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Review

Respiratory complex III dysfunction in humans and the use of yeast as a model organism to study mitochondrial myopathy and associated diseases ☆

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ABSTRACT

The bc_1 complex or complex III is a central component of the aerobic respiratory chain in prokaryotic and eukaryotic organisms. It catalyzes the oxidation of quinols and the reduction of cytochrome *c*, establishing a proton motive force used to synthesize adenosine triphosphate (ATP) by the F_1F_o ATP synthase. In eukaryotes, the complex III is located in the inner mitochondrial membrane. The genes coding for the complex III have a dual origin. While cytochrome *b* is encoded by the mitochondrial genome, all the other subunits are encoded by the nuclear genome. In this review, we compile an exhaustive list of the known human mutations and associated pathologies found in the mitochondrially-encoded cytochrome *b* gene as well as the fewer mutations in the nuclear genes coding for the complex III structural subunits and accessory proteins such as BCS1L involved in the assembly of the complex III. Due to the inherent difficulties of studying human biopsy material associated with complex III dysfunction, we also review the work that has been conducted to study the pathologies with the easy to handle eukaryotic microorganism, the yeast *Saccharomyces cerevisiae*. Phenotypes, biochemical data and possible effects due to the mutations are also discussed in the context of the known three-dimensional structure of the eukaryotic complex III. This article is part of a Special Issue entitled: Respiratory complex III and related *bc* complexes.

1. Introduction

Mitochondria are subcellular compartments present in almost all eukaryotic cells. Their inner membrane hosts the multi-subunit protein complexes of the respiratory chain (Fig. 1) that produces the power (under the form of a transmembrane electrochemical gradient of protons used for ATP synthesis) required by cells and organisms to survive. It is assumed that in humans for instance, 90% of the ATP requirement is provided by the mitochondrial respiratory function. Mitochondrial function is not restricted to ATP production, in some cases, there is no ATP synthesis at all, as is found in heat-producing brown adipose tissues for example [1]. An important mitochondrial function is also the maintenance of the cellular redox state. This latter function is well revealed in mitochondrial disorders for which an unbalanced NADH/NAD^+ ratio and lactic acidosis may be biochemical signs of the disease. Furthermore it has been shown that mitochondria are able to store an appreciable amount of calcium [2–4] to modulate calcium signals [5–7] and to induce cellular apoptosis [7–9]. In addition, membrane potential is required by mitochondrial transporters that facilitate the import into the mitochondria of the numerous proteins (>1000) synthesized in the cytoplasm. The mitochondria are the site of major steps of diverse metabolic pathways. They host an essential central part of carbon central metabolism (TCA cycle, β-oxidation of fatty acids etc.) in addition to the biosynthesis of the iron–sulphur and haem prosthetic groups.

According to these numerous essential functions, it is thus understandable that any defect in respiratory enzymes caused by genetic mutation or pharmacological interference may result in disastrous consequences with a multiplicity of presentations according to the tissues and the affected function.

A large number of mitochondrial diseases are due to mutations in the respiratory complexes [10], among which mutations in complex III (CIII) are not the most frequent in comparison with the numerous complex I-(*ND1-6* genes) or tRNA-associated mutations reported in the mitochondrial genome. They do, however, present the same diversity of pathologies compared with mitochondrial mutations in

Abbreviations: CI-V, complex I-V; cyt, cytochrome; ISP, Rieske or Iron Sulfur Protein; Sub, subunit; ROS, Reactive Oxygen Species; LHON, Leber Hereditary Optic Neuropathy; MELAS, Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes; haem b_L, low mid-point potential cyt *b* haem; haem b_H, high mid-point potential cyt *b* haem; Q_o, quinol oxidising site on the positive side of the membrane; Q_i, quinone reductase site on the negative side of the membrane; a.a., amino-acid

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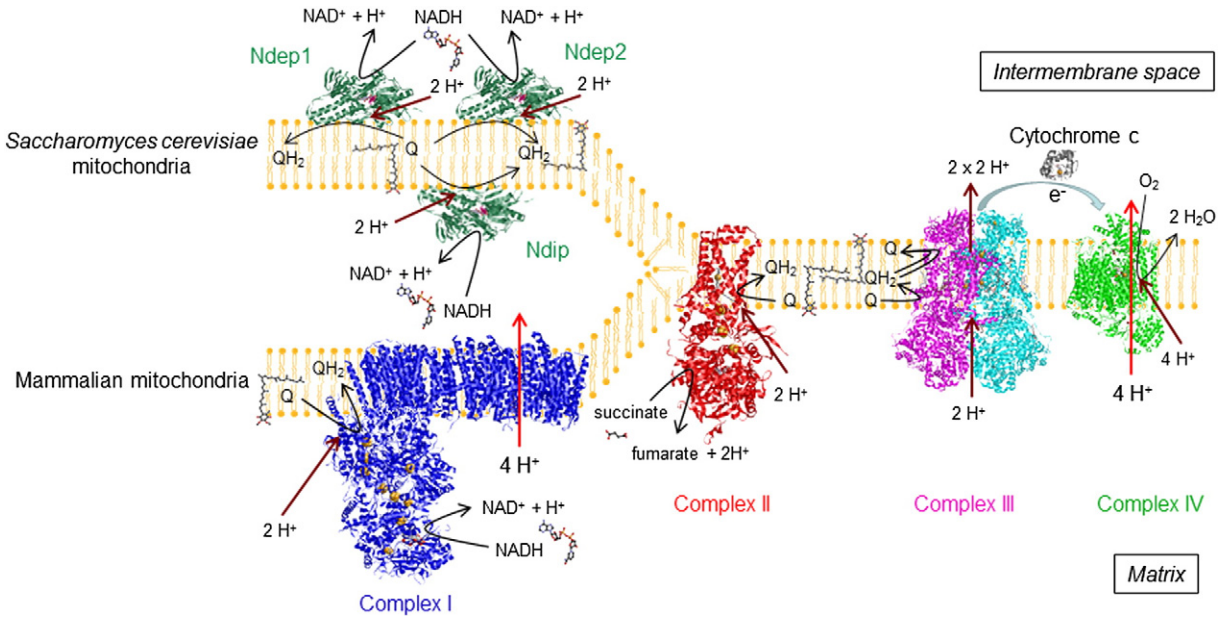

Fig. 1. Schematics of the mitochondrial respiratory chain and differences between mammalian and yeast *S. cerevisiae*. Q is quinone (UQ6 in yeast, UQ10 in mammalian), QH_2 is quinol. Brown arrows indicate chemical protons and red arrows indicate pumped protons transported across the membrane during respiratory complex activity. PDB codes are: 1OZK [129] for type II NADH dehydrogenases Ndep1, Ndep2 (external) and Ndip (internal) homology models (in dark green); 2FUG [130] and 3RKO [131] for complex I (in blue); 3SFE [132] for complex II (in red); 1PP9 [133] for complex III (dimer in magenta and cyan); 1OCR [134] for complex IV (in green); 2B4Z [135] for cyt *c* (in grey).

the other respiratory complexes. Studying CIII dysfunction is thus of prime importance, particularly to understand the pathologies associated with mutations in CIII, but also to acquire a better knowledge of the functioning of this complex in normal conditions.

1.1. Heteroplasmy and the threshold effect: the special case of complex III

Due to the high copy number of mitochondrial DNA (mtDNA) molecules in a human cell, a mitochondrial mutation might not necessarily be present in all mtDNA molecules, so that a mixture of mutated and wild-type mtDNA coexists in the same cell or mitochondrion. The percentage of mutated mtDNA, called heteroplasmy (which is not necessarily the same in all cells and tissues) clearly plays an important role in the expression of the corresponding mitochondrial mutation and the onset of a disease. It rapidly became apparent that a low proportion (between 5% and 10%) of wild-type mtDNA could be sufficient to compensate for a much greater proportion of mutated mtDNA [11]. In concordance with this result, it was observed that a rather high inhibition of a given complex is necessary to get an appreciable decrease in the rate of oxygen consumption or ATP synthesis [12]. The plot of the rate of oxygen consumption as a function of the inhibition of each complex evidences first a slight decrease until a 'threshold' after which (high inhibition) the VO_2 decreases abruptly.

This process was systematically studied for all complexes in different tissues by titrating the activity of each complex with a specific inhibitor [13]. The thresholds are different depending on tissues, on complexes and on the conditions. The study in [13] shows that the control coefficient of CIII in rat mitochondria respiring on pyruvate is nearly zero with a high threshold, greater than 80% in all tissues, except heart (around 60–70%). It means that an 80% decrease in the CIII activity will appreciably decrease the ATP synthesis in heart and not in the other tissues. With succinate, a lower threshold is observed and is different in muscle and liver [14]. The same authors showed that CIII has a low control coefficient in the measurement of the II + III activity i.e. that a rather large decrease in CIII activity (under 60%) will not change the succinate-cyt *c* reductase (CII + III) activity, so that the measurement of this activity is not a reliable assessment of a decrease in CIII activity [15,16]. This difficulty in assessing a decrease in CIII activity and the difficulty in measuring directly this activity is perhaps a reason for which CIII mutations may be difficult to identify. The existence of a threshold in the expression of a mutation affecting an enzyme activity is not restricted to oxidative phosphorylation and respiratory complexes, it is a general response of a metabolic network to an enzymatic change due to the buffer effect played by the metabolite intermediates [17].

In addition to heteroplasmy and threshold effects described above, the detection and analysis of the primary cause of the pathologies may be complicated by the fact that the respiratory chain complexes are themselves components of larger structural and functional units, the respiratory chain supercomplexes or "respirasomes" described in bacterial as well as in mitochondrial membranes [18]. Repercussions of mutations in cyt *b* of CIII have been observed in CI activity in mice and also in patients [19] however it does not seem to be a general rule since cyt *b* mutation does not always lead to decreased CI and -IV activities [20] as reported in the Part 1 below.

1.2. Complex III structure and mechanism:

CIII is a homodimeric complex with a mass of approximately 480 kDa consisting of 10–11 subunits per monomer. Three subunits form the electron-transferring catalytic core and contain the redox-active groups; haems and [2Fe2S] cluster, namely cyt *b*, cyt c_1 and the iron sulphur (or 'Rieske') protein (ISP). The atomic structure of CIII from a variety of organisms has been solved [21–24]. These structures share the same catalytic core and are very well superimposable between bacteria and eukaryotes (yeast, chicken and cow). The central subunit is cyt *b*, a predominantly hydrophobic protein consisting of eight transmembrane helices linked *via* extramembranous loops. In eukaryotes, cyt *b* is mitochondrially encoded while the other subunits are nuclearly encoded.

CIII catalyzes the transfer of electrons from ubiquinol ($CoQH_2$) to cytochrome (cyt) *c* and couples this electron transfer to the vectorial translocation of protons across the inner mitochondrial membrane. The enzyme has two discrete quinone-binding sites (Q_o or Q_P, quinol oxidation site and Q_i or Q_N, quinone reduction site), which are located

on opposite sides of the membrane and linked by a transmembrane electron-transfer pathway (Fig. 2). Cyt *b* provides both the Q_o and Q_i pockets and the transmembrane electron pathway (*via* haems b_L and b_H).

