

EXTRA VIEW



## Mitochondrial complex III Rieske Fe-S protein processing and assembly

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### ABSTRACT

Regulation of the mitochondrial respiratory chain biogenesis is a matter of great interest because of its implications for mitochondrial disease. One of the mitochondrial disease genes recently discovered associated to encephalopathy and mitochondrial complex III (cIII) deficiency is *TTC19*. Our study of *TTC19*-deficient human and mouse models, has led us to propose a post-assembly quality control role or 'husbandry' function for this factor that is linked to Rieske Fe-S protein (UQCRFS1). UQCRFS1 is the last incorporated cIII subunit, and its presence is essential for enzymatic activity. During UQCRFS1 assembly, the precursor is cleaved and its N-terminal part remains bound to the complex, between the two core subunits (UQCRC1 and UQCRC2). In the absence of *TTC19* there is a prominent accumulation of these UQCRFS1-derived N-terminal fragments that proved to be detrimental for cIII function. In this article we will discuss some ideas around the UQCRFS1 processing and assembly and its importance for the regulation of cIII activity and biogenesis.

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### Complex III overview

The mitochondrial respiratory chain (MRC) is composed of four multimeric enzymes, responsible for the redox reactions that take electrons from NADH and FADH<sub>2</sub>, ultimately reducing molecular oxygen to water. This electron transfer is coupled to proton pumping from the matrix to the intermembrane space, generating a proton electrochemical gradient, which is the driving force for the condensation of ADP+Pi by the F<sub>O</sub>-F<sub>1</sub> ATP synthase, a fifth complex, which together with complexes I to IV form the oxidative phosphorylation system (OXPHOS).

Complex III (cIII) or ubiquinol:cytochrome c oxidoreductase is the center of the MRC, transferring electrons from coenzyme Q (CoQ or just Q) to cytochrome c.

X-ray diffraction crystal structures of cIII are available from *Bos taurus* [1], *Gallus gallus* [2,3] and *Saccharomyces cerevisiae* [4] as well as a cryo-EM structure that has recently been published from *Homo sapiens* [5]. All of them are practically identical in shape and subunit composition. In all of these organisms, cIII is a tightly bound symmetrical dimer where one mitochondrial DNA (mtDNA) and nine nuclear genes encode the structural subunits forming each cIII monomer. Of all these subunits, only three possess electron transfer properties: 1) the mtDNA-encoded cytochrome b (MT-CYB in the mammalian nomenclature) that contains the two Q binding sites (Q<sub>o</sub> or Q<sub>P</sub> and Q<sub>i</sub> or Q<sub>N</sub>) and two b-type hemes with different redox potentials (*b<sub>L</sub>* and *b<sub>H</sub>*); 2) cytochrome c1 (CYC1) that contains one c-type heme and 3) the Rieske Fe-S protein (UQCRFS1), whose intermembrane space-facing hydrophilic C-terminus hosts a 2Fe-2S high-potential cluster.

