Deletion of the yeast homologue of the human gene associated with Friedreich's ataxia elicits iron accumulation in mitochondria

Françoise Foury*, Ornella Cazzalini

Unité de Biochimie Physiologique, Place Croix du Sud 2-20, 1348-Louvain-La-Neuve, Belgium

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Abstract Deletion of *YDL120*, the yeast homologue of the human gene responsible for Friedreich's ataxia, elicits decreased cellular respiration associated with decreased cytochrome *c* oxidase activity and, in certain nuclear backgrounds, mitochondrial DNA is lost. In the null mutants, the cellular growth is highly sensitive to oxidants, such as H_2O_2, iron and copper. However, only ferrous sulfate elicits loss of mitochondrial DNA. Mitochondria of the null mutants contain 10 times more iron than wild-type. The neurodegeneration observed in Friedreich's ataxia can be well explained on the basis of a mitochondrial iron overload responsible for an increased production of highly toxic free radicals.

Key words: Friedreich's ataxia; Yeast homologue; Gene disruption; Mitochondria; Iron; Copper

1. Introduction

Friedreich's ataxia is an hereditary recessive neurodegenerative disease associated with cardiomyopathy and, in some patients, with diabetes mellitus [1,2]. The gene responsible for this disease encodes a 210-amino-acid protein (frataxin) with no similarity to any protein of known function [3]. However, frataxin has homologues in *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and Gram-negative bacteria [3,4]. *S. cerevisiae* is often considered as a model for the comprehension of the molecular basis of human disease genes [5,6]. In this communication, we present a rapid functional analysis of Ydl120p, the yeast frataxin homologue discovered during the systematic sequencing of the genome, which offers interesting perspectives for Friedreich's ataxia.

2. Material and methods

2.1. Strains and media

S. cerevisiae strains used in this study were: W303-1B (*MATα ade2-1 leu2-3,112 his3-11,15 ura3-1 trp1-1*) which has received by cytoduction the mitochondrial genome of the *kar1* strain JC7 (*MATa leu1 kar1-1*); D273UK (*MATα ura3-1 lys2 met6*); CENPK2 (*MATα/MATa leu2-3,112/leu2-3,112 his3-1/his3-1 ura3-52/ura3-52 trp1-289/trp1-289*). W303ΔYDL120, D273ΔYDL120 and CENPK2ΔYDL120 are the corresponding haploid deletant strains. Rich media were composed of 2% glucose (or 3% glycerol), and 2% yeast extract KAT. Raffinose-minimum medium contained 2% raffinose, 0.67% yeast nitrogen base Difco and the required amino acids and bases.

*Corresponding author. Fax: (32) 10-473872.
E-mail: foury@fysa.ucl.ac.be

Abbreviations: ABC, ATP-binding cassette; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction

2.2. Disruption of YDL120

A disruption cassette was constructed, as described [7], by polymerase chain reaction (PCR) amplification of a kan^R gene-containing module flanked on its 5′ and 3′ sides by 40-base DNA sequences located upstream from position +261 and downstream from position +480 of the *YDL120* gene, respectively. This cassette was used to transform yeast for geneticin resistance [7]. The geneticin-resistant transformants were streaked for single colonies and purified. The correct integration of the kan^R marker at the *YDL120* target site was verified by PCR amplication of the 5′ and 3′ regions flanking the kan^R marker.

2.3. Identification of rho⁻ mutants and estimation of the mtDNA content

Glucose-grown colonies were replicated on glycerol medium; the glycerol-negative colonies were cross-replicated on glucose lawns of the rho° tester strain IL166-6C (*MATa ura1*). Diploids were selected on glucose-minimum medium and replicated on glycerol medium. Quantitation of mtDNA was as follows. Total DNA (genomic and mitochondrial) was extracted from 2-ml cell cultures by vortexing the cell suspension in the presence of glass beads and phenol. A 328-bp DNA fragment containing the mitochondrial *oli1* gene and 850 bp of the nuclear *YNL213* gene were PCR-amplified for 25 cycles, using a DNA concentration range that gave a linear answer in a 20-μl reaction medium containing 100 μM of each dATP, dGTP and dTTP and 10 μM [α-^{32}P]dCTP. After electrophoresis in a 1.4% agarose gel, DNA was revealed by autoradiography of the dried gel and quantitated with a Phosphorimager (Biorad).