The catalytic mechanism of CIII is rather complicated and although the subject of intensive research, it is far from being entirely deciphered at the molecular level. It is largely accepted that it operates according to the Q-cycle hypothesis originally proposed by P. Mitchell and modified by others (Fig. 2) [25–28]. The two electrons from a $CoQH_2$ molecule, bound to the so-called Q_o site, are not linearly transferred to two cyt *c* molecules but take two distinct pathways; only one of these routes (a high potential pathway) lead to cyt *c* via the ISP protein, localized on the external side (the positive side) of the inner mitochondrial membrane. The other electron pathway crosses the membrane, taking a low potential pathway, towards another quinone binding site (Q_i located on the other (negative) side of the membrane). Proton movements across the inner mitochondrial membrane are associated with these electron transfers, and thus energy is conserved as an electrochemical proton gradient. The "Q-cycle" mechanism results in an increased energetic yield in comparison with a linear pathway. The mechanism responsible for the electron bifurcation in Q_o, which is the basis of the catalytic mechanism, is still largely unknown at the molecular level.

The study of human CIII variants is rather difficult due to the low quantity of material available in biopsies and also due to heteroplasmy. Biochemical assays have been developed for measuring the combined activities of the different complexes (CI–V) with a limited amount of biological material from a patient (less than a hundred milligrams), the CIII activity being measured by the antimycin-sensitive reduction of cyt *c* with decylubiquinol as electron donor [29,30]. Yeast (*S. cerevisiae*) CIII presents a great advantage since the availability of biological material is not a limitation in the study. In addition, yeast mitochondria are homoplasmic for a given mitochondrial mutation. The yeast CIII is very similar to the human enzyme with 50% identity for cyt *b*. Human mutations of CIII can be introduced in yeast and their effect on the complex assembly and activity can be analyzed in details. When the respiratory defect caused by the mutation is severe, the yeast models of human disease mutations can be used to investigate compensatory mechanisms, giving hints for possible treatment.

It must be stressed that the composition in respiratory complexes of the respiratory chain is similar in yeast and human (Fig. 1) with the exception of a multisubunit, protonmotive CI (complex I, NADH-ubiquinone oxidoreductase) which is absent in *S. cerevisiae* and replaced by three type II NADH dehydrogenases (Fig. 1). One (Ndip) is directed to the matrix [31] and the two other (Nde1p and Nde2p) to the intermembrane space [32,33]. The absence of CI in yeast reinforces the role of yeast CIII in building the proton-motive force.

In this review, we will analyze the numerous mutations associated with human pathologies not only related to the mitochondrial encoded cyt *b* but also the fewer mutations in genes coding for the supernumerary subunits of the CIII (subunits 7 and 8) and the accessory proteins (BCS1L, TTC19) necessary for the CIII biogenesis, all encoded by the nuclear genome. As noted above, the yeast *S. cerevisiae* is a very useful model to study human pathologies and we will also describe the mutations introduced in the *S. cerevisiae* cyt *b* gene to mimic the human pathologies. Results are discussed in the context of the available atomic structures of the yeast and vertebrate CIII.

1.3. Part 1: Human mutations leading to a pathology directly related to the complex III

Most of the mutations found in genes coding for the membrane-bound complexes of the mitochondrial respiratory chain in man

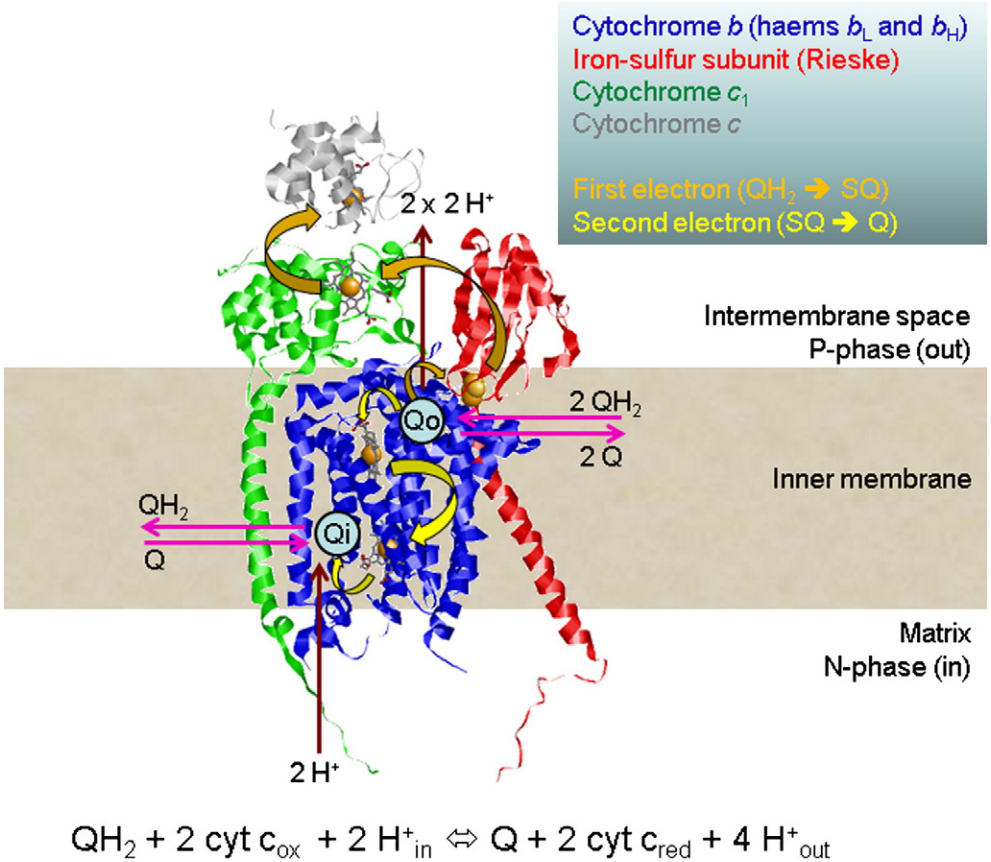

Fig. 2. Complex III catalytic mechanism. Only the three catalytic subunits (cyt *b* in blue, Rieske protein in red and cyt c_1 in green) and cyt *c* (in grey) are shown. Q_o and Q_i are quinol oxidising and reducing sites, respectively. PDB code: 3CX5 [109] (yeast CIII cocrystalized with cyt *c*). See text for details on the catalytic mechanism.

have been found in the maternally inherited mitochondrial genome which codes for the subunits ND1-6 of CI (NADH:ubiquinone oxidoreductase), cyt *b* in CIII (bc_1 or ubiquinol cyt *c* reductase), subunits 1–3 of CIV (cyt *c* oxidase) and also for the ATPase subunits 6 and 8. Relatively few mutations are associated with the nuclearly-encoded genes of the respiratory complexes. This is due to the difficulties into identifying such mutations in the whole nuclear genome and to the lower rate of appearance of nuclear genome mutations in comparison with the mitochondrial genome [34]. The reasons for the latter are twofold: firstly, the mitochondrial DNA is more exposed to reactive oxygen species (ROS) produced by the respiratory chain and hence suffers a higher degree of oxidative damage, and secondly, the DNA repair system is less efficient in mitochondria. Along with CI, CIII is a main site of ROS production and at high level, they damage cellular components and can induce cell death and are assumed to be involved in aging processes. Studies of mitochondrial genomes in human population revealed polymorphisms (supposedly neutral), which have been used as phylogenetic tools to investigate human origins and migrations. Several reports have pointed out the possible impact of haplogroups (mtDNA lineages sharing common polymorphisms) on mitochondrial physiology (for instance on aging, adaptation to colder climate, susceptibility to other mitochondrial mutations). Some haplogroup-specific polymorphisms are located in cyt *b*, the central catalytic subunit of CIII.

1.3.1. Mutations in the mitochondrial encoded cytochrome b

The review of all cyt *b* mutations described in the literature [35,36] and presented herein reveals a few common features. Firstly, it appears that skeletal muscle involvement, exercise intolerance and lactic acidosis are often reported in the pathologies. However, we should stress that these characteristics are not specific and not always encountered in cyt *b* defects and thus cannot constitute a rule for the detection of cyt *b* mutations linked to pathologies. Almost all the cyt *b* mutations are heteroplasmic and often detected in skeletal muscle tissue, with fewer in other tissues leading to the proposal that the mutations arose de novo after differentiation of the primary germ layers in cases where the mutations are not maternally inherited. An alternative explanation proposed in [37] is that sporadic mutations would segregate preferentially in muscle tissues and less in other tissues due to a negative selection.

Due to the heterogeneity of the pathologies linked to the cyt *b* mutations as seen in Table 1 (exercise intolerance, cardiomyopathy, deafness, LHON, MELAS, etc.), it is rather difficult to gather together classes of mutations and their different effects in human. We thus describe each important mutation and its associated effects in the next section which is divided in two paragraphs; the first paragraph describes the single mutations which are more likely responsible for a pathology and the second reports the cyt *b* mutations possibly linked to diseases and associated with other mutations (often in ND1 in case of LHON pathology) present in the mitochondrial genome.

1.3.1.1. Cytochrome b mutations involved in the primary cause of pathologies. In the human population, the cyt *b* gene (MTCYTB) exhibits a high level of variance. Over 100 missense changes from the consensus reference sequence (the rCRS, revised Cambridge Reference Sequence) have been reported (*mitomap.org*). Most of these variations might be silent. However, some variations, especially those located in regions involved in the catalytic activity might induce subtle changes in CIII function that are unnoticed during normal circumstances. In certain adverse conditions, these changes might affect the cell fitness and subsequently the health of the individuals bearing these variants. Mutations in cyt *b* have also been associated with mitochondrial diseases or reported in patients with specific cancers (see below).

Cyt *b* is encoded between nucleotides 14747 and 15887 on the heavy strand of the mtDNA [38]. The *MTCYB* gene in humans does not contain introns which is in contrast to yeast [39]. The gene codes for a highly hydrophobic polypeptide of mass 42.7 kDa which migrates anomalously during SDS-PAGE with an apparent mass of 30 kDa due to the presence of eight transmembrane helices (Fig. 3).

In 1970, before the sequence of human mitochondrial cyt *b* was available, Spiro et al. [40] described the first study of cyt-related inherited disorder. Two cases were studied, a 46 year-old man and his 16 year-old son affected with muscle weakness, progressive ataxia, dementia and nervous system disorder. A decreased cyt *b* content was measured in muscle mitochondria. Later Morgan-Hughes et al. [41] reported a patient with myoclonus followed by generalized weakness, ataxia, and mental confusion. Biochemical studies on muscle mitochondria revealed a defect at the cyt *b* level. A patient reported by Darley-Usmar et al. [42] showed tissues specific defects found in association with lactic acidosis and muscular weakness. Biopsies revealed low CIII activity and low level of reducible cyt *b* with diminished quantity of core proteins, ISP and subunit VI. The possible role of cyt *b* as the site of the primary defect was proposed.

