Functional study in a yeast model of a novel succinate dehydrogenase subunit B gene germline missense mutation (C191Y) diagnosed in a patient affected by a glomus tumor

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Mutations of succinate dehydrogenase (SDH) subunits B, C and D are associated to pheochromocytoma/paraganglioma (PGL) development. The mechanisms linking SDH mutations to tumorigenesis are currently unknown. We report a novel germline missense SDHB mutation (C191Y) in a patient affected by a glomus tumor. The missense mutation hits an amino acid residue conserved from mammals to the yeast Saccharomyces cerevisiae. The pathogenic significance of the human mutation was validated in a yeast model. SDH2C184Y mutant allele equivalent to human SDHBC191Y did not restore the OXPHOS phenotype of the Δsdh2 null mutant. In the mutant, SDH activity was also abolished along with a reduction in respiration. Sensitivity to oxidative stress was increased in the mutant, as revealed by reduced growth in the presence of menadione. Remarkably, the frequency of petite colony formation was increased in the mutant yeast strain, indicating an increased mtDNA mutability. Histochemistry demonstrates that SDH activity was selectively absent in the patient tumor tissue. Overall, our results demonstrate that the C191Y SDHB mutation suppresses SDH enzyme activity leading to increased ROS formation and mtDNA mutability in our yeast model. These findings further our understanding of the mechanisms underlying PGL development and point to the yeast model as a valid tool to investigate on the possible pathogenic relevance of SDH novel mutations and/or rare polymorphism.

INTRODUCTION

Pheochromocytomas (Pheos) and paragangliomas (PGLs) are neural crest-derived tumors named differently according to their location, inside or outside the adrenal gland, respectively (1). Pheos as well as abdominal and thoracic PGLs are chromaffin tumors which secrete catecholamines, although PGLs located in the head and neck (HN) area are parasympathetic in origin and not secreting. Our knowledge on the genetics of Pheos/PGLs has greatly improved, because the mutations in genes encoding three of the four subunits of the succinate dehydrogenase (SDH) (SDHB, SDHC and SDHD) have been established as an important cause of familial PGL syndromes (PGL4, PGL3 and PGL1, respectively) (2–5).

In spite of a common high degree of variability in their presentation, these syndromes may present some peculiar clinical features. PGL1 presents mainly with benign, non-secreting, multiple HN-PGLs possibly associated with Pheos and/or abdominal secreting PGLs (6,7); PGL3 is mainly characterized by single HN-PGLs (8) but the occurrence also of abdominal secreting PGLs has been recently reported (9,10). PGL4 presents generally with abdominal PGLs which, at variance...
with tumors developed in PGL1 and PGL3, are malignant in up to 30–40% of cases, leading to metastatic disease (11). Also the type of mutations vary widely, ranging from missense to nonsense mutations, from frame shift to splice site variations to genomic rearrangements. Although some type of mutations, such as nonsense or large deletions, can be assumed to be pathogenic for PGL syndromes, the cause–effect link between a missense mutation and the disease can be established only by the study of the patient’s family pedigree, the absence of the mutation in a large group of control subjects or by functional studies. Histochemical evaluation of SDH activity in tumor tissue offers a coarse indication on enzymatic activity, but human cell lines suitable to evaluate subtle functional consequences of SDH mutations are not available.

Recently, yeast model has been adopted to study the functional consequences of gene mutations on the enzymatic as well as the respiratory activity of SDH or mitochondrial complex II (12,13).

Yeast offers invaluable guidance for approaching human diseases-associated gene functions particularly concerning mitochondrial ones due to the yeast ability to survive without a functional mitochondrial respiratory chain, provided that a fermentable carbon source is made available (14–19). In contrast to humans, Saccharomyces cerevisiae genes can be easily deleted, mutated and reintroduced into yeast cells providing a considerable amount of information useful for understanding the molecular basis of diseases (20).

The mitochondrial SDH (also known as complex II or succinate:ubiquinone oxidoreductase) links the mitochondrial respiratory chain to the tricarboxylic acid cycle. Complex II is an iron–sulfur flavoprotein located in the inner mitochondrial membrane and catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone (Q) to ubiquinol.

