ATP like the corresponding wild type mitochondria (Figure 4A,B). When grown at 36°C, the P163S mutant displayed significant deficits (60%) in both respiration and ATP synthesis with respect to the wild type (Figure 4C,D). The residual respiratory activity in this mutant was efficiently (3-4 fold) stimulated by CCCP as in wild type yeast, indicating that the passive permeability to protons of the inner mitochondrial membrane was not modified in the P163S mutant. Respiration and ATP synthesis rates were much less affected in the other mutants grown at 36°C.



**Fig. 4**. *Influence of cancer-related mutations in Atp6p on yeast mitochondrial respiration and ATP synthesis*. Mitochondria were isolated from wild type yeast and the mutant strains with cancer-related mutations in the mitochondrial *ATP6* gene grown in rich galactose medium, at 28°C (A, B) or 36°C (C, D). Oxygen consumption rates were measured after consecutive addition of 4 mM NADH (state 4 respiration), 150 µM ADP (state 3) or 4 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (uncoupled respiration); they are expressed in percentage of the uncoupled respiration of wild type mitochondria (panels A and C). The rates of ATP synthesis were determined using 4 mM NADH and 750 µM ADP, in the presence/absence of 3 M oligomycin as indicated, and are expressed in percent with respect to wild type mitochondria (Panels B and D). The error bars and *P*-values indicated by \* were calculated from three independent experiments.

* 1. *Mitochondrial membrane potential*

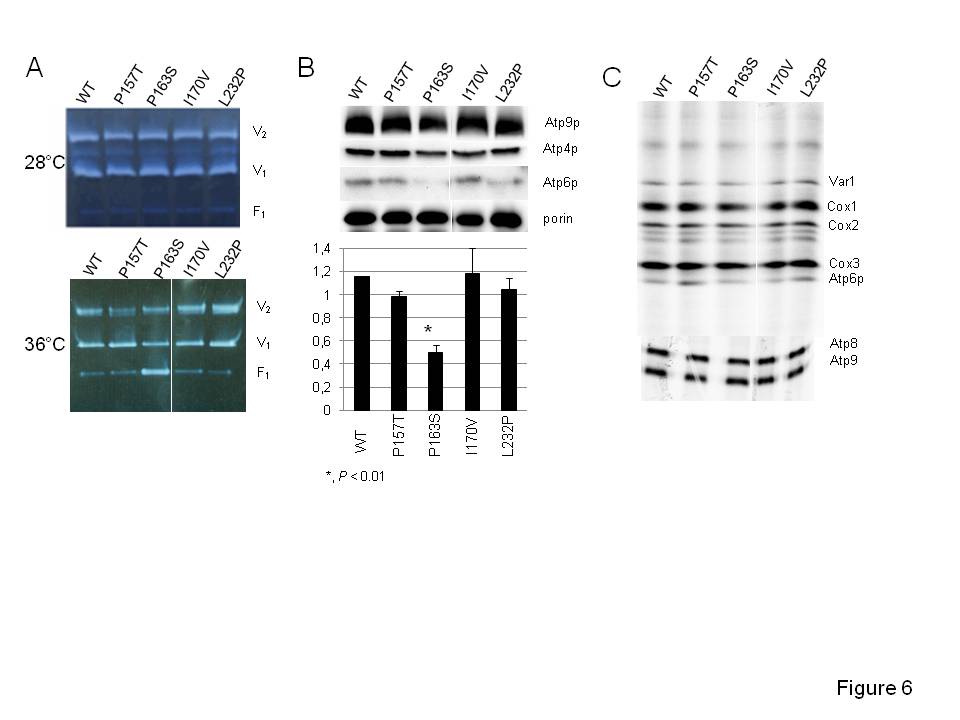
The consequences of the Atp6p mutations were further evaluated by monitoring variations in mitochondrial membrane potential (), through the fluorescence quenching of rhodamine 123. In these experiments, all the mutant mitochondria behaved mostly like the controls, except those from the P163S mutant grown at 36°C. As shown in Figure 5, these mitochondria required a much longer time to erase the drop in  induced by the addition of ADP upon energization of the mitochondrial inner membrane with ethanol. This reflects a slower phosphorylation rate of the added ADP by ATP synthase, which is consistent with the lower rate of ATP synthesis measured in these mitochondria (see above).



