Free Radical Biology and Medicine 167 (2021) 232–242

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Functional segmentation of CoQ and cyt *c* pools by respiratory complex superassembly

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ARTICLE INFO

Keywords:
CoQ
Cyt *c*
Supercomplexes
Respirasomes
Pools

ABSTRACT

Electron transfer between respiratory complexes is an essential step for the efficiency of the mitochondrial oxidative phosphorylation. Until recently, it was stablished that ubiquinone and cytochrome *c* formed homogenous single pools in the inner mitochondrial membrane which were not influenced by the presence of respiratory supercomplexes. However, this idea was challenged by the fact that bottlenecks in electron transfer appeared after disruption of supercomplexes into their individual complexes. The postulation of the plasticity model embraced all these observations and concluded that complexes and supercomplexes co-exist and are dedicated to a spectrum of metabolic requirements. Here, we review the involvement of superassembly in complex I stability, the role of supercomplexes in ROS production and the segmentation of the CoQ and cyt *c* pools, together with their involvement in signaling and disease. Taking apparently conflicting literature we have built up a comprehensive model for the segmentation of CoQ and cyt *c* mediated by supercomplexes, discuss the current limitations and provide a prospect of the current knowledge in the field.

1. Introduction

All metazoans require oxygen to produce energy and this process is carried out by the oxidative phosphorylation system (OXPHOS). The first four OXPHOS protein complexes retrieve the energy provided by reducing equivalents (i.e. Nicotinamide adenine dinucleotide hydrogen (NADH) and flavin adenine dinucleotide dihydrogen ($FADH_2$)) to couple electron transfer to H^+ translocation across the inner mitochondrial membrane (IMM). H^+ pumping creates an electrochemical gradient (negative inside) which forces the re-entrance of H^+ from the intermembrane space to the mitochondrial matrix. This process is mainly carried out by the fifth complex of OXPHOS, the ATP synthase or complex V (CV), which couples it to the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP).

The first four complexes comprise the mitochondrial electron transport chain (mETC). They are complex I (CI: NADH-ubiquinone oxidoreductase), complex II (CII: succinate:ubiquinone oxidoreductase), complex III (CIII: ubiquinol-cytochrome c reductase) and complex IV (CIV: cytochrome *c* oxidase). CI and CII catalyze the electron transfer from NADH and succinate, respectively, to ubiquinone or CoQ, yielding ubiquinol. CIII oxidizes ubiquinol to reduce cytochrome *c* (cyt *c*) and CIV performs the final step of respiration, transferring electrons from cyt *c* to oxygen, producing water. **CoQ** and **cyt *c*** are mobile electron carriers linking electron transfer among different complexes in the mETC. CoQ is a very hydrophobic 1,4-benzoquinone which, in mammals, has between 9 and 10 isoprenyl subunits, being CoQ-10 the most common form in humans and CoQ-9 predominant in rodents [1]. In mitochondria, CoQ can be reduced to form ubiquinol by CI that oxidize NADH generated in multiple redox reactions and pump protons, but also by a variety of non-proton pumping enzymes which deliver electrons to CoQ through FAD. The more representative of these enzymes is CII, which is required for the function of the TCA cycle; but they also include other relevant metabolic enzymes like the electron-transfer flavoprotein (ETF)-ubiquinone oxidoreductase, for β-oxidation; glycerol-3-phosphate dehydrogenase (mtGPDH), for the shuttling of reducing equivalents from the cytoplasm; dihydroorotate dehydrogenase, for pyrimidine synthesis; choline dehydrogenase, for glycine metabolism; sulphide CoQ reductase, for sulphur and seleno-amino acid metabolism; and proline dehydrogenase, for arginine and proline metabolism. Cyt *c* is a small, highly water soluble heme protein that becomes reduced by CIII and Erv1 in the

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https://doi.org/10.1016/j.freeradbiomed.2021.03.010
Received 14 January 2021; Received in revised form 23 February 2021; Accepted 7 March 2021
Available online 17 March 2021

CHCHD4/Mia40 protein folding pathway. To note, the extremely different partition coefficients of CoQ and cyt *c* makes the unspecific electron transfer between both impossible.