The yeast SDH, like its mammalian counterpart, consists of four nuclear encoded subunits: Sdh1p–Sdh4p in yeast and SdhA–SdhD in mammals (21). Sdh1p together with Sdh2p form the catalytic core of the complex, the site of succinate oxidation. Electrons flow through the catalytic domain to the membrane domain, consisting of Sdh3p and Sdh4p, where quinone reduction occurs. SDH complex is essential for growth on respiratory carbon sources and disruption of any one of the four genes leads to the loss of SDH activity. We report here the functional study in the yeast model of a novel SDHB germline missense mutation (C191Y) diagnosed in a patient affected by an apparently sporadic glomus tumor.

RESULTS

Clinical findings

A 36-year-old women affected by a left cervical mass suspected to be a glomus tumor was referred to our outpatient clinic by the Vascular Surgeon for endocrinological and genetic advice before surgery, according to a common clinical protocol approved by the Local Ethics Committee. She was the only daughter of a 54-year-old healthy woman and a 54-year-old man operated twice for a shoulder melanoma and treated with chemotherapy, who died at the age of 54 for metastatic melanoma. A paternal aunt had died for a bone tumor. She had two sons, 7 and 3 years old, respectively. In the patient’s past medical history, the only remarkable event was the appearance, 15 years before, of a left cervical mass which had progressively enlarged without any symptom. At the last NMR, the mass, which measured 3.5 × 2.5 cm in size, wrapped the distal part of the common carotid artery and opened up the angle formed by the internal and the external carotid arteries. The contrast medium was rapidly and intensively taken up. At clinical examination, the patient presented only the lateral cervical mass and her blood pressure was found normal. After urinary metanephrines and plasma chromogranin A had resulted normal, the patient underwent surgery for removal of the cervical mass which resulted a glomus tumor at histology. After written informed consent, the patient accepted to undergo genetic testing for mutations in the PGL susceptibility genes.

Mutation analysis

At genetic analysis, RET (exons 10, 11, 13, 14, 15, 16), VHL (all exons), SDHD (all exons) and SDHC (all exons) resulted wild-type.

A novel mutation was identified in SDHB gene represented by an heterozygous missense mutation, C191Y, a G to A substitution leading to a cysteine to tyrosine amino acid change in exon 6 (Fig. 1). This variant was not found in 125 healthy control subjects. The missense mutation found in our patient maps in a highly conserved residue throughout eukaryotes, from yeast to human (Fig. 1).

Effect of sdh2C184Y mutant allele equivalent to hsdhbC191Y mutant on oxidative growth phenotype of yeast

In order to validate the pathogenic role of the missense mutation in the human SDHB gene and to better understand the alterations caused in complex II functions by this novel mutation, we first performed complementation studies in a S. cerevisiae strain lacking SDH2 (the yeast ortholog of mammalian SDHB). The S. cerevisiae strain BY4741, which carries the deletion abolishing the SDH2 gene, BY4741Δsdh2, displays an OXPHOS negative phenotype characterized by failure to grow in media containing 2% ethanol or other obligatory aerobic compounds, as the only carbon sources (21). Δsdh2 mutant strain was transformed with wild-type and the sdh2C184Y mutant allele, equivalent to human sdhbC191Y. The results obtained and depicted in Figure 2 showed that when the Δsdh2 mutant was transformed with the plasmid carrying sdh2C184Y mutant allele no correction was obtained indicating that the sdhB mutation found in humans is deleterious in yeast.

To check whether the reduced growth of the strain carrying the pathogenic allele could result from a reduced expression of sdh2C184Y mutant, we performed northern blotting analysis. The result obtained showed that the transcript level of the mutant allele was comparable to that of the SDH2 wild-type allele (Fig. 3), indicating that the different phenotype observed was not due to different expression levels.
The oxidative negative phenotype displayed by the strain carrying the SDH2 mutant allele prompted us to evaluate SDH enzyme activity and the respiratory capacity of the mutants. Not unexpectedly on the basis of the OXPHOS negative phenotype, in the transformant carrying the sdh2C184Y allele, the SDH enzymatic activity was abolished (Table 1). However, in the presence of glucose, the mutant still displayed a detectable level of respiration (~50% of that of the parental strain) indicating that the mutation did not completely prevent electron flux. All together, these results indicated that the absence of oxidative growth should be ascribed to the absence of SDH enzyme activity, because the SDH complex participates in both the membrane-bound electron transfer chain of the mitochondrial inner membrane and the tricarboxylic acid cycle located in the matrix.