**Fig. 5.** *Influence of the cancer related atp6-P163S mutation on mitochondrial membrane potential in yeast cells grown at 36°C*. Mitochondria were isolated from wild type yeast and the *atp6*-*P163S* mutant grown at 36°C. Variation in mitochondrial membrane potential was monitored by rhodamine 123 fluorescence quenching. 0.5 g/ml rhodamine 123, 0.15 mg/ml mitochondrial proteins (*Mito*), 10 l ethanol (*EtOH*), 0.2 mM potassium cyanide (*KCN*), 50 M ADP, and 3 M CCCP were added. The shown profiles are representative of three independent experiments.

* 1. *ATP synthase assembly/stability*

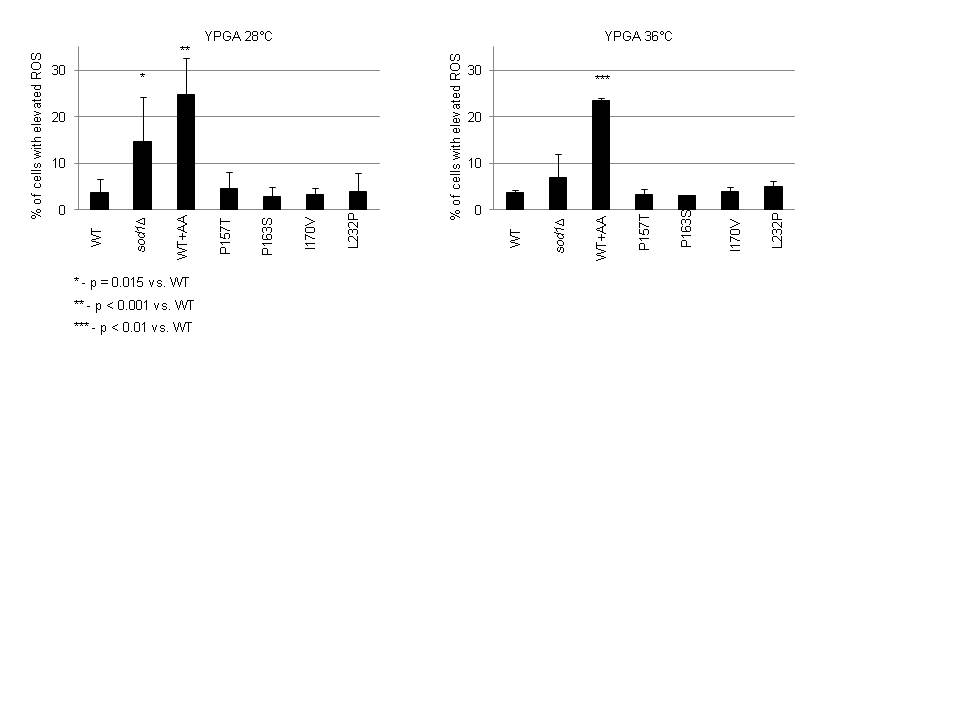
The influence of the Atp6p mutations on the assembly/stability of ATP synthase was investigated by Blue-Native-Polyacrylamide Gel Electrophoresis (BN-PAGE). In the conditions used here, ATP synthase was mainly detected as dimeric (V2) and monomeric (V1) species, and as low amounts of free F1 particles. Fully assembled F1FO complexes normally accumulated in all mutant samples, except those from the P163S mutant grown at 36°C in which higher amounts of free F1 particles were detected (Figure 6A). This could be due to a less efficient synthesis or a defective assembly of the mutated Atp6p. Pulse labeling experiments of the mtDNA-encoded proteins in P163S cells grown at 36°C revealed mostly unaffected synthesis of the mutated Atp6p (Figure 6C). However, its steady-state concentration was substantially decreased (Figure 6B). It can be inferred that at elevated temperatures, the P163S mutation partially compromises the incorporation of Atp6p into ATP synthase, followed by a rapid degradation of the unassembled protein.