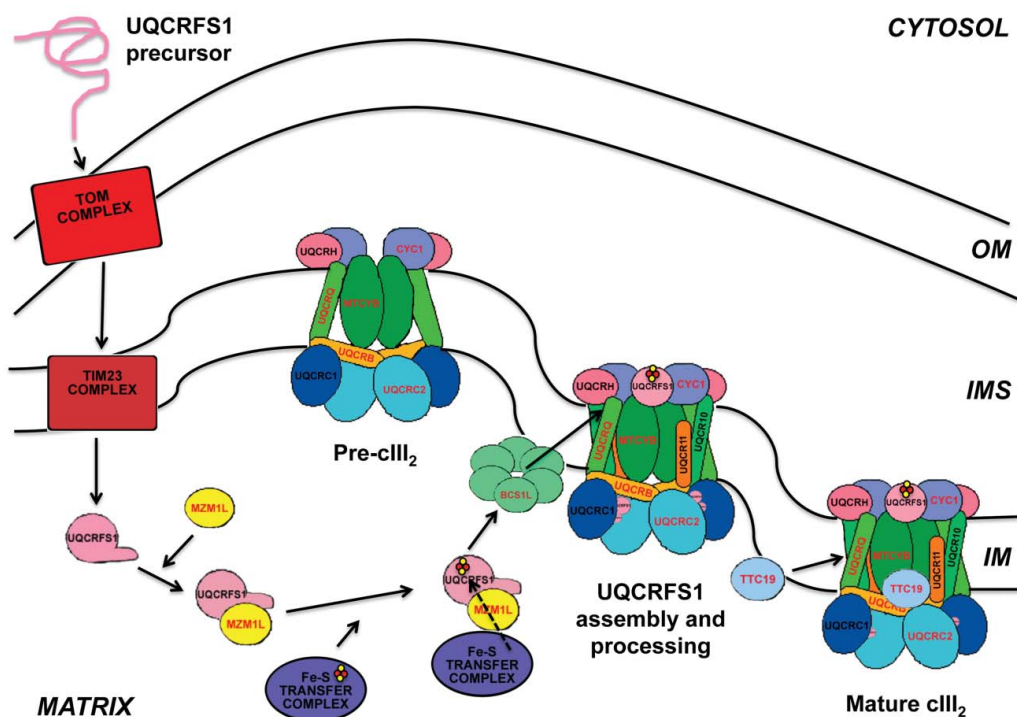
The electron transfer and proton pumping processes within cIII occur according to a 'Q-cycle' model [6,7]. UQCRFS1

receives one of the two electrons from oxidation of one ubiquinol molecule, and transfers it to CYC1. During this redox reaction, UQCRFS1 'head' moves, towards the CYC1 subunit of one of the monomers, whereas the N-terminal transmembrane domain is in close contact with MT-CYB of the other monomer [8]. The reason for this configuration may be to provide stability to the dimeric cIII<sub>2</sub> structure.

### Mammalian complex III assembly process

Most of what is known about the assembly of cIII comes from studies in yeast [9,10]. Based on the structural similarities and the fact that the first and last steps of human cIII assembly are conserved, it is assumed that the in-between process will be the same in yeast and mammals [11].

cIII assembly starts with the synthesis of cytochrome b by dedicated ribosomes, where the yeast translation factors Cbp3 (mammalian UQCC1) and Cbp6 (UQCC2) are bound to the peptide exit tunnel and direct the nascent polypeptide to translocate into the mitochondrial inner membrane [12,13]. A third factor, Cbp4 (UQCC3), is bound to cytochrome b after the *b<sub>L</sub>* heme but before the *b<sub>H</sub>* heme are incorporated into the apoprotein [14]. Pathological mutations associated with human cIII deficiency have been described in *UQCC2* [15] and *UQCC3* [16]. These early assembly factors are released when the first nuclear-encoded subunits bind to cytochrome b. Cbp3 and Cbp6 are now liberated and can bind to the mitochondrial ribosomes to act again as translational activators for cytochrome b. The rest of the structural subunits are then incorporated into the nascent complex until only Rip1 (UQCRFS1) and the smallest subunit Qcr10 (UQCR11) are missing, in an already dimeric structure named as pre-cIII<sub>2</sub> [10,11]. The incorporation of Rip1/UQCRFS1 can be