Table 1. Effect of mutations on respiratory activity and succinate dehydrogenase activity

<table>
<thead>
<tr>
<th>Allele</th>
<th>Respiration</th>
<th>SDH activity EAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>No allele</td>
<td>12.0 ± 0.72</td>
<td>3.6 ± 0.70</td>
</tr>
<tr>
<td>SDH2</td>
<td>25.5 ± 1.76</td>
<td>121.6 ± 8.85</td>
</tr>
<tr>
<td>sdh2C184Y</td>
<td>11.9 ± 1.11</td>
<td>3.8 ± 1.12</td>
</tr>
</tbody>
</table>

aAllele carried by the vector introduced into the null Δsdh2 strain.
bExpressed as μL O2/h/mg dry weight. All values are mean values of three independent experiments.
cEAU: enzyme units are expressed as nanomoles of substrate utilized per minute per milligram of protein. Values are means of three independent experiments in duplicate.

Figure 1. Mutation analysis. Wild-type (left) and patient (right) forward and reverse electropherogram showing the missense mutation C191Y in exon 6 of the SDHB gene. Below the sequencing profiles are the ClustalW interspecies alignments of the SDHB region containing the mutated amino acid found in our patient.

Figure 2. Oxidative growth phenotype. The strain BY4741 Δsdh2 was transformed with the plasmid pFL38 or with pFL38 carrying the wild-type gene SDH2 or with pFL38 carrying the sdh2C184Y mutation. Equal amounts of serial dilutions of cells from exponentially grown cultures (10⁵, 10⁴, 10³, 10² cells) were spotted onto YNB plates supplemented with either 2% glucose or 2% ethanol. The growth was scored after 5 days of incubation at 28°C.

Figure 3. Northern analysis of SDH2 in the Δsdh2 strain transformed with pFL38 plasmid carrying either wt SDH2 gene or the pathogenic sdh2C184Y allele. The same amount of RNA (20 μg) was loaded on each lane. RNA was hybridized with labeled probes for SDH2 and ACT1.

SDH enzyme activity, respiration and cytochrome content in sdh2C184Y mutant

The oxidative negative phenotype displayed by the strain carrying the SDH2 mutant allele prompted us to evaluate SDH enzyme activity and the respiratory capacity of the mutants. Not unexpectedly on the basis of the OXPHOS negative phenotype, in the transformant carrying the sdh2C184Y allele, the
The accumulation of ROS in the mutants and wild-type strains was also analyzed by incubating the cells with the fluorescent dye dihydrorhodamine123. This compound accumulates inside the cells and is oxidized by ROS to the corresponding fluorescent chromophore (23). Cells carrying sdh2<sup>2C184Y</sup> mutation as well as cells lacking SDH2 showed an intense intracellular staining for at least 30% of the population compared with the fluorescence presented in <10% of the cells carrying the wild-type gene (Fig.5B). High level of ROS is generally associated with an increase in the accumulation of mtDNA rearrangements (24,25). In S. cerevisiae, conditions that increase mtDNA mutability also increase the frequency of petite mutations (deletions of mtDNA) (26). In order to evaluate the effect of sdh2 mutations on mtDNA stability, we measured the frequency of petite mutants in haploid Δsdh2 strains carrying either the wild-type SDH2 allele, the sdh2<sup>2C184Y</sup> or the empty plasmid. The results obtained and reported in Figure 6 indicated that petite frequency was ~7-fold increased in Δsdh2 and sdh2<sup>2C184Y</sup> mutants compared with wild-type.

**Histochemical evaluation of mitochondrial electron flow**

In light of the different effects of SDH mutations on enzyme activity (27,28), we evaluated mitochondrial electron transfer into two different samples, namely in a control Pheo, taken at surgery from a patient who resulted wild-type at genetic analysis, and in the PGL tissue obtained at surgery from our SDHB-mutated patient.