Whereas cyt *c* only comprises two redox states: ferric cyt *c* (oxidized) and ferrous cyt *c* (reduced); there are three redox states in ubiquinone: ubiquinone (fully oxidized), semiquinone (partially reduced) and ubiquinol (fully reduced). Such feature of CoQ derives from its structure as its two ketone groups can undergo one electron reduction to hydroxyl groups. Thus, semiquinone is the result of one ketone group reduction and ubiquinol is the result of the reduction of the two ketone groups. This fact is relevant in mitochondrial physiology as all three redox states of ubiquinone occur in the electron transfer step catalysed by CIII, the so-called Q-cycle. In this, ubiquinol transfers subsequently two electrons to the Qo site of CIII. Once inside the complex, one is directed to reduce cyt *c* and the other is transferred to the CIII Qi site. Here, a ubiquinone molecule is partially reduced to semiquinone (Fig. 1A). Then, a molecule of ubiquinol again donates two electrons to the Qo site, one is yet again transferred to cyt *c* and the other one completes the reduction of the semiquinone bound to the Qi site, yielding ubiquinol (Fig. 1B). The radical nature of semiquinone makes it is very unstable and prone to react with oxygen to form superoxide anion; thus, the Q-cycle must be tightly coupled.

Though the IMM phospholipid composition is not involved in CoQ diffusion across membranes [2], it is critical for the organisation and function of the mETC [3]. For instance, cardiolipin (CL) participates in the reaction catalysed by complex III [4] and complex IV [5], and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are critical for complex I function and stability [6]. IMM composition varies across tissues [7]; however, phosphatidylcholine (PC) is the most abundant phospholipid in all of them, being around 40% of the total phospholipid content of the IMM. Phosphatidylethanolamine (PE) comprises the 30%, whereas phosphatidylserine (PS) and phosphatidic acid (PA) only constitute a 5%. CL, a very specific phospholipid of the IMM, can be found up to 15% of total phospholipid content. Other lipids, such as sphingolipids and cholesterol, make up the rest of lipids in the IMM. Little is known about the lateral distribution of lipids in the IMM; however, it has been suggested that their differential lateral distribution may be possible in eukaryotes as it occurs in bacterial membranes [8]. This may be possible by recruiting non-bilayer forming phospholipids, such as PE and CL, into microdomains. In addition, transmembrane asymmetry of phospholipids is known from long ago, being PE and CL more abundant, and PC less abundant, in the inner than in the outer leaflet of the IMM [9]. These microdomains may be involved in processes such as mitochondrial fusion and fission, or protein import and turnover. Notably, the differential chemo-physical properties of theses microdomains may also be implicated in kinetic processes related to membrane anchored proteins, such as cyt *c* [10], or hydrophobic, mobile molecules, such as CoQ.

2. Respiratory supercomplexes

As the individual complexes could be reconstituted independently [11] and the mobile electron carriers were shown to interconnect the electron transport between them, among other observations (for a historical review see Ref. [12]). It was proposed that the mETC complexes were randomly distributed in the IMM and that electron transfer between them followed a model of random collision [13]. This model was widely accepted until Schägger and Pfeiffer developed the blue native gel electrophoresis (BN-PAGE) and discovered that yeast and mammalian mitochondria harbour multi-complex units with fixed stoichiometries, which they termed supercomplexes [14]. This work brought back the old hypothesis of a unique enzymatic structure capable of performing all the steps in respiration, the solid model.

The discovery of supercomplexes triggered a strong debate regarding their factual biological entity [12]. After years of intense research, the use of different detergent to solubilize them [15], the finding of proteins directly involved in the formation of the Q-respirasome [15] and their posterior purification and structure resolution confirmed that they are indeed true existing molecules in eukaryotic mitochondria ([16–20]. Today, it is accepted that CI in mammalian mitochondria is mostly associated with other complexes, either attached with dimmer CIII (CI + $CIII_2$) or with dimmer of CIII and monomer or dimmer CIV (CI + $CIII_2$+CIV_{1-2} or N-respirasome). CIII is always found as a dimer ($CIII_2$) and can interact with a monomer or dimmer CIV ($CIII_2$+CIV_{1-2} or Q-respirasome). CIV mainly occurs as a monomer (CIV), though it can associate with CI [21,22], form dimers (CIV_2) [21,23], or heavier structures (CIVm and CIVn) [21]. CV is normally sole (CV) and may form dimers (CV_2) and multimers. The co-existence of free and different forms superassembled respiratory complexes lead to the proposal of the plasticity model for the structural organization of the mitochondrial electron transport chain [24,25].