In 1984, Hayes et al. [43] reported a 20 year-old Chilean girl who presented with ptosis and fatigable weakness. A high proportion of the muscle fibres contained aggregations of abnormal mitochondria and biochemical investigation demonstrated a respiratory chain deficiency localized to CIII. Birch-Machin et al. [44] reported a newborn infant with severe lactic acidosis and CIII deficiency in skeletal muscles and liver mitochondria. The issue was fatal after 3 days.

When sequencing of mitochondrial DNA became relatively routine during the 1990s, mutations could be identified at the molecular level. Bouzidi et al. [45] described a low CIII activity in muscle from a 25 year-old man with progressive exercise intolerance and later Dumoulin et al. [46] identified the first cyt *b* missense mutation shown to be pathogenic, changing the conserved Gly290 in Asp (see Table 1, Fig. 3, and Part 2 for yeast phenotype).

Andreu et al. [47] described 5 patients (from 32 to 52-year-old) with progressive exercise intolerance, lactic acidosis, in some cases myoglobinuria and biochemical evidence of CIII deficiency. They identified three nonsense mutations (p.Trp113Ter, p.Trp141Ter, p.Trp326Ter), one missense mutation in the cyt *b* gene leading to the p.Gly34Ser substitution and a deletion of 8 residues p.Gly251_Leu258del (Table 1, Figs. 3, 4 and see Part 2). There was no maternal inheritance and there were no mutations in tissues other than muscle. Andreu et al. [47] concluded that there is sporadic form of mitochondrial myopathy and the disorder is due to somatic mutations in myogenic stem cells after germ-layer differentiation.

The same research group [48] described a 38 year-old female patient who developed progressive muscle weakness with exercise intolerance and elevated arterial lactate. The family history was negative for neuromuscular disorders. A heteroplasmic (85% of the total mitochondrial DNA) cyt *b* mutation in the patient's muscle was identified and corresponds to substitution p.Gly339Glu (Table 1, Fig. 3 and see Part 2).

Andreu et al. [49] described also an infant girl with multisystem disorder (including hepatic steatosis and kidney tubular necrosis). She was the first case described with histiocytoid cardiomyopathy and died of cardiac arrest at the age of 4 weeks. The authors identified a heteroplasmic mutation in cyt *b* gene resulting in a p.Gly251Asp substitution (Table 1, Fig. 3 and see Part 2).

Keightley et al. [37] reinvestigated a 34-year-old woman followed for 17 years, originally reported in [50], with symptoms of lactic acidosis and exercise intolerance associated with severe CIII deficiency. She responded to therapy with menadione and ascorbate. Gradually, she developed a mitochondrial encephalomyopathy. Keightley et al. [37] identified a nonsense mutation resulting in a truncated cyt *b* (p.Gly166Ter, Table 1). The heteroplasmic mutation was present at 87% in skeletal muscle and only 0.7% in blood. The mutation was also present in other tissues, including hair roots, indicating that it must have arisen either very early in embryogenesis or in the maternal germline.

Table 1
Human mutations leading to pathology and a possible dysfunctional complex III.

Mutations in mitochondrial encoded cyt *b*	Mitochondrial nucleotide change	Mutation in human	Causing diseases or associated with symptoms/polymorphism	Residue in yeast/mutation	Depicted in Fig. 3	References
	14787del	I14-frameshift	MELAS/Parkinsonism	V13		[52,53]
	C14792G	H16D	Associated with ND6, Leigh syndrome	S15		[68]
	G14831A	A29T	LHON, ND1 associated/polym.	Y28		[69]
	A14841G	N32S	LHON, ND1 associated	N31		[70]
	G14846A	G34S	Exercise intolerance	G33S	+	[47]
	T14849C	S35P	Optic dysplasia, exercise intolerance, cardiomyopathy	S34	+	[55]
	T14864C	C40R	MELAS	C39	+	[63]
	G15024A	C93Y	Possible deaf helper mutation	V92		[71]
	G15043A	G99-synonymous	Associated with Major Depressive Disorder/polym.	A98		[72]
	G15059A	G105Ter	Exercise intolerance and myoglobinuria	G104		[121]
	G15077A	E111K	DEAF associated/polym.	T112		[73]
	G15084A	W113Ter	Exercise intolerance	W114		[47]
	G15150A	W135Ter	Exercise intolerance	Y136		[58]
	G15168A	W141Ter	Exercise intolerance	W142		[47]
	G15170A	G142Ter	Exercise intolerance, lactic acidosis	G143		[59]
	T15197C	S151P	Exercise intolerance	S152P	+	[58]
	G15242A	G166Ter	Encephalomyopathy	G167		[37]
	G15243A	G166E	Cardiomyopathy	G167E	+	[51]
	G15257A	D171N	LHON associated factor/polym.	S172		[66,67]
	T15287C	F181L	Possible deaf factor/polym.	L182		[74]
	A15395G	K217E	LHON associated factor	R218		[75]
	C15452A	L236I	Cardiomyopathy/polym.	M237		[51,122]
	G15497A	G251S	Obesity, exercise intolerance/polym.	G252		[123,124]
	G15498A	G251D	Cardiomyopathy,deafness/polym.	G252D	+	[49,76]
	15498del24	G_{251}DPDNYTL-del	Exercise intolerance	G_{252}HPDNYIP-del	+	[47]
	A15533G	N263D	Lactic acidosis, seizures, mental delay; possibly associated with unknown nuclear mutation	V264		[64]
	A15579G	Y278C	Multisystem disorder	Y279C	+	[54]
	G15615A	G290D	Exercise intolerance	G291D	+	[46,125]
	T15621G	L292R	Possible LHON helper mutation	I293		[126]
	T15635C	S297P	Neonatal polyvisceral failure	A298	+	[20]
	T15635G	S297A	Possible LHON helper mutation	A298		[126]
	A15662G	I306V	Multi-disorder mitochondriopathy/polym.	F307		[77]
	T15674C	S310P	LHON/polym.	S311		[78]
	T15693C	M316T	Cardiomyopathy, possible left ventricular noncompaction/polym.	T317		[79]
	G15699C	R318P	Exercise intolerance, encephalopathy	K319P	+	[60]
	G15723A	W326Ter	Exercise intolerance	F327		[47]
	G15761A	G339Ter	Exercise intolerance	G340		[61]
	G15762A	G339E	Mitochondrial myopathy, exercise intolerance	G340E	+	[48]
	G15773A	V343M	Possibly synergistic, associated with LHON/polym.	V344		[80]
	C15800T	Q352Ter	Exercise intolerance, myopathy	Q353		[62]
	G15812A	V356M	Synergistic, associated with LHON/polym.	F357		[66,77]
Mutations in nuclear genes coding for CIII subunits	Gene/Function/ Locus	Mutation in human	Disease	Equivalent in yeast	Depicted in Fig. 3	References
	UQCRB/Subunit 7 13.5 kD/8q22.1	Change the last seven a.a. and addition of 14 a.a. at C-ter.	Hypoglycemia, CIII activity deficiency	Qcr7/Subunit 7 14.5 kD	+	[85]
	UQCRQ/Subunit 8 9.9 kD/5q31.1	S45F	Severe neurologic defects	a.a. Q55 in Qcr8/ Subunit 8 10.9 kD	+	[86]
Mutations in nuclear genes coding for biogenesis factors	Gene/Function/ Locus	Mutation in human	Disease	Mutation in yeast (human numbering)/ Yeast complementation	see Fig. 5	References
	BCS1L/CIII-FeS subunit assembly factor/2q35	G35R R45C T50A R56Ter R73C S78G P99L I106Ter R114W G129R R144Q R155P R183H, C R184C S277N	Leigh, GRACILE or Bjornstad syndromes, Encephalopathy, hepatic failure and tubulopathy, muscle weakness, optic atrophy.	G35R[a] S78G[a] P99L[a] R114W[a] R155P[a] R183H[a] R184C[a] R291Ter[a] Q302E[a] R306H[a] P99L[b] R155P[b] S277N[b] V353M[b] K192P[c]		[94–97, 100–105,127]

Table 1 (*continued*)

Mutations in nuclear genes coding for biogenesis factors	Gene/Function/ Locus	Mutation in human	Disease	Mutation in yeast (human numbering)/ Yeast complementation	see Fig. 5	References
		R291Ter Q302E R306H V327A V353M F368I		S314N[c] R73C[d] R183C[d] R184C[d] F368I[d] F368I + R73C[d] G129R[e]		
	TTC19/FeS CIII assembly, only metazoan/17p12	Q173Ter L219Ter	Neurodegenerative disorder, CIII activity decreased in muscle	Ortholog unknown		[108]

The list of cyt *b* mutations is derived from MITOMAP (A Human Mitochondrial Genome Database. http://www.mitomap.org, 2011) and updated. We used the HGVS (Human Genome Variation Society) nomenclature in the text but for space and clarity reasons, we use a simpler nomenclature in the Tables and Figures.
Abbreviations: DEAF: Maternally inherited DEAFness or aminoglycoside-induced DEAFness; GRACILE: Growth Retardation, Amino aciduria, Cholestasis, Iron overload, Lactic acidosis and Early death; LHON: Leber Hereditary Optic Neuropathy. MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes; polym.: reported as polymorphism in the MITOMAP data base (http://www.mitomap.org/bin/view.pl/MITOMAP/PolymorphismsCoding).

[a] Yeast Δ*BCS1* complemented by the human *BCS1L* containing the mutations [94].
[b] Yeast Δ*BCS1* complemented by the human *BCS1L* gene containing mutations [95].
[c] Yeast Δ*BCS1* complemented by the yeast *BCS1* gene containing mutations [95].
[d] Yeast Δ*BCS1* complemented by the human *BCS1L* gene containing mutations [100].
[e] Yeast Δ*BCS1* complemented by the human *BCS1L* gene containing mutation [105].