To analyze SDH activity, we adopted two different histochemical techniques: the Thiazolyl Blue Tetrazolium Bromide (MTT) assay, where MTT is mainly reduced by thenoyltrifluoroacetone (TTFA), again indicating specificity of the assay. Of note, staining was scattered throughout the tissue in small granules resembling mitochondria (Fig. 7B). Staining was completely prevented when 100 μM nitropropionic acid (NPA, a prototypical SDHA inhibitor) was added to the reaction buffer (data not shown), suggesting specificity of the signal. Remarkably, tumor tissue from the SDHB-mutated patient was totally negative for SDH activity when measured by means of the MTT assay (Fig. 7B). Histochemistry revealed that NADH dehydrogenase and COX activities were unaffected and similar in both tumor samples (Fig. 7B). The DCIP assay carried out in control Pheo tissue revealed that enzyme activity was prevented by the SDHA inhibitor NPA and not by the SDHD blocker thenoyltrifluoroacetone (TFA), again indicating specificity of the assay. Of note, in the SDHB-mutated tumor tissue, SDHA activity was dramatically reduced (Fig. 7C).

**DISCUSSION**

We report on a 36-year-old women affected by a left cervical glomus tumor who, at genetic analysis, was found to be a

The mitochondrial cytochrome profile is an index of the structural integrity of the respiratory-chain complexes. The measurement was performed in the Δsdh2 strain transformed with the empty vector pFL38, with the wt SDH2 or the sdh2<sup>2C184Y</sup> allele. The strain transformed with the sdh2<sup>2C184Y</sup> allele displayed a significant reduction of the cytochrome aa<sub>3</sub> similar to that of the strain transformed with pFL38 empty plasmid (Fig. 4) indicating that the bottleneck of respiratory structures was at the complex IV level.

**Figure 4.** Reduced versus oxidized cytochrome spectra of Δsdh2 strain transformed with wild-type SDH2, sdh2<sup>2C184Y</sup> mutant allele and pFL38 plasmid without insert. The peaks at 550, 560 and 602 nm (vertical bars) correspond to cytochromes c, b and aa<sub>3</sub>. The height of each peak relative to the baseline of each spectrum is an index of cytochrome content.
carrier of a missense SDHB germline mutation. As the most frequent clinical phenotype associated to SDHB mutations is characterized by catecholamine secreting retroperitoneal PGLs (11), the clinical picture presented by our patient had to be considered unusual. Moreover, as the SDHB mutation was so far not described and the family pedigree was not informative, we wondered whether this novel mutation might be considered pathogenic for PGL4 syndrome.

Given that the missense mutation found in our patient hits an amino acid residue that is conserved from mammals to the yeast S. cerevisiae, we have set up a yeast recombinant system to study the pathogenicity of sdh2C184Y corresponding to sdhBCl191Y missense mutation found in the patient. The construct, expressing mutant version of SDH2, was generated and analyzed for the ability to complement defective growth on non-fermentable carbon sources of a mutant strain carrying a SDH2 deletion. Experimental evidence obtained by using this model supports the deleterious effects of the sdhBC191Y mutation. Indeed, complementation studies on an sdh2 deficient yeast strain showed that the mutant allele sdh2C184Y transformed into the sdh2 null mutant failed to restore the yeast OXPHOS competence. In addition, SDH measurements indicated that sdh2C184Y mutation alone was sufficient to prevent SDH complex to correctly perform its activity. The mutant still displayed a detectable level of respiration, though reduced, indicating that it remains respiration competent, in agreement with what previously observed with a mutant of Sdh1 subunit (31). Being compromised the correct flux of metabolites through the Krebs cycle, the mutant was however unable to perform a respiration based life cycle.

The mutation caused a significant reduction of the level of cytochromes aa3. A decrease of complex IV activity has been recently reported by Smith et al. (13) in both sdh1 and sdh2 null mutants as a consequence of the loss of complex II + III activity due to the loss of SDH activity. However, another possible explanation is that, in addition to its enzymatic activity, SDH complex, which resides into the inner mitochondrial membrane, plays a structural role that contributes to maintain the integrity of respiratory complexes in the inner mitochondrial membrane where respiratory cytochromes also are embedded (32).
The mutated C residue at position 184 in yeast corresponds to C154 in the *Escherichia coli* protein that was described as required for the stabilization of the [4Fe–4S] center, the central FeS cluster in the electron transport system of the SDH complex (33). According to structural analysis performed on *E. coli* SdhB protein (33), the cysteine to tyrosine mutation could have dramatic effects on SdhB function for at least two reasons: (i) the steric hindrance of the aberrant tyrosine residue is not compatible with the shape of the protein, resulting in a number of clashes between the Y-side chain and other protein regions; (ii) the geometry and physico-chemical properties of the tyrosine side chain do not allow interactions either with FeS clusters or with other residues involved in FeS stabilization.