2.4. Preparation of mitochondria and measurement of respiratory activities

Cyanide-sensitive cellular respiration was measured with a Clark electrode at 30°C in a 3 ml of medium containing 25 mM potassium phosphate, pH 6.5, and 2% glucose. Mitochondria were prepared after lysis of spheroplasts obtained by the action of zymolyase as described [8]. Oligomycin-sensitive ATPase, cytochrome *c* oxidase and NADH cytochrome *c* reductase were measured in submitochondrial particles as described [9].

2.5. Measurement of the mitochondrial iron pool

Non-heme iron was measured using bathophenanthroline sulfonate as a chelator in the presence of dithionite as described [10]. Total iron cellular content was measured after boiling the cells for 2 h in the presence of 1% sodium dodecylsulfate and 10 mM MES, pH 4.5.

3. Results

3.1. Deletion of YDL120 is associated with mitochondrial defects

The N-terminal region of the 174-amino-acid protein encoded by the *YDL120* gene (Genbank, accession number Z74168) has the typical features of a mitochondrial targeting presequence with regularly spaced basic, hydroxylated and hydrophobic residues. The PSORT program (http://psort.nibb.ac.jp/form.html) predicts with a very high probability ($P = 0.9$) that Ydl120p localizes to mitochondria.

The *YDL120* gene was disrupted in three strains of different nuclear backgrounds, the haploid strains D273UK and W303-1B and the diploid strain CENPK2. W303ΔYDL120 mutants

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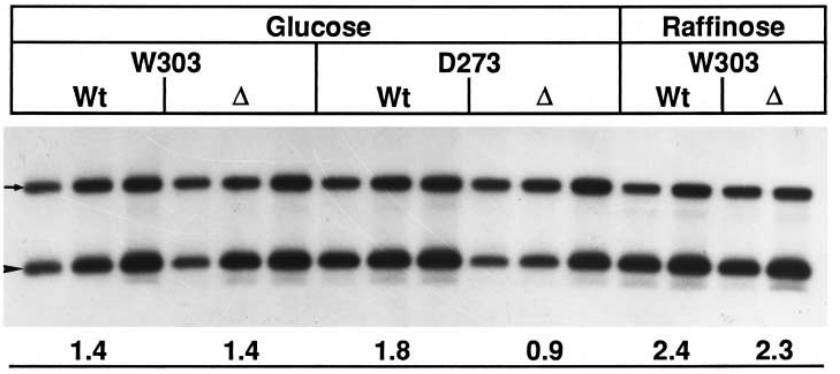

Fig. 1. Estimation of the mitochondrial DNA content by quantitative PCR. Total DNA was extracted as described under Section 2 from cells first refreshed on glycerol-rich medium and then grown in liquid glucose-rich or raffinose-minimum medium for about 10 generations. The ratio of the mitochondrial to nuclear DNA content was estimated by quantitation of the radioactivity of the DNA bands with a Phosphorimager and the average values are given below the autoradiogram. The DNA concentrations used for glucose and raffinose grown strains were 0.15, 0.3, 0.6 μl and 0.15, 0.3 μl, respectively. Wt=wild-type; Δ=null mutant. The arrow and head arrow indicate the *YNL213* (850 bp) and *oli1* (328 bp) DNA bands, respectively.

had no obvious growth defect phenotype on glucose, raffinose and glycerol containing media. However, W303ΔYDL120 cellular respiration was 60% of that of the wild-type for cells grown on raffinose-minimum medium and 10% for cells grown on glucose-rich medium (data not shown). The mtDNA content was estimated after PCR amplification of the mitochondrial *oli1* gene, using the nuclear *YNL213* gene as an internal standard. While the mtDNA content of W303ΔYDL120 was not affected in glucose and raffinose media, a 2-fold decrease was observed in D273ΔYDL120 (Fig. 1).