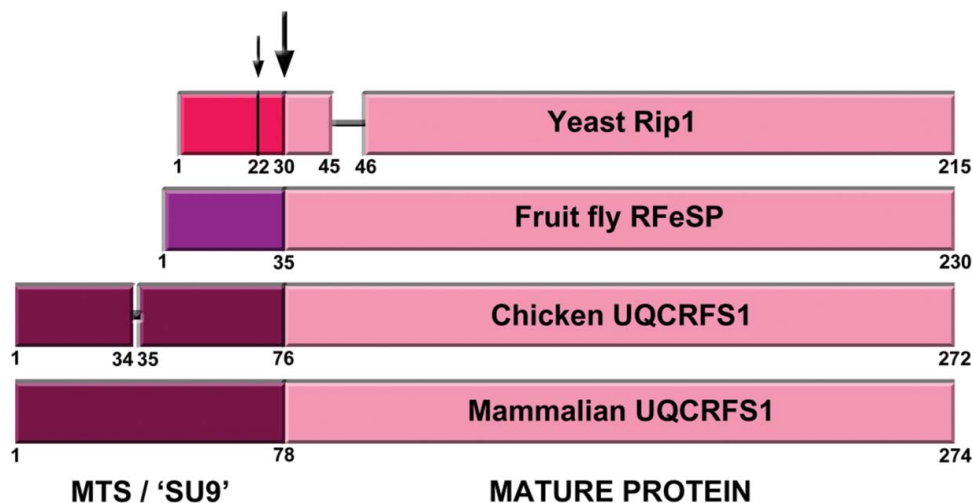
**Figure 1.** UQCRFS1 pathway from synthesis of the precursor in the cytoplasm, transport into the mitochondrial matrix through the TOM/TIM23 import system, incorporation of the [2Fe-2S] clusters in the matrix and translocation to pre-cIII<sub>2</sub> in the inner membrane (IM). According to our model, the N-terminal MTS is cleaved off *in situ* by the UQCRC1+UQCRC2 MPP activity. See main text for more details.

considered as the crucial cIII<sub>2</sub> maturation step because it is then that the enzyme becomes catalytically active. As depicted in Figure 1, UQCRFS1 is synthesized as a precursor protein in the cytosol and imported to the mitochondrial matrix following the TOM and TIM23 pathway, similarly to other proteins containing cleavable mitochondrial targeting sequences (MTS) [17]. In the matrix, UQCRFS1 binds to MZM1L/LYRM7 [18–20], the LYR-motif containing chaperone responsible for its stabilization and for the recruitment of the Fe-S transfer complex in the matrix [21,22]. Once UQCRFS1 has acquired its 2Fe-2S cluster, BCS1L translocates and incorporates it to pre-cIII<sub>2</sub> in the IM [23–25]. This function is therefore essential for cIII<sub>2</sub> activity and mutations

in BCS1L are the most frequent cause of mitochondrial disease associated to cIII deficiency (reviewed in ref. [11]). Pathological mutations have also been described in LYRM7 [26–29] and in TTC19, encoding a third factor necessary for UQCRFS1 metabolism [30,31], which is not present in yeast and will be discussed in more detail below.

### Bovine cIII subunit 9: the UQCRFS1 mitochondrial targeting sequence

One interesting observation that was pointed out as the main difference between the bovine cIII and the yeast and chicken