Remarkably, we report for the first time that the OXPHOS phenotype of the Δsdh2 strain is associated with mtDNA instability, having observed a 7-fold increase in the frequency of petite in the *sdh2*Δ C184Y and in the null Δsdh2 mutants compared with the wild-type strain. In *S. cerevisiae*, conditions that increase ROS production also increase mtDNA mutability and consequently the frequency of petite mutations (24, 25). Indeed, we observed a significant increase in the sensitivity to menadione and enhanced endogenous ROS production, both in the null mutant and in that carrying the C184Y mutation that could account for petite mutability.

An increase of ROS production but not of mitochondrial DNA damage was previously described in a *sdh2* null mutant (13). However, a couple of reasons may account for this apparent contradiction: (i) our experiments were performed at 37°C, a temperature that is known to increase mitochondrial DNA mutability (34–35), instead of 30°C and (ii) our mitochondrial mutability assay could be more sensitive than the PCR mtDNA amplification that the authors themselves consider a relatively insensitive assay (13).

All together, our results on oxidative growth, SDH activity, respiration, cytochrome profile and mtDNA mutability obtained with *sdh2*Δ C184Y point mutation are identical to those obtained with the Δsdh2 null allele indicating for this missense mutation a complete loss of function.

To investigate the functional impact on human mitochondrial electron transport of C191Y mutation, SDH activity was evaluated in tumor tissues by means of histochemical techniques. Our data demonstrate that in the tumor carrying the C191Y mutation electron transfer to complex IV is impaired, if the donor is succinate and not if electrons originate from NADH. Conversely, mitochondria from a patient affected by a sporadic pheo are able to transfer electrons from succinate to complex IV. These results, along with the finding that COX activities are similar in the two patients, indicate that in the *SDHB*-mutated PGL mitochondrial respiration on succinate is selectively inactivated. This is in keeping with the structural changes that the C191Y mutation induces at the level of the FeS cluster of SDHB. Indeed, the mutation we found in C191 residue of the FeS cluster containing domain causes a complete impairment of the electron transport from the substrate-binding domain (SDHA) to the SDHD–quinone system, likely due to problems in binding or orientation of the FeS clusters.

Of note, we were not able to demonstrate a loss of heterozygosity in tumor tissue (data not shown). This finding,
together with the lack of SDH activity at histochemistry, which is consistent with a null phenotype of the mutant tumor, indicates that the SDHB mutation either has a dominant negative effect or that inactivation of the normal allele occurs by other mechanisms, such as methylation of the promoter or post-transcriptional events.

Evidence that SDHA activity is almost abrogated in tumor tissue with the C191Y mutation was unexpected due to the putative selective impairment of SDHB activity. This result, together with the data obtained in yeast is in keeping with the observation that succinate-oxidizing SDH subunit is the SDHA/SDHB complex, rather than SDHA alone (13). A SDHB mutation, therefore, would result in a complete inability to transform succinate into fumarate. Accordingly, in a recent paper, Guzy et al. (28) reported that loss of SHDB inactivates succinate oxidation and triggers increased ROS production.

Our study supports a role for the yeast model as a relevant tool to investigate on the functional consequences of SDH subunit mutations in patients affected by PGL syndromes. This model might be of clinical relevance to establish the pathogenic significance of novel mutations or even rare polymorphisms. In conclusion, our results demonstrate that the C191Y mutation inactivates SDH activity both in yeast and in the patient’s tumor tissue and demonstrate that C191 residue plays a crucial role for the enzymatic activity of the catalytic complex SdhA/SdhB (Sdh1/Sdh2). The SDHB missense mutation leads to an enhanced endogenous ROS production and to mtDNA mutability in our yeast model. Whether this mutability might also occur in human cells and plays a causative role in tumor development cannot be assessed in the present study and deserves further experiments in human SDH-mutated tumor tissues.