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**Fig. 6.** *Influence of cancer-related mutations in the mitochondrial ATP6 gene on the assembly/stability and synthesis of Atp6p in yeast*. Mitochondria were prepared from wild type yeast and the strains with cancer-related mutations in the mitochondrial *ATP6* gene grown at 28°C or 36°C. (A) BN-PAGE analyses of mitochondrial proteins. Mitochondrial protein complexes were extracted with 2% digitonin and 100 g of protein samples were run in BN gels. Dimeric (V2) and monomeric (V1) F1FO complexes, and free F1 were revealed in gel by their ATPase activity. (B) SDS-PAGE analyses of mitochondrial proteins isolated from strains grown at 36°C. Samples of 20 µg of mitochondrial proteins were run in a 12% polyacrylamide SDS-containing gel and probed with antibodies against the indicated proteins. Densitometry of Atp6p content in two independent experiments normalized to porin is shown. (C) Mitochondrial protein synthesis. Proteins encoded by mtDNA were labeled in whole 36°C-grown cells with [35S]-(methionine+cysteine) for 20 min in the presence of cycloheximide to inhibit cytosolic protein synthesis. Following the labeling reactions, total protein extracts were prepared from the cells (0.2 OD at 650 nm) and separated by SDS-PAGE. For a better resolution of Cox3p and Atp6p, a 12% polyacrylamide gel containing 6 M urea was used (up). The Atp8p and Atp9p were separated using a 16.5% polyacrylamide gel (bottom). The gels were dried and analyzed with a PhosphorImager. The results shown in panels A,B, D are representative of two independent experiments.

* 1. *ROS levels in cells*

Defects in mitochondrial oxidative phosphorylation often lead to an increased production of reactive oxygen species, because of a higher rate of electron diversion to oxygen from their normal pathway ([Houstek et al., 2006](#_ENREF_39" \o "Houstek, 2006 #32)). Given the importance of ROS in cell-signaling pathways during tumorigenesis ([Gupta et al., 2012](#_ENREF_38" \o "Gupta, 2012 #117)), the impact of the studied *atp6* cancer mutations on the accumulation of ROS in cells was evaluated, using dihydroethidium as a probe ([Stephens et al., 2003](#_ENREF_86" \o "Stephens, 2003 #98)). As controls, we analyzed yeast cells deleted for the *SOD1* gene, which encodes a cytosolic copper-zinc superoxide dismutase that enables detoxification of superoxide ([Rexroth et al., 2012](#_ENREF_74" \o "Rexroth, 2012 #99)) and wild type cells treated for 45 minutes with 20 µM Antimycin A, an inhibitor of complex III ([Dzierzbicki et al., 2012](#_ENREF_28" \o "Dzierzbicki, 2012 #123)). As expected, higher ROS levels were observed in *sod1* cells and in wild type cells treated with Antimycin A, whereas none of the analyzed mutants showed an increased accumulation of ROS with respect to untreated wild type yeast cells (Figure 7).



**Fig. 7.** *Influence of cancer-related atp6 mutations on the accumulation of ROS in yeast cells*. Cells cultured to OD=1 in rich glucose medium at 28°C were treated with zymolyase to remove their wall and then incubated with 10 M dihydroethidium overnight at 4°C. Cells displaying elevated ROS levels were scored by flow cytometry (FL1 channel, 10000 cells). The error bars and P-values (\*) were calculated from three independent experiments.