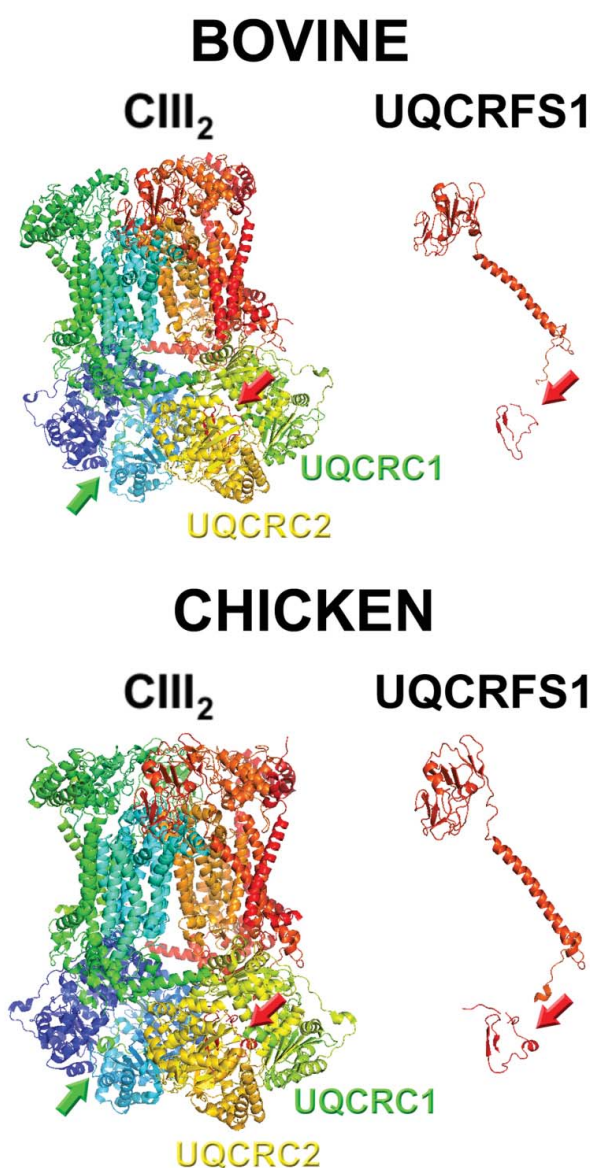
**Figure 2.** Graphic alignment of the Rieske protein precursors from yeast (*S. cerevisiae*), fruit fly (*D. melanogaster*), chicken (*G. gallus*) and mammals (bovine, mouse and human). Numbers indicate relevant residue positions. The two yeast cleavage sites, at positions 22 and 30 are indicated with arrows. The main cleavage site, conserved in all organisms is indicated with the big arrow. Different color MTS indicates the lack of homology of these sequences, while the homology in the mature protein sequences is in very high.

enzymes was that the bovine complex contained eleven subunits instead of the ten found in the latter organisms. This extra subunit with a molecular mass of 8 kDa was called 'subunit 9' or SU9 [32,33] and its sequence was determined to be the 78 N-terminal amino acids of the UQCRFS1 precursor, i.e. its MTS [34]. As a norm, proteins directed to the mitochondrial matrix contain an N-terminal amphipathic helix of variable length, which is recognized by the TOM complex and directs the precursor protein to the TIM23 complex to be translocated to the mitochondrial matrix. Here the precursor is then processed by the matrix processing peptidase (MPP), which cleaves off the MTS and gives rise to the mature protein [35]. Normally, the MTS peptides are proteolysed by Cym1/PITRM1 or hPreP, as their accumulation inside mitochondria could have a highly toxic effect [36]. Therefore, the presence of the UQCRFS1 MTS incorporated inside cIII as a structural subunit represents an anomaly and the reason why this happens is unknown.

Yeast Rip1 has a much shorter MTS than mammalian and avian UQCRFS1: 30 amino acids vs. 78 and 76, respectively (Figure 2). Interestingly, TTC19 orthologs exist in organisms with long UQCRFS1 MTS, while absent in yeast. The processing of yeast Rip1 is also different from bovine UQCRFS1. Rip1 is processed in two steps, first, the 22 N-terminal amino acids are cleaved by the MPP, generating an intermediate form of Rip1 (iRip1), and, in a second step, the mitochondrial intermediate peptidase (MIP) removes the next octapeptide to give rise to the mature form of the Rieske Fe-S protein (mRip1) [37]. This processing was proposed to happen once Rip1 is inserted into cIII<sub>2</sub> [38]. In the case of the bovine enzyme, a single-step processing of UQCRFS1 seems to be carried out by MPP after it is incorporated into cIII<sub>2</sub> [34], where SU9 remains bound to the complex and localized between the two core subunits (UQCRC1 and UQCRC2), as determined in the crystal structure (Figure 3) [1]. When chicken cIII<sub>2</sub> was isolated and crystallized for the first time, no SU9 was identified [2], leading to the idea that there was no such UQCRFS1-derived subunit in the avian enzyme.