A much more severe phenotype was observed for CENPK2. Tetrad analysis showed that all meiotic segregants associated with *YDL120* deletion grew extremely slowly on glucose-rich medium (Fig. 2A) and did not grow on glycerol. Growth on glycerol was not restored in the diploid progeny produced in a cross of the meiotic segregants with a rho° tester strain. Moreover, no mtDNA was detected after PCR experiments (Fig. 2B). These data show that in CENPK2ΔYDL120 mtDNA was lost.

Antimycin A-sensitive NADH cytochrome *c* reductase, cytochrome *c* oxidase and oligomycin-sensitive ATPase activities were measured in submitochondrial particles of strains W303-1B and W303ΔYDL120 grown in glucose-rich and raffinose-minimum media. The NADH cytochrome *c* reductase and oligomycin-sensitive ATPase activities of the null strain grown on glucose were 25–30% of that of the wild-type; however, the cytochrome *c* oxidase activity was less than 2% of that of the wild-type (Table 1). In submitochondrial particles from raffinose-grown cells, the cytochrome *c* oxidase activity and content in *a*-type cytochromes were about 2-fold decreased (data not shown) while the other activities were normal (Table 1). Thus, the deletion of *YDL120* elicits a specific defect in cytochrome *c* oxidase and in particular reduces heme A content. This cytochrome *c* oxidase defect cosegregated in tetrads with the *YDL120* deletion (data not shown).

3.2. *Deletion of YDL120 is associated with sensitivity to H_2O_2, iron and copper*

The pathology observed in Friedreich's ataxia is quite similar to that observed in patients with a deficiency in vitamin E, an antioxidant assumed to protect the nervous system from oxidative damage [11]. We found that W303ΔYDL120 and D273ΔYDL120 were more sensitive to oxidant reagents such

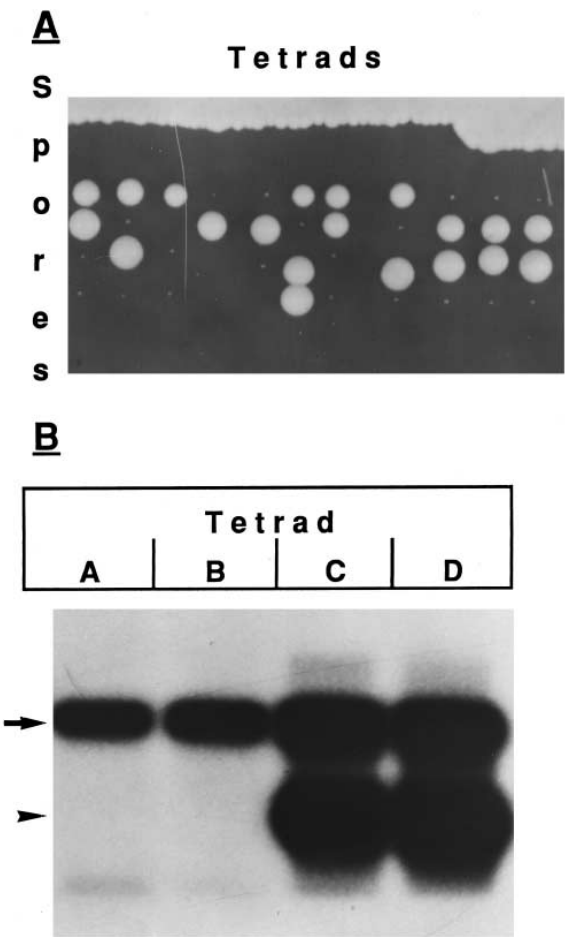

Fig. 2. Meiotic segregation of the *ydl120* null mutation in CENPK2 background. A: Eight complete tetrads are shown with two wild-type and two slow-growing spores. The latter are resistant to geneticin and thus correspond to the *YDL120* deletion. Picture was taken after 5 days at 28°C. B: Agarose gel autoradiography after PCR amplification of the mitochondrial *oli1* and nuclear *YNL213* genes from glucose grown *ydl120* null spores (A,B) and wild-type spores (C,D). Conditions are the same as in Fig. 1.