1. **Discussion**

We previously investigated seven mutations (9176T>G, 8851T>C, 8993T>G, 9191T>C, 9176T>C, 8993T>C, and 9185T>C) in the yeast mitochondrial *ATP6* gene equivalent to mutations found in individuals with neuropathy, ataxia and retinitis pigmentosa (NARP), Leigh Syndrome (LS), or bilateral striatal lesions in childhood (BSLC) ([Kucharczyk et al., 2010](#_ENREF_46" \o "Kucharczyk, 2010 #39); [Kucharczyk et al., 2013](#_ENREF_47" \o "Kucharczyk, 2013 #37); [Kucharczyk et al., 2009a](#_ENREF_48" \o "Kucharczyk, 2009 #42); [Kucharczyk et al., 2009b](#_ENREF_49" \o "Kucharczyk, 2009 #41); [Rak et al., 2007a](#_ENREF_71" \o "Rak, 2007 #1); [Vindrieux et al., 2013](#_ENREF_94" \o "Vindrieux, 2013 #86)). All these mutations significantly decreased the rate of mitochondrial ATP synthesis in yeast, by 30 to >95% compared to the wild type, due to functional impairment or a less efficient incorporation of the *a*-subunit into ATP synthase. Importantly, these findings in yeast correspond to the reported severity of these mutations in humans, which likely reflects a high level of evolutionary conservation within the affected regions of the *a*/6 subunit ([Baracca et al., 2000](#_ENREF_5" \o "Baracca, 2000 #11); [Baracca et al., 2007](#_ENREF_6" \o "Baracca, 2007 #34); [Carrozzo et al., 2000](#_ENREF_13" \o "Carrozzo, 2000 #72); [Carrozzo et al., 2004a](#_ENREF_14" \o "Carrozzo, 2004 #79); [Cortes-Hernandez et al., 2007](#_ENREF_20" \o "Cortes-Hernandez, 2007 #4); [De Meirleir et al., 1995](#_ENREF_23" \o "De Meirleir, 1995 #94); [Dionisi-Vici et al., 1998](#_ENREF_25" \o "Dionisi-Vici, 1998 #120); [Houstek et al., 2006](#_ENREF_39" \o "Houstek, 2006 #32); [Mattiazzi et al., 2004](#_ENREF_56" \o "Mattiazzi, 2004 #53); [Morava et al., 2006](#_ENREF_64" \o "Morava, 2006 #38)).

The present study on the four cancer-related *ATP6* variants (8914C>A, 8932C>T, 8953A>G and 9131T>C) using yeast as a model reveals a complete different picture. Indeed, while these mutations affect relatively well-conserved residues of the *a*-subunit, only one of them (8932C>T) had a significant impact on ATP synthase characterized by a less efficient assembly/stability of the *a*-subunit in yeast cells grown at elevated temperatures. In this respect, it is interesting to note that all the residues affected by these mutations (P157, P163, I170, and L232 respectively (yeast numbering) are in quite remote positions from the contact zone near the middle of the membrane where protons are exchanged between the *a*-subunit and the *c*-ring (Figure 2B). However, the detrimental consequences of the 8932C>T mutation indicate that the P163 residue, located in a loop connecting the last two membrane embedded helical segments of the *a*-subunit (*a*MH4 and *a*MH5), is important for an efficient folding of the protein whereas the three other residues may be considered as not critical, neither for the assembly nor for the functioning of ATP synthase. The almost normal production of ROS in the mutants provides another line of evidence that the analyzed mutations have only mild if any effect on the functioning of ATP synthase.

Since the discovery of somatic mtDNA mutations in tumor cells, multiple studies (reviewed in ([Senyilmaz and Teleman, 2015](#_ENREF_79" \o "Senyilmaz, 2015 #539); [Wallace, 2012](#_ENREF_96" \o "Wallace, 2012 #8))) have focused on establishing a causal relationship between those changes and the alterations in energy metabolism that are a hallmark of cancer cells. The picture that emerges from recent studies suggests that perturbations in mitochondrial OXPHOS can contribute to cancer progression by activating retrograde signaling pathways that modulate nuclear gene expression in a conductive way towards tumorigenesis ([Gaude and Frezza, 2014](#_ENREF_34" \o "Gaude, 2014 #80); [Guha and Avadhani, 2013](#_ENREF_37" \o "Guha, 2013 #533); [Senyilmaz and Teleman, 2015](#_ENREF_79" \o "Senyilmaz, 2015 #539); [Srinivasan et al., 2015](#_ENREF_84" \o "Srinivasan, 2015 #532)). Nevertheless, functional mitochondrial genome is essential in cancer cells, as the ρ0 derivatives devoid of mtDNA generally exhibit reduced tumorigenicity ([Cavalli et al., 1997](#_ENREF_16" \o "Cavalli, 1997 #25); [Morais et al., 1994](#_ENREF_62" \o "Morais, 1994 #24); [Weinberg et al., 2010](#_ENREF_101" \o "Weinberg, 2010 #529)). In cybrid cell lines injected into athymic mice mild mtDNA mutations increase tumorigenicity, while those that cause severe mitochondrial dysfunction prevent tumor formation ([Cruz-Bermudez et al., 2015](#_ENREF_22" \o "Cruz-Bermudez, 2015 #534)).