**MATERIALS AND METHODS**

**Mutation analysis**

After obtaining informed consent in agreement with the guidelines of our Ethics Committee, DNA was extracted from peripheral blood leukocytes using the commercial kit NucleoSpin Blood L (Macherey-Nagel, Düren, Germany) following the manufacturer’s instructions. Searching for germline mutations, we analyzed SDHD (all exons), SDHB (all exons) and SDHC (all exons), RET (exons 10, 11, 13, 14, 15, 16), VHL (all exons) genes. All the gene coding regions and exon–intron boundaries were amplified by PCR using the appropriate primers as previously described (7). PCR products purified using a PCR purification kit (Qiagen, Milan, Italy) following the protocol instructions and semi-quantified in a 2% agarose ethidium bromide gel using DNA molecular weight markers. The corresponding modified primers used to generate mutated alleles were:

S2M forward CGAATGTATTCTGTGTCATATGGCTCT GATCGGACATGACATTCG.

For the assay of sensitivity to menadione (Sigma-Aldrich, Seelze, Germany) on plates, cells grown to the mid-logarithmic phase in YNB + glucose 1% were diluted sequentially and spotted onto plates containing 20 μm menadione.

**Construction of yeast mutant alleles**

sdh2<sup>C184Y</sup> mutant allele, which is the correspondent of the human <sup>C191Y</sup> was produced by site-directed mutagenesis, by using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). SDH2 wild-type gene cloned in the pFL38 centromeric plasmid (37) was used as template DNA. To obtain this plasmid, we amplified a DNA fragment of 1530 bp (SDH2) containing the ORF and the 5’ and 3’ flanking regions (495 and 300 bp, respectively) by PCR, using genomic DNA of strain BY4741 as template and the appropriate forward and reverse primers containing restriction sites at their 5’ ends. The sequences of the oligonucleotides used for cloning SDH2 are:

HSDH2 forward CGCGAAGCCTTGTAGGTGCAATG GCCACCC and SSDH2 reverse CCCCGTCGCCACCTTTC TCGCCTATGATG.

The corresponding modified primers used to generate mutated alleles are:

S2M forward CGAATGTATTCTGTGTCATATGGCTCT GATCGGACATGACATTCG.

In bold are the base changes. In order to maximize the expression of these variants, the preferred yeast codon (38) was used in the oligonucleotide sequence used for mutagenesis: TAT for tyrosine. Both wild-type and mutated inserts were sequence-verified on both strands.

**RNA preparation and northern analysis**

Total RNA was prepared by extraction with hot acidic phenol (39), from cells grown in YNB supplemented with 2% glucose. Northern analysis was carried out as previously described (40). The SDH2 probe was a 1000 bp fragment obtained by PCR amplification with pFL38/SDH2 plasmid as template. The primers used for the amplification were Fins2 (CGCGAGCTAGTACACCCGAG) and SSDH2. The amount of DNA loaded on the gel was estimated by hybridization with an actin gene probe (ACT1). All the probes were labeled with [α-<sup>32</sup> P]dCTP using the rediprime DNA labeling system (Amersham).

**Yeast strains, media and general genetic methods**

Yeast strains used were BY4741 (MATa; his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and its isogenic <sup>sdh2</sup>Δ<sup>kanMX4</sup> mutant. Cells were cultured in yeast nitrogen base (YNB) medium [0.67% YNB without amino acids (ForMedium™, UK)] supplemented with 1 g/l of drop-out powder (36) containing all amino acids except those required for plasmid maintenance. Various carbon sources were added at 2% (w/v) (Carlo Erba Reagents Italy). Media were solidified with 20 g/l agar (ForMedium™). YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (ForMedium™). For the cytochromes absorption spectra, respiration and mitochondria extraction cells were grown to late-log phase in the YNB medium supplemented with 0.6% glucose.

For the assay of sensitivity to menadione (Sigma-Aldrich, Seelze, Germany) on plates, cells grown to the mid-logarithmic phase in YNB + glucose 1% were diluted sequentially and spotted onto plates containing 20 μm menadione.