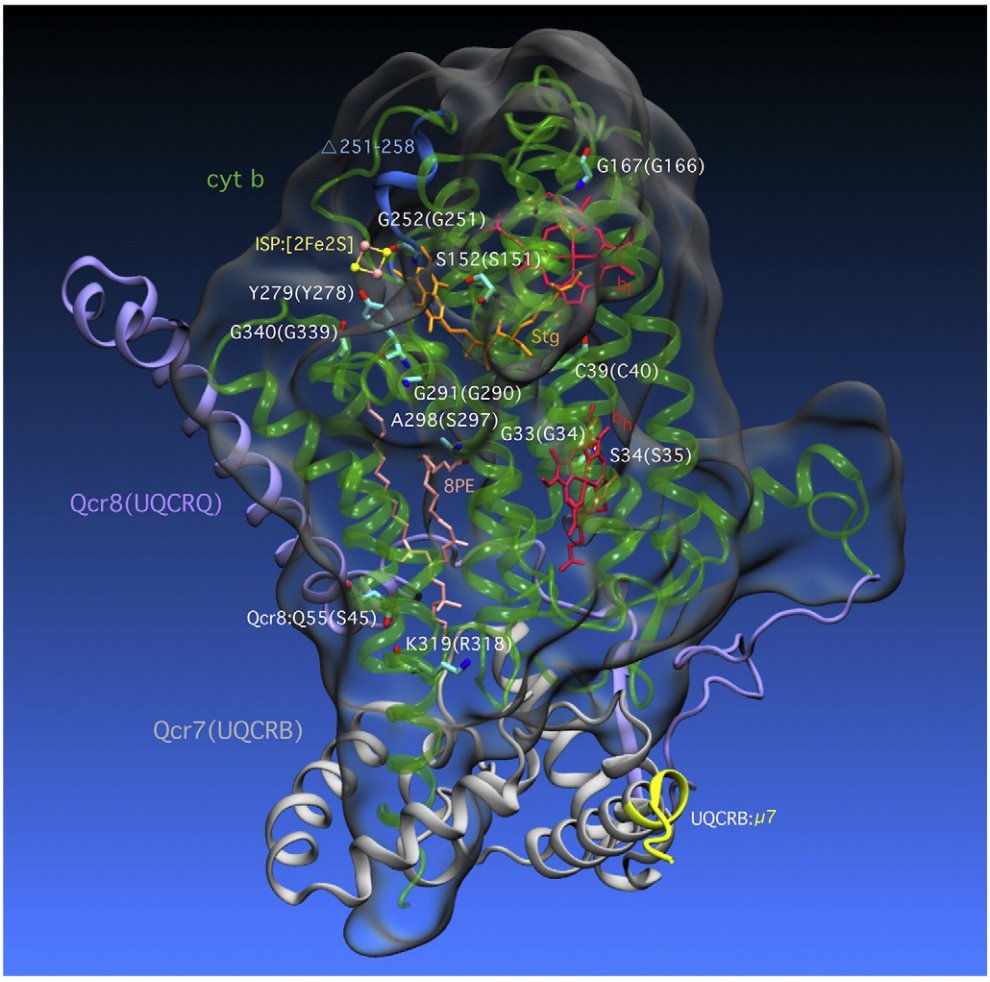

Fig. 3. Disease-associated mutations within cytochrome *b* and the Qcr7, 8 subunits of the complex III. This figure was prepared using the coordinates for yeast (*S. cerevisiae*) CIII (3CX5.PDB, [109]). Represented in cartoon form are CIII subunits cyt *b* (green), Qcr7 (grey) and Qcr8 (purple). A glass van der Waals surface has been added to cyt *b* to aid visual clarity. Residues associated with human disease mutations are shown for cyt *b* (except nonsense mutations leading to a truncated cyt *b*) and Qcr8 using yeast notation, with the corresponding human residues given in parentheses. The seven-residue deletion ΔGly251-Leu258 at the Q_o site is represented in blue cartoon form. The seven-residue addition to the C-terminus of Qcr7 (μ7) is indicated in yellow. Also shown are Q_o-bound stigmatellin (orange), cyt *b* haems b_L and b_H (red), cyt *b*-bound 3-SN-phosphatidylethanolamine (8PE in pink) and the [2Fe2S] cluster from Rieske ISP protein (polypeptide backbone not shown). Refer to Table 1 and text for more details.

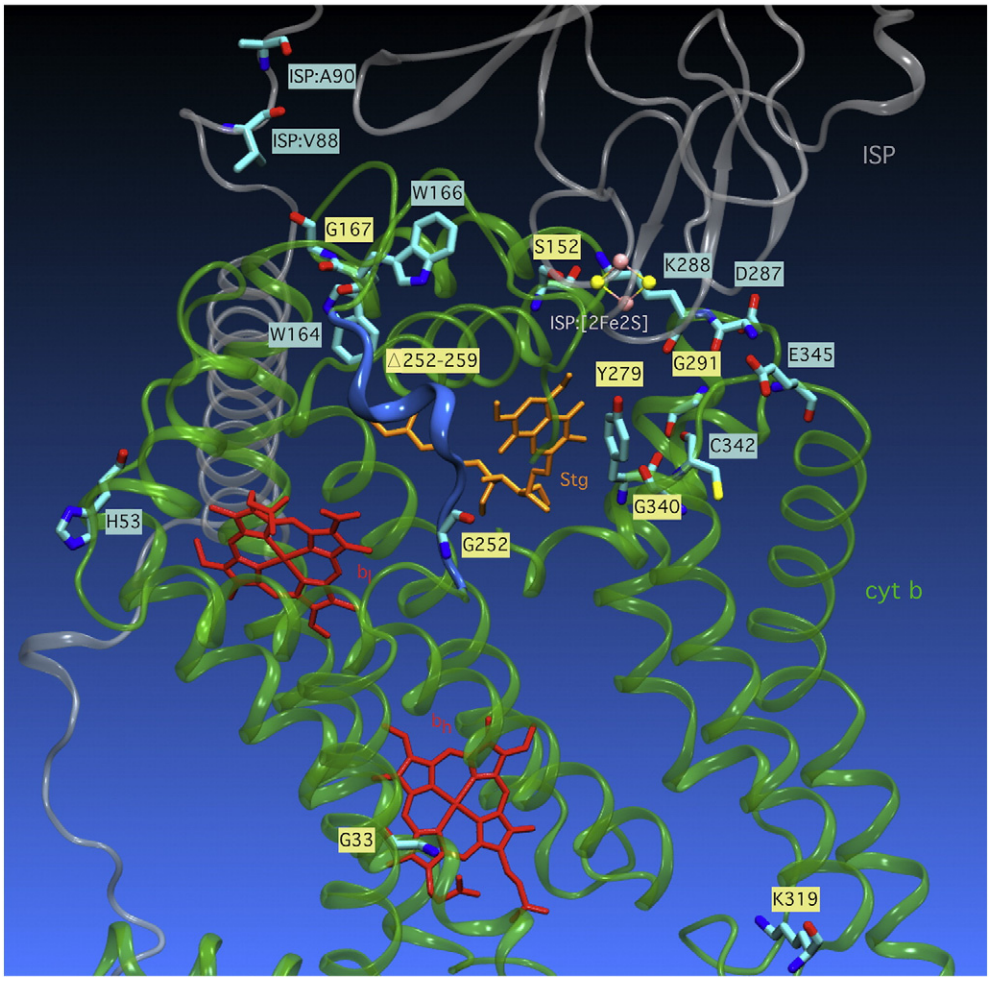

Fig. 4. Yeast cytochrome *b* mutations and associated reversions studied as models for human pathologies. This figure was prepared using *S. cerevisiae* CIII coordinates 3CX5.PDB. The polypeptide backbone of cyt *b* is represented in green with the Rieske ISP in grey. Residues associated with human pathologies upon mutation are shown with yellow labels (yeast notation). Suppressor mutations (reversions) are indicated with blue labels. Also shown are stigmatellin (orange), cyt *b* haems b_L and b_H (red) and the ISP [2Fe2S] cluster. Refer to Table 2 and text for more details.

In the same position cyt *b* Gly166, Valnot et al. [51] described a *de novo* cyt *b* mutation leading to the substitution p.Gly166Glu (Table 1, Figs. 3, 4 and see Part 2) in one patient with severe hypertrophic cardiomyopathy. It is noteworthy that out of the five isolated patients with CIII deficiency, only one carried a cyt *b* mutation and this study supports an autosomal rather than a maternal inheritance in the majority of their patients with CIII deficiency [51].

De Coo et al. [52] identified a heteroplasmic 4-bp deletion leading to a truncated protein (p.Ile14fs, Table 1). The young boy patient had an akinetic rigid syndrome and a mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). He developed parkinsonism and myoclonus. More than 95% of the mtDNA in muscle was mutated. Rana et al. [53] found defective synthesis of the cyt *b* protein, decreased amount of ISP and subunit VI, defective oxidative phosphorylation, defective CIII activity and increased hydrogen peroxide production, indicative of increased electron transfer bypass reactions to oxygen.

Wibrand et al. [54] described a patient who, besides severe exercise intolerance, had also multisystem disorder (deafness, mental retardation, retinitis pigmentosa, cataract, growth retardation, epilepsy) with a selective decrease of CIII activity in muscle. The heteroplasmic mutation in muscle (88%) and leukocytes (15%) changed a highly conserved Tyr to Cys at position 278 (Table 1, Figs. 3, 4 and see Part 2).

Schuelke et al. [55] described a 25-year-old patient with neurologic symptoms starting in infancy. He suffered from septo-optic dysplasia, retinitis pigmentosa, exercise intolerance, hypertrophic cardiomyopathy. The mutation m.14849T>C was heteroplasmic in muscle (69%) and fibroblasts (12%) resulting in the p.Ser35Pro substitution within cyt *b* (Table 1, Fig. 3). Analysis of this p.Ser35Pro substitution in the atomic structure of the bovine CIII in the presence of the Q_i site substrate quinone (1NTZ.pdb [56]) shows that residue Ser35 is located in transmembrane helix A on the Q_i site and is only 4.5 Å from the substrate ubiquinone hydrophobic tail and in close proximity (4–5 Å) to the edge of haem b_L. Substitution of a Ser by a Pro likely distorts this transmembrane helix and interferes with ubiquinone and/or haem binding. Such mutation was not reported in any other organism (see for review [57]) and could be easily studied in yeast in order to better understand the cyt *b* defects at a molecular level.

Legros et al. [58] reported two original deleterious mutations (substitutions p.Trp135Ter and p.Ser151Pro, see Table 1, Figs. 3, 4 and Part 2). The heteroplasmic mutations were only found in muscle tissue where they represented 60–80% of the mtDNA molecules. Both patients had an intolerance to exercise from their late childhood with a CIII activity below 20% in muscle associated with a reduced amount of cyt *b* protein but activity was found normal in leukocytes and fibroblasts.

Bruno et al. [59] described a 40-year-old woman developing progressive exercise intolerance, lactic acidosis and muscle cramps beginning at the age of 30 years. Histochemical and biochemical studies revealed ragged-red cyt *c* oxidase positive fibers and combined deficiency of CI

and CIII activities in muscle. The mutation m.15170G>A leads to a stop codon (p.Gly142Ter, Table 1). The mutation was very abundant in muscle (>90%) but undetectable in lymphocytes and fibroblasts.

A p.Arg318Pro substitution of a highly conserved amino acid was reported in a 38-year-old woman suffering from migraine, muscle pain, sensorineural deafness [60] associated with a severe reduction in the activities of both CI and CIII in skeletal muscle. (Table 1, Figs. 3, 4 and part 2).

Mancuso et al. [61] reported the cyt *b* m.15761G>A heteroplasmic mutation (73% in muscle) leading to a stop codon (cyt *b* p.Gly339Ter, Table 1) and thus a truncated protein with a loss of the last 41 residues corresponding to the *gh* loop and the last transmembrane helix H. The 19-year-old woman suffered from exercise intolerance, vomiting and lactic acidosis.