On the other hand, it is clear that many of the somatic mtDNA variants found in cancer samples are passenger mutations that do not offer any particular advantage in tumor progression. Such mutations are fixed by genetic drift in a rapidly expanding clonal population of proliferating cancer cells, and are thus a result rather than a cause of the carcinogenic process. Many such variants correspond to polymorphic sites and mutational hotspots ([Vega et al., 2004](#_ENREF_92" \o "Vega, 2004 #528)). In a recent comprehensive study of 1675 tumor–normal pairs from 31 tumor types, no evidence of positive selection was found for any of the 1907 identified somatic mtDNA substitutions ([Ju et al., 2014](#_ENREF_41" \o "Ju, 2014 #536)). The mutational signature of tumor somatic substitutions is similar to that of germline mutations in primate population evolution, indicating that the mtDNA replication and repair mechanisms are not significantly altered in cancer cells, and genetic drift is the main force behind their accumulation. The surplus of non-synonymous mtDNA substitutions is a general feature of the young branches of the human phylogenetic tree ([Kivisild et al., 2006](#_ENREF_44" \o "Kivisild, 2006 #535)), a description that also fits the rapidly evolving cancer cell populations. While the existence of driver mtDNA mutations that contribute to cancer progression seems to be well documented, the majority of somatic mitochondrial genome variation in tumors appears to be neutral.

The results obtained in the present study indicate that none of the studied *ATP6* variants are likely to have a significant effect on the functioning of the mitochondrial ATP synthase. The 8914C>A, 8953C>G and 9131T>C variants are likely neutral substitutions, like the majority of somatic mtDNA substitutions in cancers ([Ju et al., 2014](#_ENREF_41" \o "Ju, 2014 #536)). In fact, the 8953A>G variant was reported as a polymorphism in one individual ([Loo et al., 2011](#_ENREF_53" \o "Loo, 2011 #527)), and a 9130C>A variant leading to an amino acid substitution (L202M) in the same residue as 9131T>C (L202P) is reported in two sequences in the MITOMAP database ([Lott et al., 2013](#_ENREF_54" \o "Lott, 2013 #160)).

Interestingly, 8932C>T is the only variant that gave an observable phenotype (albeit only at an elevated temperature) when modeled in yeast, and it is also a known polymorphic site, reported in 126 mtDNA sequences in the MITOMAP database. While it is still likely that this variant is a neutral polymorphism in humans, and that the effect observed in yeast is due to the differences between these two systems, it has to be noted that not all human mtDNA polymorphisms are truly neutral. Substitutions in mtDNA, including in the ATP synthase subunit genes were found to be under positive selection, hypothetically related to cold climate adaptations ([Mishmar et al., 2003](#_ENREF_60" \o "Mishmar, 2003 #540); [Ruiz-Pesini et al., 2004](#_ENREF_77" \o "Ruiz-Pesini, 2004 #525)). The temperature-sensitive phenotype in yeast mutants raises the intriguing possibility that P163S plays a role in human cancer cells or climate adaptation, although further research is clearly warranted.

There are, however, other possible explanations of the reported results. Cancer can be viewed as an evolutionary process where metabolic adaptation occurs from the interplay of mutation generation, genetic drift, and clonal selection ([Gerlinger et al., 2014](#_ENREF_35" \o "Gerlinger, 2014 #571); Lipinski et al., 2016). Parallels between putative roles of mtDNA mutations in climate adaptation and in tumor progression could therefore hint at effects that could not be revealed by simple phenotype analysis. In tumorigenesis, as well as in evolution, the selective effect of a mutation is often dependent on epistatic interactions with other genetic factors, as well as with the environment. Mutations that appear to be neutral could thus reveal their selective role in the context of specific genetic changes and/or environmental conditions. As yeast genetics offers unparalleled possibilities such as the combination of different mitochondrial and nuclear mutations, as well as phenotypic high-throughput screening, and multigenerational evolutionary experiments, models like the ones described here could provide an excellent starting point to look for these interactions and to work towards a systems level understanding of the role of mitochondrial genome changes in cancer and evolution.

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