**Cytochrome spectra and respiration**

Differential spectra between reduced and oxidized cells of a suspension of cells at 60 mg/ml (wet weight) were recorded...
at room temperature, using a Cary 219 spectrophotometer. Oxygen uptake was measured at 30°C using a Clark-type oxygen electrode in a 1 ml stirred chamber containing 1 ml of air-saturated respiration buffer (0.1 M phthalate–KOH, pH 5.0), 10 mM glucose (Oxgraph System Hansatech Instruments, England) starting the reaction with the addition of 20 mg of wet weight of cells as previously described (41).

**Isolation of mitochondria and enzyme assay**

Preparation of mitochondria was carried as previously described (41). The SDH activity was expressed as nmol/min/mg protein. The SDH DCIP assay was conducted as described (30) with minor modifications. The initial reaction rate at 600 nm was recorded after addition of mitochondria to the cuvette containing 0.15 mM dichlorophenolindophenol, 2.5 mM phenazine methosulfate, 100 mM sodium azide, 0.1 M phosphate buffer, pH 7.6 and 15 mM succinate (sodium salt) (Sigma-Aldrich). Protein concentration was determined by the method of Bradford (42).

**Measurement of intracellular oxidation levels**

The oxidant-sensitive probe dihydrorhodamine123 (Molecular Probes) was used to measure intracellular oxidation levels (23). Cells cultured in YNB supplemented with 1% glucose were treated with dihydrorhodamine123 and visualized by fluorescence microscopy (excitation λ at 505 nm, blue/emission λ at 534 nm, green); each aliquot was treated with Calcofluor White (M2R) (blue) to evidence cell wall in order to count total cells (43). For each strain, the number of stained cells over total cells in 20 optical fields were counted in order to calculate the percentage values.

**Mitochondrial DNA mutation frequency**

SDH2-defective yeast strain (Δsdh2) exhibits growth defect on a non-fermentable (respiratory) carbon source being the growth completely prevented in YNB but not in YP (data not shown). However, when mutations in mtDNA, either rho− (carrying deletions of mtDNA) or rho+ (devoid of mtDNA) occur, the mutant strain become unable to growth also on YP-ethanol. Taking advantage of this different phenotypes, in order to counterselect the petite cells that could be present in the population, strains transformed with wild-type or mutated SDH2 allele were grown for 48 h in YP medium supplemented with 2% ethanol. Then, the strains were inoculated in YNB medium supplemented with 2% glucose. After 15 generations of growth at 37°C, cells were plated on YP agar plates supplemented with 2% ethanol plus 0.2% glucose at a dilution giving ~200 cells/plate. Frequency was defined as the percentage of colonies showing the petite phenotype after 5 days incubation at 28°C. For each strain at least 4000 clones were analyzed.

**Histochemistry**

Frozen tumor tissue was cut in a cryostat into 20 μm sections which were placed in a well of a 24-well plate (3 sections/well), SDH activity was evaluated by incubating the slices for 30 min in a 60 mM phosphate buffer (pH 7.4) containing Cobaltous chloride 500 mM (Sigma-Aldrich), Thiazoly Blue Tetrazolium Bromide (MTT+) 250 μM (Sigma-Aldrich), sodium succinate 0.015–15 mM (Sigma-Aldrich) (29). NADH dehydrogenase activity was evaluated by using the same buffer containing 0.0015–15 mM NADH (Sigma-Aldrich) instead of succinate. The SDH DCIP assay was conducted as described (30). COX activity was evaluated by incubating slices for 1 h in a medium containing Tris–HCl 50 mM pH 7.6 (Calbiochem, Inc., La Jolla, CA, USA), 1 mg/ml cytochrome c type IV, 5 mg/ml DAB hydrochloride (Sigma-Aldrich). Reactions were stopped by removing the buffer and PFA fixation. To visualize the staining, a Nikon 2000TU microscope equipped with a CCD camera was used.

**Miscellaneous**

Transformation of yeast strain was obtained by the lithium acetate method (44). Restriction-enzyme digestions, E. coli transformation and plasmid extractions were performed using standard methods (45).

Sequence alignment for mutation analysis was performed with ClustalX (46) and BLAST Align Tool (47).

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Conflict of Interest statement. None declared.

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