Another truncated cyt *b* protein with the subsequent loss of transmembrane helix H was also reported in [62] with the cyt *b* nonsense mutation m.15800C>T (substitution p.Gln352Ter). The affected 24-year-old woman had exercise intolerance with muscle cramps and lactic acidosis. The mutation was heteroplasmic in muscle (45%) but absent in the other tissues examined. This mutation is associated with a combined deficiency in CI and CIII activities.

Recently, Emmanuele et al. [63] described a novel mutation m.14864T>C in *MTCYB* (a.a. change p.Cys40Arg, Table 1 and Fig. 3) in a 15-year-old girl affected with migraines, epilepsy, sensorimotor neuropathy, and stroke-like episodes (MELAS). The mutation was heteroplasmic in muscle, blood, fibroblasts, but absent in her asymptomatic mother. Residue Cys40 is highly conserved and located in the middle of transmembrane helix A, between haems b_L and b_H. Mutation to the much larger and cationic Arg residue is likely to be deleterious to the correct folding and (thermodynamically unfavorable) insertion of helix A into the hydrophobic interior of the lipid bilayer.

All the cyt *b* mutations described above are found to be heteroplasmic. However, Fragaki et al. [20] described an unusual fatal multisystemic presentation and the first case of a homoplasmic mutation m.15635T>C in all tissues tested leading to the replacement of a conserved Ser297 into Pro (Table 1 and Fig. 3). The patient was a new born boy died at 24 h of age of polyvisceral failure. Since her mother did not exhibit any mutation, the mutation is supposed to have arisen de novo very early in embryogenesis. A CIII activity deficiency was detected but other respiratory chain complex (CI, CII, CIV) and ATPase activities were unaffected. Ser297 is located in the middle of transmembrane helix F and replacement with a bulkier Pro could distort the local environnement of helix F and likely modifies drastically the overall cyt *b* folding.

1.3.1.2. Cytochrome b mutations associated with other mitochondrial mutations. A number of mutations in cyt *b* may not be the primary cause of human disease but are associated with other mutations present in either mitochondrial genes (for example in the LHON pathology) or rarely in nuclear genes [64]. These type of mutations described below are reported in Table 1 but are not depicted in Fig. 3 if the direct involvement of these mutations in pathology is not demonstrated but they mainly act as secondary and helper mutations or can be defined later as polymorphisms.

LHON (Leber Hereditary Optic Neuropathy) is a maternally-inherited form of acute visual loss and blindness. Two major cyt *b* alleles have been described to contribute to LHON pathology. The first substitution (p.Asp171Asn) (Table 1) is located in a hydrophilic region at the end of the amphipathic cd_2 helix, at less than 10 Å from the ISP protein. Substitution p.Asp171Asn may possibly affect the docking of the ISP with cyt *b* during the catalytic cycle. Substitution p.Asp171Asn was found to be pathogenetically significant but was not found alone associated with LHON [65]. The m.15257G>A mutation (p.Asp171Asn) is always associated with mutations in the mitochondrially encoded *ND5* [66,67] or *ND6* genes of CI. The second cyt *b* mutation corresponds to substitution p.Val356Met associated with the primary LHON mutations in ND5 and ND6 subunits of CI or associated together with the cyt *b* p.Asp171Asn substitution described above [66,67]. Val356 (Phe357 in yeast, see Table 1) is located in the transmembrane helix H and its side chain is facing outside the CIII towards the membrane bilayer and away from interdimeric contacts, so it is difficult to predict the possible repercussions of this secondary mutation on the function of CIII. For these two reported cases of cyt *b* mutations associated to the LHON pathology, the involvement of both CI and CIII mutations indicates that the clinical signs might be the consequences of the synergetic effects of mutations and an overall decrease in mitochondrial energy production. However, these two cyt *b* substitutions (p.Asp171Asn and p.Val356Met) are now reported as polymorphisms in the Mitomap database. In this case of multiple mutations, yeast may provide an excellent model since it is homoplasmic for a given mitochondrial DNA mutation and thus can be used to study the effects of each individual mutation. This could reveal that a given mutation leads to a real phenotype or has no effect and is more likely a polymorphism.

Cyt *b* p.His16Asp substitution (Table 1) was reported by Ronchi et al. [68] in addition to mutation in ND6 leading to a Leigh syndrome in a 15-month-old female patient but to date this mutation does not seem to be associated with pathology since the mother, homoplasmic for the cyt *b* mutation, was not affected by the pathology. Residue His16 (Ser15 in yeast) is located with loop *a* in the amino terminal region of cyt *b* in the matrix side.

Fauser et al. [69] found in LHON patients the m.15257G>A mutation (cyt *b* change p.Asp171Asn) already reported in LHON pathologies (see above) and described a novel mutation m.14831G>A (cyt *b* change p.Ala29Thr, Table 1) but the real pathogenicity of this mutation was not proven since it is associated with a second ND1 mutation and a 16S rRNA polymorphism. Residue Ala29 is located at the beginning of the transmembrane helix A in the Q_i site.

Yang et al. [70] reported on a Chinese family carrying the novel heteroplasmic m.14841A>G mutation (cyt *b* replacement p.Asn32Ser, Table 1) and affected by the LHON pathology with visual impairment. The authors suggest that this mutation may play a synergistic role with a ND1 mutation, increasing the severity of the LHON pathology. Residue Asn32 is located 6 Å from both the Q_i site quinone molecule and a propionate of haem b_H on the negative side of the membrane, at the amino-terminal region of the transmembrane helix A.

Tang et al. [71] described a case of maternally hearing loss in a Chinese 16-year-old man. The primary mutation is m.7505T>C in $tRNA^{Ser}$ but the mutation m.15024G>A leading to the cyt *b* substitution p.Cys93Tyr (Table 1) may have a modifying role in deafness expression. Residue Cys93 lies in the middle of transmembrane helix B and substitution with the larger Tyr side chain introduces a steric hindrance with proximal residues.

In a study related to the Major Depressive Disorder, Rollins et al. [72] reported a m.15043G>A mutation (not changing the nature of cyt *b* residue Gly99) associated with two others mutations in mitDNA, a putative haplotype associated with risks factors for psychiatric disorders. The cyt *b* mutation is reported as a polymorphism (Mitomap database).

Lévêque et al. [73] reported a maternal inherited m.15077G>A mutation (cyt *b* substitution p.Glu111Lys) as one of the putative pathogenic variants in hearing impairment (polymorphism in Mitomap database).

Ballana et al. [74] have identified a three-generation family segregating two heteroplasmic mtDNA mutations: the deafness-related m.1555A>G mutation in the 12S *rRNA* gene and a new mutation m.15287T>C (substitution p.Phe181Leu) in the cyt *b* gene. Phe181 is located in the Q_o region, in transmembrane helix D and at the interdimeric interface with the *trans* dimeric cyt *b* molecule. Mutation to Leu may thus be disruptive for the stable association of the dimer, although a Leu residue is naturally present in the corresponding yeast position (Leu182, Table 1) and this mutation may be a polymorphism (Mitomap database).

A Han Chinese family affected with vision loss (LHON) was studied in [75]. In addition to a ND4 and others mitDNA mutations, the cyt *b* m.15395A>G mutation (substitution p.Lys217Glu, Table 1) may act synergistically with the primary LHON mutation. Lys217 is located in the cyt *b de* loop, on the matrix side at the interface with subunit 8 of the CIII. Introduction of a negatively charged Glu instead of the positively charged Lys could destabilize the interaction between cyt *b* and Subunit 8.

Sensorineural hearing loss is one of the most frequent clinical features in patients with mitochondrial diseases caused by mutations in mitDNA. Kato et al. [76] reported a m.15498G>A mutation (substitution p.Gly251Asp, Table 1, Figs. 3, 4 and Part 2) possibly linked to deafness. This mutation was already encountered in a case of cardiomyopathy [49].

An interesting study [64] reported on a patient affected with seizures, lactic acidosis, mental delay and behavior abnormalities associated with a decrease in CIII activity in patient's skeletal muscle. Mutant (m.15533A>G leading to cyt *b* change p.Asn263Asp, Table 1) transmitochondrial cybrids restored normal activities and the proband's mother, clinically unaffected, also harbored the homoplasmic mutation. These results demonstrate the possible pitfalls in the detection of pathogenic mitochondrial mutations, and highlight the role of the genetic mtDNA background in the development of mitochondrial disorders. This case raises the possibility of a primary unknown nuclear mutation responsible for the disease and the lower CIII activity.

Finsterer et al. [77] studied the case of a 33-year-old man with major renal insufficiency and severe multi-disorder mitochondriopathy. One novel heteroplasmic mutation m.15662A>G (>75% in skeletal muscle) was detected (cyt *b* substitution p.Ile306Val, Table 1) in addition to mutations in *ND1* gene and the cyt *b* mutation m.15812G>A (substitution p.Val356Met already observed in LHON [66]). Ile306 residue is located at the C-terminal region of transmembrane helix F, facing the solvent on the matrix side of the complex. Mutation to Val is a conservative change, but could be deleterious for the interaction with the nearby helix *bc* loop. It should mentioned that the patient did not exhibit any alteration in CIII activity. The residue Ile306 is not conserved during evolution and the corresponding mutation m.15662A>G is reported as a polymorphism in the Mitomap database but the cumulative effects of the four transitions in the mitochondrial DNA are not excluded to explain the pathology.

Abu-Amero et al. [78] investigated a 16-year-old patient with LHON and reported a m.15674T>C mutation (cyt *b* substitution p.Ser310Pro, Table 1) in addition to a pathogenic mutation in the $tRNA^{Gln}$. The cyt *b* mutation is reported to be pathogenic but one cannot exclude the primary role of the $tRNA^{Gln}$ mutation. Residue Ser310 is located in the *fg* loop, on the negative side of the membrane and mutation to a Pro residue seems compatible without any drastic changes in this cyt *b* region, however an unpredicted detrimental effect is not excluded, perhaps mediated by perturbed interaction with nearby subunit Qcr7.

Tang et al. [79] were looking for mitochondrial mutations associated with the heart left ventricular noncompaction condition. They described a 16-year-old patient with left ventricular trabeculation associated with the rare mutation m.15693T>C (substitution p.Met316Thr, Table 1) which could be potentially pathogenic, however this mutation is reported as a polymorphism (Mitomap database). Met316 residue is located in the cyt *b fg* loop on the negative side of the membrane. As with the S310P variant, mutation of Met316 to Thr may affect the local fold of the *fg* loop leading to destabilisation of the interaction with nearby Qcr7.

A possible secondary role for the cyt *b* p.Val343Met substitution (mutation cyt *b* m.15773G>A, Table 1) was reported [80] in Italian patients with LHON pathology and myoclonus. Two homoplasmic LHON mutations in *ND4* and *ND1* genes were present in these patients. Results suggest that the LHON phenotype may relate to the synergic role of the mtDNA variants. Val343 is located in the *gh* loop on the positive side of the membrane. Simulating the p.Val343Met substitution in the atomic structure of CIII does not seem to indicate a particular steric hindrance or electrostatic interaction with proximal residues and this mutation is reported as a polymorphism (Mitomap database).