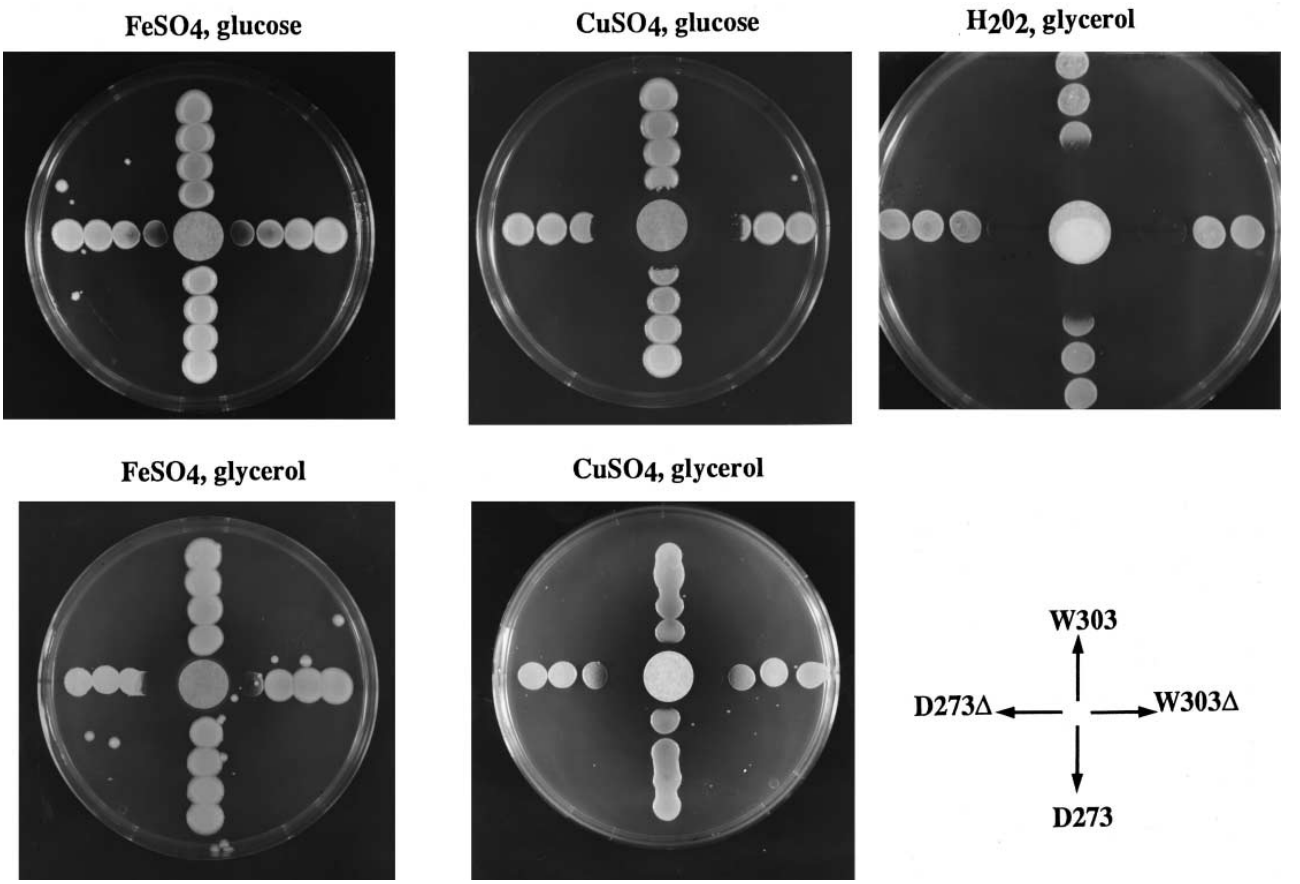

Fig. 3. Iron, copper and H_2O_2 toxicity in the *ydl120* mutants. A concentration gradient was generated by dropping 50 μl of 1 M $FeSO_4$ and $CuSO_4$, or 25 μl of H_20_2 on a filter paper in the center of a Petri dish containing glucose- or glycerol-rich medium. Four 7-μl drops of confluently growing cells of W303-1B, W303ΔYDL120, D273UK, D273ΔYDL120 were deposited at regular distances from the center of the plate. Pictures were taken after 4 days at 28°C, excepted for the strains grown in the presence of H_2O_2 and $CuSO_4$ on glycerol medium (2 days).