UQCRC1 and UQCRC2 are homologous to the two MPP subunits ( $\beta$ -MPP and  $\alpha$ -MPP, respectively), and in plants they still preserve their processing properties [39]. Mammalian cIII<sub>2</sub> was considered to have lost its MPP activity. However, when the isolated enzyme was treated with detergents it showed this activity [40]. The same phenomenon was observed by reconstitution of the system with recombinant UQCRC1 and UQCRC2; interestingly, MPP activity was inhibited when stoichiometric amounts of 'SU9' were formed by adding N-terminal UQCRFS1 peptides as substrate [41].

Taking all this information together, it is plausible that UQCRFS1 is processed *in situ* by UQCRC1+UQCRC2 MPP activity when incorporated into pre-cIII<sub>2</sub> (Figure 1). This would explain why the N-terminus of the UQCRFS1 precursor is found between these two subunits in the cIII<sub>2</sub> structure and inhibits its MPP activity. This mode of action was also postulated based on the structural features of vertebrate cIII<sub>2</sub> [42].



**Figure 3.** Structures of full complex III dimer (cIII<sub>2</sub>) and of isolated UQCRFS1 (of only one of the monomers), from bovine (PDB ID: 1BGY) and chicken (PDB ID: 3H1J). The arrows indicate the presence of the UQCRFS1 fragments in the cavity between UQCRC1 and UQCRC2. Images were generated with MacPyMol.

### N-terminal UQCRFS1 peptides bound to mammalian and avian CIII<sub>2</sub>

After the identification of the first pathological mutations in TTC19 [30] our laboratory got interested in determining the molecular function of this factor, related with cIII<sub>2</sub> biogenesis. For that reason we have studied TTC19-deficient cells and a knock out (Ttc19<sup>-/-</sup>) mouse model, whose characterization was recently published [31]. In this report, by importing radioactively labeled UQCRFS1 in isolated mouse liver mitochondria and analyzing its incorporation into cIII<sub>2</sub> in a time course, we found that UQCRFS1 is processed right after its import and assembly, and the peptides derived from the N-terminus of the UQCRFS1 precursor remain bound to the complex. However, we did not find evidence of a unique cleavage point, giving rise to only two fragments corresponding to the mature UQCRFS1



and the 78-amino acid long SU9. Contrariwise, we observed the formation of the mature UQCRFS1 and the appearance of three main species of approximately 4, 8 and 12 kDa in size. In the steady state, these small UQCRFS1-derived peptides were readily detectable in the Ttc19<sup>-/-</sup> tissues while they were practically absent in the Ttc19<sup>wt</sup> samples. By mass spectrometry we were able to find tryptic peptides spanning from positions 8 to 204 of the UQCRFS1 precursor in the lower part of a SDS-PAGE (corresponding to sizes of 14 kDa and smaller), whereas in the higher part of the gel (30–14 kDa) we could only find peptides within the sequence of the mature protein (from residues 84 to 274) [31].

When checking the twenty-two bovine and sixteen chicken cIII<sub>2</sub> crystal structures available in the RCSB PDB protein data bank (<https://www.rcsb.org>) [43] there are peptides derived from UQCRFS1 MTS in all of them (examples in Figure 3). In the original chicken structure, density corresponding to an amino acid chain sitting between UQCRC1 and UQCRC2 was detected and a chain was built in the structure, but the identity of this peptide remained unknown (PDB ID 1BCC, 2BCC, 3BCC) [3]. This issue was resolved in the later chicken structures (see Table 1). Even though all the structures contain these peptides, the sequences in each of them are very variable and in none of them the full UQCRFS1 MTS, from amino acids 1 to 78, is found (Table 1). This could reflect heterogeneity in the fragments that are contained in each of the analyzed cIII<sub>2</sub> crystals [42].