Yet, the roles ascribed to these super-structures are still under intense investigation and proposals range from structural to functional, or a combination of both. In the next sections we will summarize the current knowledge about the possible roles of supercomplexes in mammalian cells.

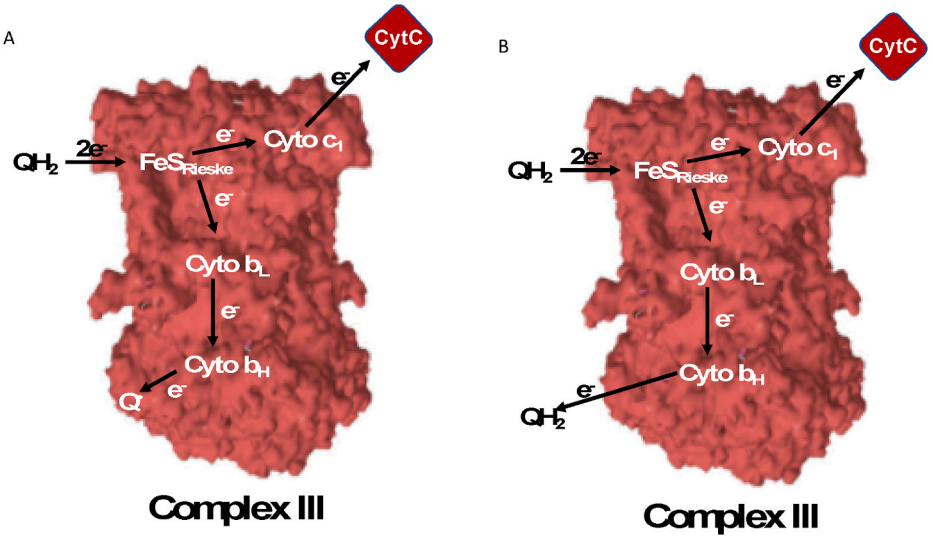

Fig. 1. - Complex III Q cycle. Schematic representation of the electron transfer pathway between ubiquinol, complex III and cyt *c*. A) two-electron transfer from ubiquinol to the Rieske iron-sulphur cluster which subsequently derives one electron to cytochrome *c*1 and the other one to cytochrome b_L. Cytochrome *c*1 donates its electron to cytochrome *c* and cytochrome b_L to cytochrome b_H. Cytochrome b_H transfers its electron to ubiquinone, forming semiquinone. B) Again, two-electron transfer from a new ubiquinol to the Rieske iron-sulphur triggers the same chain of events and in the last step cytochrome b_H now transfers its electron to the semiquinone, forming ubiquinol again.

3. ROS production

One of the first hypothesis regarding the role of respiratory supercomplexes was their function as ROS modulators [14]. Treatment of either bovine heart mitochondria or liposome-reconstituted supercomplex I + III_2 with dodecyl maltoside, a detergent capable of disrupting supercomplex into their individual complexes, provided experimental support to the hypothesis of that the physical association between CI and CIII limited the production or ROS by CI at the molecular level [26]. A similar scenario was found in cultured cells [27] in which it was observed that ablation of RISP diminished supercomplex stability and that it associated with higher ROS levels. Later, other studies have also spotted the relationship between decreased supercomplex assembly and increased ROS levels in live cells and tissues [21,28–31]. Notably, all these studies altered the interaction between CI and CIII; therefore, CoQ most probably has a role in ROS production by supercomplexes which may be related to the vicinity of CI and CIII catalytic sites or IMM fluidity.

It has been recently observed that Na^+ modulates ROS production during acute hypoxia through the regulation of IMM fluidity [32]. Thus, Na^+ is able to promote ROS production when combined CII + CIII activity is enabled, independently of the sole CII or CIII activities. Interestingly, this increase in ROS production was parallel to the reduction in combined CII + CIII enzymatic activity and respiratory capacity, whereas combined CI + CIII activity and respiration remained unchanged. This observation reinforces the idea of that supercomplex formation, particularly in between CI and CIII, limits the production of ROS, in this case, promoted by Na^+:phospholipid interaction.