as H_2O_2 and diamide, both on glucose- and glycerol-rich media (Fig. 3). Iron and copper are potentially toxic by their capacity to generate, via the Fenton reaction, oxygen radicals in the presence of H_2O_2 and O_2^-. Copper sulfate, particularly at concentrations higher than 0.5 mM, inhibited cellular growth of *ydl120* null mutants more strongly than wild-type both on glucose- and glycerol-rich media (Figs. 3 and 4). No induction of rho^- mutants was observed (Fig. 4). An intense brownish color typical of copper sulfide mineralization on the cell surface [12] was observed in the null mutants. Ferrous sulfate was also inhibitory (Figs. 3 and 4) with a more pronounced effect on glycerol than on glucose medium (Fig. 3). Interestingly, 45% and 98% rho^- mutants were produced in W303ΔYDL120 after the addition of 0.25 mM and 1 mM $FeSO_4$, respectively, to the culture medium (Fig. 4). No rho^- mutants were observed in the wild-type strains. Inhibition of the cellular growth by methyl viologen, menadione, cadmium, zinc, cobalt and manganese was not increased in the null *ydl120* mutants.

Iron concentration was measured using bathophenanthroline disulfonate as a chelator of ferrous ions [10]. The mitochondria of the rho^+ W303ΔYDL120 and rho° CEN-PK2ΔYDL120 strains grown on glucose contained 8–12 times more iron than wild-type (Table 2). Iron accumulated in mitochondria specifically since the total cellular iron content was only slightly increased in the null mutants (Table 2).

Table 1
Mitochondrial ATPase and respiratory activities in wild-type strain and *ydl120* null mutants

Conditions	Specific activity (μmol/min/mg protein)					
	Oligomycin-sensitive ATPase		NADH cytochrome *c* reductase		Cytochrome *c* oxidase	
	Wild-type	Mutant	Wild-type	Mutant	Wild-type	Mutant
Raffinose	3.6	3.0	0.9	0.8	0.8	0.3
Ethanol	3.0	1.8	1.0	0.9	0.6	0.3
Glucose	1.3	0.4	0.7	0.2	0.4	0.006

The wild-type W303-1B and mutant W303-1BΔYDL120 strains were grown in glucose, raffinose and ethanol media. The experiments have been repeated 2–3 times. The concentration of oligomycin was 20 μg/ml. ATPase is expressed in μmol Pi/min/mg protein and NADH cytochrome *c* reductase and cytochrome *c* oxidase are expressed in μmol cytochrome *c* reduced (or oxidized)/min/mg protein.