In our *in organello* assays, after import and incorporation into cIII<sub>2</sub> of radioactive mouse Uqcrfs1, we observed that the 8 and 12 kDa fragments that are generated immediately after incorporation, disappeared with time and this clearance was much more efficient in wild-type compared to Ttc19<sup>-/-</sup> mitochondria. The abnormal accumulation of the N-terminal peptides was associated with aberrant electrophoretic mobility of

cIII<sub>2</sub> in blue-native gel electrophoresis (BNGE), defective enzymatic activity and increased reactive oxygen species (ROS) production. Therefore, we concluded that during the incorporation and *in situ* UQCRFS1 processing, several peptides containing its MTS are produced and remain bound to cIII<sub>2</sub>. However, in order to preserve cIII<sub>2</sub> structural integrity and function, these peptides (at least those of 8 kDa and 12 kDa) must be removed. TTC19 is involved in the removal of these N-terminal UQCRFS1 peptides, as discussed in the next section.

### Role of TTC19 in complex III biogenesis and function

Since the first *TTC19* mutations were published in 2011 [30], a significant number of cases have been subsequently reported [44,45]. The biochemical profile of these patients, showing isolated cIII deficiency, underscores the importance of TTC19 for a correct biogenesis of this complex. Analysis of cIII<sub>2</sub> assembly state in TTC19-deficient skin fibroblasts and muscle biopsies revealed the presence of UQCRC1 and UQCRC2-containing subassemblies [30,31], which suggested an involvement of TTC19 in the first steps of cIII<sub>2</sub> assembly. However, the availability of the Ttc19<sup>-/-</sup> mice and more detailed kinetic analyses in human cells, revealed that the assembly process *per se* was not particularly altered in the absence of TTC19, including a normal incorporation of UQCRFS1 [31]. TTC19 is loosely bound to fully assembled cIII<sub>2</sub>, as this interaction is disrupted when treating the mitochondrial membranes with a relatively mild detergent such as n-Dodecyl- $\beta$ -D-maltoside (DDM), but not if the samples are exposed to the even milder detergent Digitonin. Moreover, TTC19 only binds to cIII<sub>2</sub> if UQCRFS1 is incorporated into the complex [31], suggesting a role in cIII<sub>2</sub> biogenesis or maturation after assembly is completed. In fact, because the absence of TTC19 impairs the removal of the small UQCRFS1-derived fragments, we assigned a quality control or “husbandry” role for TTC19, in maintaining the structural and functional integrity of cIII<sub>2</sub>.

**Table 1.** UQCRFS1 MTS sequences found in the published cIII<sub>2</sub> structures

PDB ID	Reference	UQCRFS1 positions found
BOVINE STRUCTURES		
UQCRFS1 MTS: Positions 1 to 78		
1BGY, 1BE3	[1]	From 48 to 78
1L0L, 1L0N, 1NTK, 1NTM, 1NTZ, 1NU1, 1SQB, 1SQP, 1SQQ, 1SQV, 1SQX, 2FYU	[53–56]	From 1 to 57
1PP9, 1PPJ, 2A06	[57]	From 32 to 42 and from 48 to 78
1QCR	[58]	From 21 to 48
4D6U, 4D6T	[59]	Monomer 1: From 50 to 56 and from 64 to 77 Monomer 2: From 62 to 63 and from 65 to 78
5KLV	[60]	From 20 to 27; from 44 to 61 and from 63 to 70
5NMI	[61]	From 49 to 78
CHICKEN STRUCTURES		
UQCRFS1 MTS: Positions 1 to 76		
1BCC, 2BCC, 3BCC	[3]	—
3H1L, 3H1H, 3H1I, 3H1J, 3H1K	[62]	From 45 to 63
3CWb, 4U3F	[63,64]	From 46 to 75
3TGU	[65]	From 2 to 8 and from 46 to 75
3L70, 3L71, 3L72, 3L73, 3L74	[66]	From 45 to 75
HUMAN STRUCTURE		
UQCRFS1 MTS: Positions 1 to 78		
5XTE	[5]	From 1 to 57

### Prospects

The imminent questions derived from our recently published work [31] are: 1) whether UQCRC1+UQCRC2 possess MPP activity *in vivo* and what is their role in UQCRFS1 processing, 2) what proteases are working in collaboration with TTC19 in the removal of UQCRFS1 N-terminal peptides produced during assembly-processing and 3) how this process intervenes in UQCRFS1 turnover, to warrant cIII<sub>2</sub> fitness.