1.3.2. Somatic cytochrome b mutations

Finally, several acquired somatic mitochondrial mutations (not shown in Table 1) have been found in cancer pathologies, including homoplasmic mutations. For example, Fliss et al. [81] reported a 21-bp deletion at nucleotide 15642 (corresponding to human cyt *b* residues p.Leu299_Pro305del) in a patient with bladder cancer. This region of cyt *b* is located in the transmembrane helix F and this deletion is likely to be severely deleterious to the fold of cyt *b* and by consequence the assembly of the intact CIII.

In a colorectal cancer, Polyak et al. [82] found 3 somatic mutations in the mitochondrial genome. Two occurred in the cyt *b* gene leading to p.Arg80His or p.Phe276Leu substitutions and one mutation in the *COXI* gene. Arg80 is located at 3.5 Å from the propionates of haem b_L in the CIII 3D-structure and mutation with the positively charged side-chain of His could modify the properties of haem b_L. Phe276 (Pro277 in yeast) is located in the *ef* loop just after the $P_{270}EWY_{273}$ motif conserved through evolution and important for catalysis of quinol substrate in the Q_o site.

A likely cause for the accumulation of mitochondrial mutations in cancer cells is the production of ROS in this organelle by the respiratory chain. ROS oxidatively damage the rapidly mutiplying mtDNA molecules and this is coupled with the lower level of DNA repair in mitochondria compared to the nucleus [83]. These findings suggested that mitochondrial mutations may contribute to tumor growth as pointed out early on by Warburg [84] who suggested that alterations of oxidative phosphorylation in tumor cells play a causative role in cancerous growth.

1.3.3. Mutations in the nuclear encoded structural subunits of the complex III

To our knowledge, only two nuclear genes coding for intrinsic CIII subunits have been reported to contain mutations in human and lead to a pathology. The first report in 2003 by Haut et al. [85] described the case of a young Turkish girl born of consanguineous parents (both healthy and heterozygous for the mutation) with a homozygous 4 bp deletion in exon 4 of the *UQCRB* gene located in chromosome 8. The deletion is predicted to change the last seven amino acids and to add a stretch of 14 residues at the C-terminal region of the subunit 7 of 13.5 KD (Table 1 and Fig. 3). This C-terminal region of subunit 7 is accessible from the outside of the CIII (Fig. 3) and is in contact with Core protein I of the trans monomer in the CIII dimeric structure, on the matrix side of the membrane. The patient suffered from hypoglycemia and liver dysfunction associated with a CIII activity deficiency and a lower amount of cyt *b* as determined spectrophotometrically.

The second case was reported by Barel et al. [86] with a consanguineous Israeli Bedouin kindred presenting an autosomal-recessive phenotype with severe psychomotor retardation, dystonia, ataxia and marked dementia. Reduced CI and III activities were found in muscles. A single homozygous missense mutation (p.Ser45Phe) in UQCRQ (encoding CIII subunit VIII in chromosome 5) was detected and is shown in Fig. 3 to be localized in the Q_i site region, facing cyt *b* and a phosphoethanolamine molecule which is located 4 Å from the Ser45 side chain in the yeast CIII atomic structure. The introduced bulky Phe residue may destabilize interactions with this lipid molecule which could modify the conformation of this region important for catalysis on the Q_i site, as it has already been observed for the importance of lipid molecules in the CIII [87]. Note that in the closest relative to human CIII 3D-structure, the bovine CIII 3D-structure (pdb 2A06) displays also this phosphoethanolamine molecule in the same

position as in the yeast structure and might explain the reduced CIII activities found in patients.

1.3.4. Mutations in the nuclear encoded auxiliar subunits required for the complex III assembly

The synthesis and assembly of the respiratory chain complexes is a rather complicated process (especially in eukaryotes), requiring additional proteins encoded by the nuclear genome and imported into the mitochondrion. There are as many as thirty different factors involved in the assembly process of yeast CIV (cyt *c* oxidase) [88]. To date, thirteen different factors have been described and reviewed in [89] specific for the CIII assembly in yeast, namely Cbs1, Cbs2, Cbp1 and Cbp3 as translational activators of COB mRNA, Cbp2 as a splicing factor of COB pre-mRNA, Cbp4 and Cbp6 interact with the newly synthesized cyt *b*, Cyc2 is involved in the cyt *c* and c_1 maturation process, Cyt2 as haem lyase for cyt c_1, Bca1 a recently discovered factor for early step in CIII assembly [90], Mzm1 a matrix protein for the ISP protein assembly [91], TTC19 a membrane protein only found in metazoans and Bcs1 an AAA-ATPase necessary for the ISP insertion. To date, only the latter two nuclear genes, *BCS1L* and *TTC19*, were shown in man to contain mutations which lead to pathologies. However most of the CIII deficient cases remain to be elucidated at the molecular level. It is likely that more mutations in already known assembly factors will be described as well as in yet unknown assembly factors which remain to be discovered.

1.3.4.1. The case of BCS1L. Bcs1 was initially identified by Tzagoloff et al. [92] as a inner membrane protein necessary for yeast respiratory growth. The human *BCS1L* gene encodes a homolog of *S. cerevisiae BCS1* gene involved in the assembly of CIII. *BCS1* is present in all eukaryotes but not in bacteria. The Bcs1 protein shares sequence similarity with members of the AAA-ATPases superfamily. The predicted 419-amino-acid human protein is 50% identical to the yeast Bcs1. Bcs1 is a translocase of the FeS subunit of the CIII across the inner membrane and is involved in its topogenesis rather than a chaperone protein. Bcs1 is a membrane-bound protein with one transmembrane helix (Fig. 5) and its AAA-domain is facing the matrix side where Bcs1 and the ISP subunit may interact. Bcs1 is likely to form an oligomeric ring structure (often hexameric for other AAA-ATPases) with a central pore in the membrane necessary to translocate ISP, a reaction requiring ATP hydrolysis (see for review [93]).

More than 20 different mutations have been mapped on the *BCS1L* gene [94] and linked to human diseases (Table 1 and Fig. 5). The first mutations found in the *BCS1L* gene were described by de Lonlay et al. [95] who reported six patients from four different families of Turkish origin with CIII deficiency. Two patients and one foetus had mutation leading to the p.Ser277Asn transition. The pathologies of the patients were neonatal tubulopathy, hepatic failure, and encephalopathy. Two other unrelated patients from consanguineous families had acidosis, hepatic failure and neurologic symptoms, consistent with a Leigh syndrome: the mutation caused the substitution p.Pro99Leu (Table 1, Fig. 5). A fifth newborn patient had two mutations in *BCS1L* leading to both p.Arg155Pro and p.Val353Met substitutions. Yeast complementation studies with the yeast or human gene containing the corresponding mutations (Table 1) confirmed the deleterious effects of these missense mutations [95].

The GRACILE syndrome (Growth Retardation, Amino aciduria, Cholestasis, Iron overload, Lactic acidosis and Early death) affected Finnish patients with the *BCS1L* homozygous mutation leading to the substitution p.Ser78Gly [96] (Table 1, Fig. 5). Contrary to the Turkish patients described above, the Finnish patients had normal CIII activity and no neurological problems. However they exhibited marked hepatic iron overload. This high level of free plasma iron could arise from the loss of the [2Fe2S] cluster from the ISP. Visapää et al. [96] also described three British infants who had a different pathology with CIII deficiency and neurological symptoms. They are associated with *BCS1L* mutations leading to substitutions p.Val327Ala, p.Arg56Ter, p.Ser78Gly, p.Arg144Gln as well as mutations in the introns splicing sites.

Very recently, Lynn et al. [97] described a male infant who died at three months of age and presenting with GRACILE-like syndrome. He was affected with acidosis, hypoglycaemia, aminoaciduria and iron overload but had no neurological symptoms. A strong defect in CIII activity was detected and to a lesser extent at the level of CI and CIV. Both the nonsense mutation c.166C>T leading to substitution p.Arg56Ter and inherited from the father and the c.-588T>A mutation in the 5′ untranslated first intron region upstream from the start codon and inherited from the mother, were already reported in British infants described above [96].

A mouse model carrying a homozygous mutation leading to the substitution p.Ser78Gly described by de Lonlay et al. [95] was developed recently and represents the first example of viable higher eukaryotic model of CIII deficiency mimicking a human mitochondrial disorder of nuclear origin [98]. The mouse model exhibits similar symptoms with the human patients.

Bjornstad syndrome is the least severe disease associated to *BCS1L* mutations. It is an autosomal recessive disease characterized by sensorineural hearing loss and pili torti (flattened and twisted hair shafts). Lubianca et al. associated the Bjornstad syndrome with the locus 2q34-36 [99]. Hinson et al. [94] identified mutations in *BCS1L* resulting in p.Arg183His substitution in eight related individuals from consanguineous parents. In a four-year-old child, Hinson et al. [94] described a Bjornstad syndrome with mild mitochondrial CIII deficiency and found heterozygosity for 2 missense mutations leading to substitutions p.Arg184Cys and p.Gly35Arg (Table 1 and Fig. 5). Five additional *BCS1L* sequence variants were identified: one affected a splice site (leading to a truncated protein I106Ter), one lead to a truncated protein (p.Arg291Ter) and three missense mutations (p.Arg114Trp, p.Gln302Glu, p.Arg306His) in combination (or not) with the substitution p.Arg306His found in the case of Norwegian ancestry. All *BCS1L*

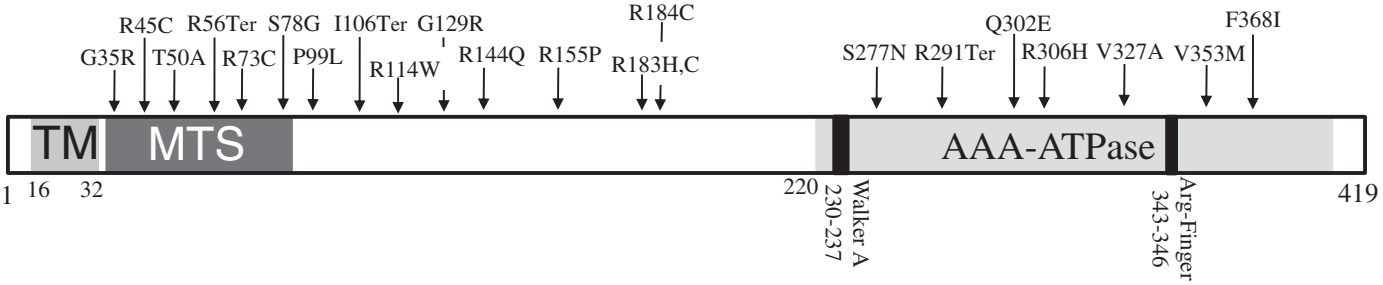

Fig. 5. Amino-acid changes leading to pathologies in the human BCS1L. Schematic representation of the human BCS1L protein (a.a. 1–419) with the different domains: TM: TransMembrane helix (a.a. 16–32); MTS: Mitochondrial Targeting Signal; AAA-ATPase domain of the AAA type family proteins (a.a. 220–419) including the Walker A motif (a.a. 230–237) and the Arginine Finger (Arg343–Arg346). Amino-acid substitutions alone or in combination (see text and Table 1 for more details) and causing a pathology in human are indicated by an arrow.

mutations disrupted the assembly of CIII and mitochondrial respirasome and the clinical severity of the mutations was also correlated with the production of reactive oxygen species.