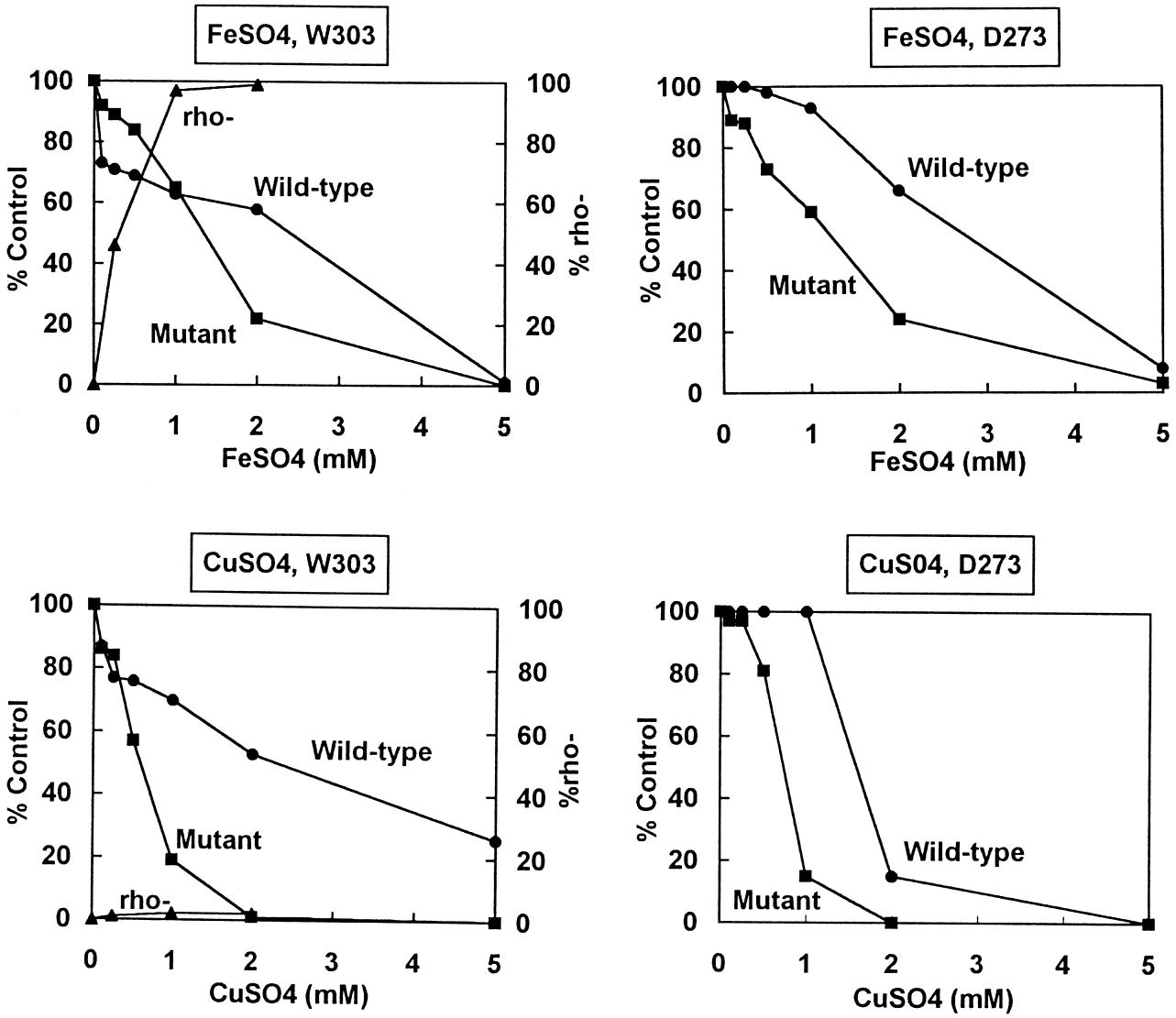

Fig. 4. Inhibition of cellular growth and induction of rho$^-$ mutants by $FeSO_4$ and $CuSO_4$ in wild-type and *ydl120* null mutants. W303-1B, W303ΔYDL120, D273UK, D273ΔYDL120 were grown for 16 h in liquid glucose-rich medium and the cells were counted in the exponential phase of growth. Rho$^-$ mutants were scored based on their 'petite' phenotype. $FeSO_4$ was added from a fresh 0.5 M stock solution in 0.1 N HCl and 5 μM β-mercaptoethanol.

Table 2
Accumulation of iron in mitochondria of wild-type and *ydl120* null mutants

Conditions[a]	Iron (nmol/mg protein)[b]	
	Wild-type	ΔYDL120
W303 (rho$^+$)		
Intact cells	1.7 ± 0.1	2.3 ± 0.5
Mitochondria	5.0 ± 1.0	60.0 ± 10
Supernatant[c]	3.0	4.5
CENPK2 (rho°)		
Mitochondria	2.5	19.5
Supernatant[c]	1.0	2.0

[a]Iron concentration was measured in the W303 and CENPK2 nuclear backgrounds. A *YDL120* wild-type rho° strain in the CENPK2 background was obtained after ethidium bromide treatment.
[b]Standard deviation is given when two independent cultures were studied.
[c]This fraction corresponds to the post-mitochondrial supernatant.

Iron accumulation cosegregated with *ydl120* deletion in tetrads (data not shown). Thus the primary site of the toxic action of $FeSO_4$ seemed to be the mitochondrion.