As for the first question, in plants, MPP is integrated into cIII<sub>2</sub>, thus carrying out the processing of the precursor proteins in addition to act as an MRC complex [39]. However, in *S. cerevisiae* and in mammals, there is a soluble MPP localized in the matrix. Only two reports cited above [40,41], have demonstrated an MPP activity incorporated into purified bovine cIII<sub>2</sub> and performed by UQCRC1+UQCRC2. It would be necessary to demonstrate *in vivo* whether this activity is necessary for the *in situ* processing and proper incorporation of UQCRFS1.

Concerning the second question, we performed co-immunoprecipitation studies that revealed specific interactions of TTC19 with cIII<sub>2</sub>, in the context of the respiratory

supercomplexes (i.e. cI+cIII+cIV) [46] and with the components of the so-called SPY complex [47]. This is a mitochondrial quality control “proteolytic hub” that includes both the i-AAA protease YME1L and the rhomboid protease PARL. Logically the SPY complex is the first candidate to be explored in relation to its role in UQCRC1/cIII<sub>2</sub> quality control.

Regarding the third question and taking together the information reported above, this process can be considered as a regulatory mechanism of cIII<sub>2</sub> maturation and activity. Even in situations in which UQCRC1 cannot be incorporated, there is an assembly of pre-cIII<sub>2</sub>, which contains all the subunits except UQCRC1 and most probably the smallest UQCRC1 [20,25]. Therefore, the pre-cIII<sub>2</sub> can be considered as an assembly checkpoint previous to the final activation, which must be somehow regulated. Accordingly, UQCRC1+UQCRC2 could control the amount of UQCRC1 that must be incorporated into cIII<sub>2</sub> by recognizing the UQCRC1 MTS and processing it upon incorporation. The presence of the N-terminal UQCRC1 fragments in between UQCRC1 and UQCRC2 would inhibit their MPP activity and the incorporation of any new UQCRC1. The removal of the UQCRC1 N-terminal fragments would reactivate cIII<sub>2</sub> MPP activity and promote the removal of old, possibly damaged, UQCRC1 from the pre-cIII<sub>2</sub> scaffold, so that new UQCRC1 can then be processed and incorporated again. This would keep the complex functional. The interchange of catalytically active subunits that can be oxidatively damaged in large enzymatic complexes is a mechanism demonstrated in chloroplast Photosystem II [48–50] and in mammalian mitochondrial cI [51], where there seems to be an interchange of old damaged subunits for newly imported ones. If this model is correct, the turnover of UQCRC1 should be faster than that of the other cIII<sub>2</sub> subunits, which is in fact the case in mouse tissues [52].

To determine whether the rate of UQCRC1 incorporation and turnover, as well as the N-terminal fragment clearance change depending on the metabolic conditions is a question of great interest to understand the relevance of this process in the adaptation to different energetic demands. This could be tested in differentiated tissues, where mitochondria in general and cIII<sub>2</sub> in particular could play a different metabolic role. It is also possible to analyze UQCRC1 turnover in cultured cells grown in OXPHOS-dependent (galactose as carbon source) and independent (glucose as carbon source) media. If this were to be changed, it would reflect how cIII<sub>2</sub> maturation must adapt to an increased MRC activity.

These questions can be experimentally addressed; their elucidation will contribute to clarify the complex regulatory mechanisms of OXPHOS and their implication in health and disease.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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