Fernandez-Vizarra et al. [100] reported two isolated cases of young patients heterozygous for two mutations in *BSC1L*. The first case was a Moroccan girl with encephalopathy, lactic acidosis, psychomotor regression, hypotonia, deafness, brittle hair and CIII activity deficiency. Although the parents were unrelated, two proximal mutations were present in *BCS1L*, one mutation was inherited from the mother (substitution p.Arg184Cys, Table 1, Fig. 5) and one from the father (substitution p.Arg183Cys). Studies with yeast model showed that both individual mutations reduced mitochondrial respiratory activity and affect the incorporation of ISP in the CIII. The second case was an Italian girl from healthy unrelated parents, deceased at four-year-old. Her pathologies were encephalopathy, lactic acidosis, psychomotor delay, hypotonia, seizures, failure to thrive, brittle hair and CIII activity deficiency. Two mutations were detected in the *BCS1L* gene and lead to the substitutions p.Arg73Cys and p.Phe368Ile (Table 1 and Fig. 5). Introduction of the mutations in yeast *S. cerevisiae* lead to the conclusion that the replacement p.Phe368Ile is more detrimental than p.Arg73Cys.

De Meirleir et al. [101] investigated two Spanish patients that died prior to the age of three months. They had lactic acidosis, severe failure to thrive, liver dysfunction, renal tubulopathy and accumulation of iron in aggregates of macrophages which could be explained by the lack of incorporation of iron into the [2Fe–2S] cluster of ISP protein. An isolated biochemical CIII deficiency was detected in liver. Sequencing *BCS1L* gene revealed a missense mutation leading to substitution p.Arg45Cys and a nonsense mutation p.Arg56Ter (Table 1, Fig. 5). Each parent was heterozygous for one of the mutations. The missense mutation in combination with an absence of BCS1L function due to the second allele is responsible for the CIII deficiency in this family.

Ramos-Arroyo et al. [102] reported another Spanish infant carrying the substitutions p.Arg45Cys and p.Arg56Ter in BCS1L. Her symptoms were neonatal severe hypotonia and food intolerance and she developed lactic acidosis, renal tubulopathy with glucosuria and aminoaciduria. At the age of four months, she showed nystagmus, microcephaly and hypertonia prior to death at the age of six months. Muscle tissue exhibited impaired CIII activity. This child did not show iron overload as previously observed in patients reported in [101] and as has been observed in GRACILE syndrome described above. Ramos-Arroyo et al. [102] proposed that phenotypic variability in individuals with the same *BCS1L* mutations in a nuclear gene may be related to the tissue specificity of the expression of the mutant gene.

Blázquez et al. [103] described an infantile encephalomyopathy with unusual phenotype in a 4-year-old Spanish boy affected with different symptoms such as psychomotor retardation, dysmorphic features, failure to thrive, hypotonia, lactic acidosis, mild sensorineural hearing loss and hepatic dysfunction. However iron metabolism, renal function and hair were normal. Mitochondrial CIII deficiency was detected in muscles and fibroblasts associated with lower amounts of both BCS1L protein and CIII. They identified a homozygous mutation in *BCS1L* gene resulting in a p.Thr50Ala substitution in the mitochondrial targeting signal (Table 1, Fig. 5). Each unaffected parent was heterozygous for the mutation.

Morán et al. [104] studied fibroblasts from six CIII-deficient patients harboring mutations in the *BCS1L* gene (corresponding to isolated or combined substitutions p.Pro99Leu, p.Arg56Ter, p.Arg45Cys, p.Arg184Cys, p.Thr50Ala, Table 1 and Fig. 5). Cells exhibited slow growth in glucose, increased H_2O_2 levels, variable enzyme deficiencies and assembly defects of CIII but also of CI and CIV. Apoptotic cell death, structural alterations such as abnormal mitochondrial morphologies and fragmentation of the mitochondrial networks were also described in patient's fibroblasts. The patients had cytosolic accumulation of the BCS1L protein, suggestive of an impaired mitochondrial import, assembly or stability defects of the BCS1L complex.

A 20-year-old Kenyan woman patient was described by Tuppen et al. [105]. She was a 'floppy' infant whose condition worsened during childhood with increasing muscle weakness and optic atrophy. A CIII deficiency due to a homozygous BCS1L mutation leading to the substitution p.Gly129Arg (Table 1, Fig. 5) was demonstrated. The yeast *S. cerevisiae* carrying the human BCS1L substitution p.Gly129Arg is not able to grow on respiratory substrates.

1.3.4.1.1. Yeast as a model for BCS1 studies. In addition to be a very useful model for studying pathologies of mitochondrial origin (see part 2), yeast offers also the opportunity to mimic human pathologies of nuclear origin.

The two following studies have been carried out in the yeast *S. cerevisiae* to better understand the function of Bcs1 and more precisely the role of specific residues, some of them implicated in changes leading to pathologies in human. The use of yeast points out similarities but also differences between the yeast and human Bcs1 due to the fact that the same mutations do not always produce the same phenotype and biochemical results as seen below.

Conte et al. [106] reported in yeast *S. cerevisiae* that the gradual expression of Bcs1 leads to the formation of supercomplexes CIII–IV. The yeast substitution p.Arg81Cys allows CIII and supercomplex assembly in yeast whereas the corresponding human p.Arg45Cys substitution (Table 1 and Fig. 5) was instead the cause of a severe CIII deficiency [101]. However, yeast variants p.Lys192Pro and p.Phe401Ile (corresponding to human p.Arg155Pro and p.Phe368Ile substitutions associated with diseases, Table 1 and Fig. 5) severely altered the CIII structure and activity.

Using yeast, Nouet et al. [107] have performed a structure–function analysis of Bcs1 by randomly generating a collection of respiratory-deficient mutants. They showed that most mutations are in the C-terminal region of Bcs1. Intragenic and extragenic compensatory mutations were isolated. They showed that residues located at the junction between the Bcs1-specific and the AAA domains are important for the activity and stability of the protein and that the yeast residue Phe342 is important for interactions with partners or substrate proteins.

1.3.4.2. Gene TTC19. The second gene recently described and involved in assembly of CIII which carries human mutations is *TTC19* encoding for the tetratricopeptide repeat domain 19 (with 5 TPR domains mediating protein–protein interactions) of 45.5 kDa (mature size 35 kDa). It is located in the mitochondrial inner membrane and has been shown to physically interact with CIII. This protein is present in all metazoan but absent in other eukaryotes (plants, fungi) and prokaryotes. *TTC19* gene contains at least 10 exons and is encoded by chromosome 17 in human.

Ghezzi et al. [108] identified in two 37 and 24-year-old siblings from north-eastern Italy a homozygous mutation in the *TTC19* gene resulting in a p.Leu219Ter substitution truncating the protein (Table 1). They had a slowly progressive neurodegenerative disorder with onset in late infancy. A third unrelated subject from the same alpine area had a similar disease course. Muscle biopsies showed a marked isolated reduction of mitochondrial CIII activity. In addition, the authors identified a man with adult-onset mitochondrial CIII deficiency with a different homozygous mutation in the *TTC19* gene resulting in a p.Gln173Ter substitution (Table 1). The patient was clinically normal until 42 years old and then rapidly developed progressive neurological failure and died 3 years later. Muscle biopsy showed marked CIII deficiency. Ghezzi et al. [108] reported also the knockdown of the fly (*Drosophilia*) TTC19 ortholog which resulted in reduced life span, low fertility and locomotor impairment in the adult fly.

The proposed function for the mitochondrial targeted TTC19 protein is a chaperone involved in the early step of CIII assembly but questions remain regarding its direct function in interaction with

CIII, its absence in non-metazoan organisms, the non-affected CIII activity in the *TTC19* null mutant in *Drosophila melanogaster* larvae, and the relatively late-disease onset in patients. This suggests age and tissue dependent regulation of *TTC19* [108].

1.4. Part 2: Use of the yeast model to study cytochrome b mutations associated with human disease

A number of mutations in the human cyt *b* have been linked with diseases in man (Table 1, Fig. 3). Nonsense or frameshift mutations that result in truncated cyt *b* almost invariably abolish complex assembly. The precise effect of the missense mutations is often more difficult to predict or determine. Their characterisation in human tissues or cells is frequently hampered by the limited amount of tissue available and by the lack of cell lines harboring the mutation to be studied. The yeast *S. cerevisiae* is a convenient model for such a study as it has well-known advantages. Cells can survive in the absence of respiration by using the fermentation process as an energy source. Therefore the absence of one or more respiratory enzymes is not lethal. The yeast bc_1 complex (and especially the catalytic core) is very similar to its counterpart in humans. The atomic structure of the yeast enzyme has been determined [23,109,110]. Many genetic, biochemical and genomic tools are available. Of particular interest for such study, is the mitochondrial transformation technique. Using that method, engineered mutations can be introduced into mitochondrially-encoded genes, such as the cyt *b* gene. The mutants are then analyzed by a broad range of methods.

Using that approach, the impacts of human diseases were analyzed to determine the effects at the structural and mechanistic levels, and to explore possible compensation mechanisms. From respiratory growth deficient mutants, respiratory competent clones could be selected and the secondary mutations (suppressors or compensatory mutations) identified.

1.4.1. Mutations in the Q_i site region and perturbation of the overall bc_1 complex assembly

p.Gly33Ser. Gly33 is located within transmembrane helix A, a hydrophobic environment at the Qi site, close to haem b_H. The introduction of a Ser chain of the mutation p.Gly33Ser could perturb the local environment of the haem. The mutation had severe effect on the bc_1 structure as it was observed that the level of cyt *b* was decreased by 50%, spectral properties of cyt *b* were altered and the level of ISP was dramatically lowered, which was likely to result from a major perturbation of cyt *b* folding (Table 2, Fig. 4) [111].

p.Lys319Pro. Arg319 is very highly conserved in the vertebrate, plant and bacterial sequence data. It is replaced by Lys in fungi. Arg/Lys319 is located at the C-terminal region, a β-turn linking helices F2 and G and probably acts as a backbone H-bond donor to Asp316. Mutation of p.Lys319Pro would remove this H-bond and may disrupt the geometry of the turn. As judged by the lower bc_1 content in mutant cells, the mutation hampers the assembly of the complex [60] (Table 2, Fig. 4).