4. Discussion

More than 10 years ago, Friedreich's ataxia had been associated with mitochondrial dysfunction, mainly on the basis of decreased activity of several enzymes of the mitochondrial matrix [13]. However, these data were controversial and not developed further. Recently, the coding sequence of the small hydrophilic yeast Ydl120p and human frataxin proteins was published but this did not shed light on their function. However, a typical mitochondrial targeting presequence was detected in the N-terminal region of Ydl120p, suggesting that this protein localizes to mitochondria. The same conclusion was previously reached on the basis of phylogenetic studies showing that the *cyay* gene, which is the prokaryotic homologue of the eukaryotic *frataxin* gene, is only found in the

gamma-subdivision of purple bacteria which are the closest neighbours of mitochondria [4]. Finally, the mitochondrial localization of frataxin has recently been demonstrated in human cells by immunofluorescence studies (Koutnikova, H., Foury, F. and Koenig, M., submitted to publication).

Deletion of the *YDL120* gene is associated with mitochondrial dysfunction, the severity of which varies from one strain to another. In W303ΔYDL120, cytochrome oxidase activity is decreased concomitantly with cellular respiration, while in CENPK2ΔYDL120, the mitochondrial genome cannot be maintained even under normal growth conditions. These data raise the possibility that Ydl120p plays a role in the maintenance of the mitochondrial genome as previously suggested by the observation that skin fibroblasts in Friedreich's ataxia are more sensitive to ionising radiation [14,15]. Another possibility would be that Ydl120p controls the expression of the mitochondrial genome, more especially at the level of cytochrome oxidase. In this respect, it would be interesting to determine whether the translation of the three mitochondrially encoded cytochrome oxidase subunits is specifically decreased in null mutants. However, as discussed below, although an indirect regulatory function of the mitochondrial genome by *YDL120* is quite plausible, we do not think that this is the primary function of this gene. The most striking trait of the null *ydl120* mutants is that they accumulate in mitochondria 10–15 times more iron than the wild-type. Iron accumulates in the mitochondria of both rho$^+$ and rho$^-$ null mutants, i.e. in the absence of mtDNA. Moreover, the cellular growth of the rho$^-$ CENPK2ΔYDL120 mutants is much slower than that of a rho$^-$ wild-type strain, suggesting that Ydl120p plays a vital function in the cell which is not directly linked to the expression of the mitochondrial genome.

What might be the function of Ydl120p? Iron is an essential component of the mitochondrial metabolism, present in the heme of the cytochromes and in FeS center proteins. Thus iron must be imported into mitochondria. Its concentration, however, must be tightly regulated as it is a highly reactive species which generates very toxic radicals. The mechanism of iron transport in mitochondria is not known (for a review, see [16]). In particular, it is unknown whether an efflux of iron from mitochondria exists. It has been shown that rat liver and *S. cerevisiae* mitochondria contain substantial amounts of free reduced iron localized in the matrix and loosely bound to the inner membrane [10,16–18]. In intact yeast mitochondria iron reduction is an energy-requiring process depending on external NADH [19]. Our data strongly suggest that Ydl120p is involved in the control of mitochondrial iron homeostasis. The loss of the mitochondrial genome observed after the addition of ferrous ions to the culture medium in null *ydl120* rho$^+$ mutants suggests that the maintenance of mtDNA is tightly controlled by the concentration of mitochondrial iron. In this respect, it must be mentioned that mutants in the Atm1p mitochondrial ATP binding cassette (ABC) transporter also accumulate iron in mitochondria and lose mtDNA [20].

We have also shown that copper is toxic in the null mutants. However, the frequency of rho$^-$ mutants is not increased. Large amounts of copper sulfide precipitate on the cell surface of the null mutants, a phenomenon which is considered as a detoxification mechanism [12]. These data suggest that the observed toxicity is not related to copper accumulation in mitochondria but probably results from an increased copper uptake from the external medium in agreement with the observation that the uptakes of iron and copper, although distinct, are intimately linked [21],

Association of Friedreich's ataxia with mitochondrial dysfunction and iron deposits has been mentioned more than 10 years ago [13,22] but was never seriously taken into consideration. It can be now predicted that Friedreich's ataxia is a mitochondrial iron disease. In conclusion, this work has brought the proof that *S. cerevisiae* can be a good model to approaching the molecular basis of human disease genes of unknown and unpredictable function.

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