The effect of Na^+ on IMM fluidity could be ablated by knocking out the gene encoding for the mitochondrial Na^+/Ca^{2+} exchanger (NCLX) [32]. It has been also shown that NCLX knock-out promotes larger production of ROS during reperfusion [33]. As ROS during reperfusion are mainly produced by mitochondrial CI through reverse electron transport (RET) and with succinate as the electron source [34], it is possible that Na^+ decreases CoQ transfer during reperfusion through its interaction with the IMM. This suggests an additional role of mitochondrial Na^+ in lowering ROS in reperfusion through the decrease in IMM fluidity.

SCAF1 regulates the interaction between CIII and CIV [15,21,23,35] and it also mediates the interaction between CIII and CIV inside the N-respirasome [21]. Enriched N-respirasome fractions from C57BL/6 mouse heart mitochondria, which lack the supercomplex assembly factor-1 (SCAF1), produce more ROS under the CIII substrate CoQ_1H_2 than their wild type (i.e. SCAF1 back) counterparts [21]. Interestingly, however, N-respirasomes from different strains showed the same rate of ROS production under the CI substrate NADH. This can be interpreted as that CI limits the turnover of the respirasome, which then not only impacts on the supercomplex enzymatic rate [17,21], but also on ROS production by downstream redox centres [21].

4. Superassembly and complex I stability

Although the individual complexes can be reconstituted independently and their isolated activity can be achieved *in vitro*, the stability of complex I depends on the presence of both CIII [36,37] and CIV [38–40]. This effect was initially proposed to be caused by a severely hampered stability [36]. However, it has been also suggested that, in addition, the absence of CIII prevent the incorporation of the N-module and stall the final step in CI assembly, leading to the hypothesis that CIII acts as a platform for the maturation of CI [41,42]. Importantly, human and mouse cells with defects in CI structural subunits (NDUFS4, NDUFS6 or NDUFA2) or the assembly factor NDUFAF2, showed an impaired N-module assembly while harbouring normal CIII and CIV. Therefore, only the assembly of CI at the level of N-module incorporation was affected and, in such cases, conspicuous incomplete free CI subcomplex (i.e. without N-module) is revealed more abundant than the same subcomplex attached to CIII, in the BN-PAGE [43–46]. If the normal assembly process of CI implied the interaction with CIII previous to N-module assembly, it would be expected that all CI in those mutants was attached to CIII, and this is not always the case. Moreover, this model does not explain why the absence of CIV and cyt *c* also decreases CI stability [38,40,47]. Finally, a systematic evaluation of the CI assembly concluded that, in normal circumstances, CI is fully assembled before it is incorporated into supercomplexes [48]. Nevertheless, relevant for this model are the accumulated evidence that indicate that the turnover of the CI N-module is independent and much faster than the rest of the complex [46,49,50]. Therefore, the fact that the N-module turnover is much more rapid than the rest of CI and the observation that the superassembly of CI with CIII stabilized individual CI in N-module lacking mutants [44] allows the reconciliation of all observations with the proposal of supercomplex being a platform for CI stability. It is plausible that the N-module is attached to both free and superassembled CI-subcomplex without the mandatory requirement of SC formation to finish the assembly CI. Then, since the CI subcomplex degrades more slowly than the N-module and that the interaction of partially assembled CI with CIII stabilizes it, CI degradation may be accelerated when CIII or CIV are not present (i.e. CI is not incorporated into the supercomplex). Interestingly, neither CIII or CIV inhibitors interfere with CI assembly [36,38,42,51].