1.4.2. Mutations and compensatory mutations in the Q_o site region of cytochrome b

p.Ser152Pro: Ser152 is located at the entrance of the Q_o site, at the C-terminal end of helix cd_1, immediately preceding a β-turn leading to helix cd_2 and may be hydrogen-bonded to Lys288 located at the P-side of helix F1 (Fig. 4). Mutation to Ala results in a marked resistance to atovaquone associated with loss of function as the bc_1 complex activity was decreased to 20% of wild type control [112]. p.Ser152Ala has no major effect on the enzyme assembly but would result in the loss of H-bonding capacity, which could result in slight structural rearrangement of the Q_o pocket, a decreased affinity of atovaquone for the site and a lessened catalytic activity. Mutation to Pro had more severe effect and results in the loss of the ISP from the bc_1 complex and a nearly inactive enzyme (Table 2) [111]. The introduction of a Pro might be expected to distort the local fold of the region between cd_1 and cd_2, which is in close proximity with the [2Fe2S] domain of the ISP when this subunit is docked in the *b*-'proximal' position. Such a change is likely to be deleterious to the stability of the complex.

Table 2
Yeast cytochrome *b* mutations studied as model for human pathologies (see Fig. 4).

Mutations	Respiratory growth	Complex III level (%WT)	Complex III activity (%WT)	Suppressor mutations	References
G33S	Deficient	55	12	None	[111]
S152P	Deficient	55	6	ISP A90D	[111]
G167E	Deficient	75	22	E167 + W164L E167 + W166L	[113]
Δ252–259	Deficient	60	0	None	[111]
G252D	Competent	90	100		[111]
Y279C	Weak growth	75	58		[111]
G291D	Deficient	50	11	D291 + D287h D291 + H53D ISP V88A/G/D ISP A90T/D	[111]
K319P	Deficient	50	50		[60]
G340E	Deficient	Decreased	0	Deletion of E340 E340A E340V E340 + C342G E340 + E345G E340 + K288N	[117,128]

p.Gly167Glu: Gly167 is located in the extramembranous cd_2 helix of cyt *b*. The residue is in close proximity of residues forming the flexible 'tether' of the ISP, a region essential for the macroscopic movement of the cluster-containing headgroup of the subunit. Gly167 is highly conserved between organisms. It may be that a Gly at this position is required for the proper folding of the hinge region. It seems likely that replacement of Gly167 by the bulkier and charged residue could interfere with the movement of the ISP, which would in turn alter the catalytic activity of the complex. The mutation could also affect the binding of the ISP to the bc_1 complex. Experimental data showed that the mutation had severe effect on the bc_1 complex activity and that the mutant enzyme was sensitive to detergent and unstable in assay conditions. It was suggested that the ISP was progressively lost during the assay or its assembly was progressively distorted causing further inhibition of the enzyme activity [113]. The bc_1 complex activity was partially restored by mutations located close to the primary mutation, p.Trp164Leu and p.Trp166Leu, which are likely to reduce the steric hindrance caused by p.Gly167Glu (Table 2, Fig. 4).

p.Gly252_Pro259del: The deletion of eight amino acids may be expected to severely disrupt the assembly of the complex (Table 2, Fig. 4). However the mutant enzyme was still partially assembled, as judged by the optical signal for cyt *b* but the level of ISP was dramatically decreased [111]. The mutation is located in a surface loop connecting helices E and *ef* loop on the P side of the membrane, and is not directly in contact with the ISP. The alteration of the folding of cyt *b* caused by the shortening of this loop is likely to disrupt the stable binding of the ISP. The small population of ISP-containing enzyme was inactive. This could be explained by the major alteration of the catalytic site caused by the deletion.

p.Gly252Asp: The mutation p.Gly252Asp had no effect on yeast growth or bc_1 activity under standard conditions (Table 2). It has been reported that the yeast p.Gly252Asp mutant was thermosensitive, as the mutant bc_1 complex was found to be partly inhibited when the cells were grown at higher temperature (36 °C) [114]. Examination of the yeast bc_1 3D-structure (1P84.pdb [110]) reveals a tightly-bound phospholipid molecule at the P-side interface between cyt *b* and c_1.

The lipid headgroup is stabilized by polar interactions with the side-chains of His185 of cyt c_1 and Ser268 of cyt *b*, with the fatty acyl chains in hydrophobic contact with Trp273. An additional hydrogen bond is provided by the imidazole ring of His253 (cyt *b*) to an acyl ester oxygen atom of the bound lipid. Mutation p.Gly252Asp may disrupt this latter hydrogen bonding association, or distort the local fold at the lipid–protein interface. The possible interference of Asp252 with structural lipid could, in consequence, destabilize the enzyme assembly under some conditions.

p.Tyr279Cys: Tyr279 is in close proximity to the highly conserved '$P_{271}EWY_{274}$' motif region at the N-terminal region of the *ef* helix and within 3.5 Å of residue His181, a ligand of the ISP [2Fe–2S] cluster. The mutation p.Tyr279Cys has a moderate effect on the respiratory growth competence, little effect on bc_1 complex level and does not hinder the stable binding of the ISP (Table 2, Fig. 4). p.Tyr279Cys affects the quinol binding as EPR spectra showed an altered signal indicative of a lower occupancy of the Q_o site [111]. The positioning of ubiquinol at the Q_o site has been suggested to be influenced by hydrogen bonding interactions with the side-chain hydroxyl group of Tyr279 [110]. Interestingly, the same change is responsible for acquired resistance to atovaquone in the malaria agent, *Plasmodium falciparum* (p.Tyr268Cys, in *P. falciparum*). In yeast, p.Tyr279Cys causes also high level of atovaquone resistance [115]. The equivalent mutation in *R. capsulatus* bc_1 (p.Tyr302Cys) is associated with ROS production, intra-subunit crosslinking between the ISP and cyt *b*, and [2Fe–2S] cluster damage [116].

p.Gly291Asp: Gly291 is located in a transmembrane helix F1 (Fig. 4), a region of the molecule in the close vicinity of the Q_o site. Replacing Gly by Asp in that hydrophobic environment is likely to have severe consequence and to impair the folding of cyt *b* at the Q_o site, hindering the assembly of the ISP into the complex. The bc_1 complex level was indeed decreased (Table 2) and the level of ISP was dramatically lowered [111]. The respiratory function is partially restored by a second mutation in cyt *b*, p.Asp287His. Residue Asp287 is located in the Q_o region, 3 Å from the primary mutation. It is likely that the replacement of Asp by His reduces the electrostatic repulsion introduced by p.Gly291Asp and restores a correct folding in the Q_o region. A long-distance compensatory mutation in cyt *b* was also found: p.His53Asp. His53 is located in the loop connecting helices A and *ab* near the hinge region of the ISP (the ISP which reacts with the other monomer). Mutation to Asp may increase the stability of binding of the ISP to the complex.

p.Gly340Glu. Gly340 is located in the C-terminus of the transmembrane helix G, in close proximity to the Q_o site (Fig. 4). The residue is located within a predominantly hydrophobic pocket. The introduction of a charged Glu is expected to be destabilizing. Indeed the mutation completely abolishes the assembly of the bc_1 complex (Table 2). Removal of the anionic side-chain by the secondary mutations p.Glu340Ala, p.Glu340Val or by the in-frame deletion of Glu340 restores the respiratory growth competence. Second-site suppressor mutations were also observed that partially compensate for the deleterious primary mutation: p.Cys342Gly, p.Glu345Gly and p.Lys288Asn (Table 2) [117]. These second mutations might remove some source of destabilising electrostatic and/or steric hindrance around Glu340.

1.4.3. Compensatory mutations in the Rieske Iron Sulphur protein

Interestingly, other compensatory mutations partially restoring the bc_1 complex function altered by p.Ser152Pro and p.Gly291Asp were found in the hinge region of the ISP. The [2Fe–2S] prosthetic group of the ISP is located within its soluble C-terminal domain, on the P-side of the inner mitochondrial membrane (Fig. 4). This domain is connected to the transmembrane helix via a flexible hinge region (sequence T_{85}ADVLAMAK$_{93}$ in *S. cerevisiae*) that makes that the movement of the prosthetic group domain possible. This ISP hinge region is a "hot-spot" for selecting secondary mutations restoring cyt *b* Q_o site deficiency [118]. Several compensatory mutations at residues V88 and A90 were observed (ISP p.Val88Ala/Gly/Asp and p.Ala90Thr/Asp). The secondary mutations restored a more stable binding of the ISP on the mutated bc_1 complex. However the catalytic activity was still low.

Suppressor analysis was extended to another cyt *b* mutant, p.Ala144Phe. Ala144 is located in helix cd_1. p.Ala144Phe results in an assembled but partially inactive enzyme with altered quinol binding, which was presumably caused by minor changes in the local structure of cd_1 helix in the Q_o site. Several suppressors were found in the hinge region of ISP between residues 85 and 92 [119]. It was shown that ISP mutations at residues 85, 92 and 93 compensated p.Ala144Phe, but not p.Ser152Pro and p.Gly291Asp. It was suggested these ISP mutations altered the structure of the hinge region and allowed a greater reach of the ISP [2Fe–2S] domain and a more efficient docking on the Q_o site distorted by p.Ala144Phe. From the suppressor analysis, it could be suggested that the hinge region would play a role in the stabilization of the subunit on the complex and in a correct positioning of the [2Fe2S] domain on cyt *b* for an optimal Q_o site catalysis.

2. Concluding remarks

As shown in Table 1, most of the human mutations present in CIII and biogenesis factors are found in the mitochondrial encoded cyt *b* (both on the Q_o and Q_i sites, Fig. 3) which is the catalytic core of the enzyme and in the accessory factor *BCS1L* encoded by the nuclear genome. It is interesting to note the total absence to date of mutations in the nuclear genes encoding the ISP and cyt c_1 that are essential for the electron transfer activity in the bc_1 complex, in comparison with more than 20 mutations reported in *BSC1L* gene. It is predicted that in the future, more mutations present in the nuclear genes encoding subunits of the bc_1 complex will be described and studied in mammals as well as in yeast which is an experimentally tractable and reliable model for human pathologies as exemplified by several studies reported here. Our compilation of data in this review may motivate additional biochemical and genetics studies carried out in *S. cerevisiae* (or other model organisms like the yeast *Yarrowia lipolytica* used for the study of human CI-related pathologies [120]) in order to mimic defects found in human pathologies which would be otherwise difficult to study at a molecular level using human tissue samples. The unicellular eukaryotic yeast presents the advantages of a fermentative growth in case of respiratory deficient mutants, the unlimited amount of biological material available for study, the homoplasmic nature of the mitochondrial mutations introduced by the biolistic method, the in-depth biochemical and biophysical study of the effects of each mutation. In addition, yeast allows the study of the repercussions of combined and individual mutation in the mitochondrial genome in case where multiple mutations are present in human pathology for example in LHON. Due to the difficulty to localize and detect nuclear mutations in comparison with the relatively more simple sequencing of the entire mitochondrial genome, it is also predictable that more mutations will be discovered in the future in nuclear encoded accessory factors necessary for the import, correct folding, insertion of metal cofactors and incorporation of the CIII subunits in the final and active membrane complex. Also, interest in mitochondria and the somatic mutations detected in the mitochondrial genome with regard to cancer has revived due to their role in apoptosis and other aspects of tumor biology which are extensively studied these days.

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