In a remarkable observation, the overexpression of the alternative oxidase (AOX) from *Emericella nidulans* in CIII or CIV deficient cells promoted the stabilization of CI [21,42,51] as it did the growth of the cells in low oxygen or in the presence of CI inhibitors [51]. CIII mutants recovered CI in the form of the individual complex (CI in AOX expressing cells), whereas CIV mutants recovered the expression of CI in the form of both individual CI and supercomplex CI + $CIII_2$, being CI + $CIII_2$ the most abundant form (CI + $CIII_2$ in AOX expressing cells [21,51]). Guarás et al. showed that due to the absence of CIII or CIV $CoQH_2$/CoQ ratio increased dramatically and promoted the backflow of electrons from $CoQH_2$ to CI by RET. RET produced the overoxidation of CI Cys residues at the level of the N-module which, in turn, triggered the degradation of the N-module in the short term followed by the degradation of the entire CI at later stages [51]. Notably, this could be reversed upon AOX overexpression, by inhibition of CI interaction with ubiquinol (by piericidin A or rotenone) or by a drastic and long-term reduction in oxygen, all interventions that prevent RET. This interpretation has been recently questioned arguing that that Antimycin A (a CIII inhibitor) do not de-stabilize CI as much as the ablation of CIII. They reasoned that this inhibitor should be able to produce RET [42]. However, this reasoning is inaccurate. It is well stablished that Antimycin A does not induce RET as its primary mechanism of ROS production, instead it produces ROS at the Qo site in CIII due to the disturbance of the Q-cycle [52]. Thus, CIII take electrons from $CoQH_2$ and form superoxide. In fact, by generating superoxide through CIII electrons are leaked preventing the growth of the QH_2/Q ratio as much as it does if no CIII is present. Therefore, the probability of RET decrease. Nevertheless, it is still possible that this drug produced RET at some extent which may explain the partial decrease in CI caused by Antimycin A [51]. Strikingly, while human muscle lacking CIII showed a defect in amount and activity of CI [36,53,54], *Cox10*^{KO} mouse muscle which is unable to assemble CIV neither shows any defect in CI assembly nor activity [55]. Therefore, CI stability becomes compromised *in vivo* in the absence of CIII and the impact of AOX, under these circumstances, remains to be proven.

5. Segmentation of the CoQ and cyt *c* pools

A fundamental question regarding the functionality of the supercomplexes is which would be the impact, if any, in the kinetic behaviour of the mETC in general and in the dynamics of the mobile electron carriers CoQ and cyt *c* in particular. Data from different laboratories contributed to the general acceptation of the fluid model supporting the concept that electron transport is a diffusion-coupled kinetic process and

all redox components (i.e. of the mETC) are independent lateral diffusants [13]. In particular, it was proposed that CoQ freely diffuses along the IMM after measuring the fluorescence quenching of different CoQ fluorescent analogues of various lengths in lipid vesicles and submitochondrial particles (SMPs) [56,57]. These studies were designed to determine the mobility of external CoQ analogues across membrane bilayers and did not take into account the existence of supercomplexes (i.e. they had not been discovered). Therefore, though they provide a valuable information on the chemo-physical nature of the IMM, they lack the resolution to solve the problem of whether naturally occurring CoQ freely diffuses between the supercomplexes and the bulk IMM.

The old solid model for the organization of the mtETC, on the contrary, supported the notion of that a physically associated, macromolecular assembly of electron-transferring complexes was the functional respiratory unit in the IMM [58]. This idea was brought back by the discovery of supercomplexes by Schägger and Pfeiffer [14], which prompted to the belief of the respirasome to be the true respiratory entity and all the free complexes observed to be mere breakdown products. However, kinetic data regarding this functional aspect of the respirasome are lacking.

An intermediate, satisfactory explanation for all these data is the plasticity model, which proposed that the mETC is organized bearing both functional individual complexes and supercomplexes [24,25]. The plasticity model takes into account the multifaceted metabolic needs of the cell and hypothesizes that all complexes and supercomplexes are active and dedicated to a panoply of metabolic requirements. Data in the literature are growing on behalf of this model. Mouse fibroblasts with constitutively abnormal low expression of CIII showed that all remaining CIII was sequestered by CI. The functional consequence was a reduced CII + CIII activity and CII-based respiration, whereas CI + III activity and respiration remained normal. In addition, fasting of mice to promote fatty acid oxidation promoted a decrease in maximal CI and CI + III activities, whereas CII and CII + CIII activities remained unchanged [15]. Balsa and co-workers described that as cells were grown in galactose and forced to rely on mitochondrial metabolism, respirasome levels increased through the expression of SCAF1, matching the content of supercomplexes to the metabolic needs of the cell [59]. Similarly, a recent paper by García-Poyatos and colleagues has provided evidence that the absence of the Q-respirasome through genetic deletion of SCAF1 is sufficient to restrain zebra fish growth, diminish female fertility and promote abnormal fat deposition. As the phenotype was reversed by doubling food supply, but not by high fat diet, it is possible that specific metabolic pathways, determining nucleic acid or protein synthesis are affected by the sole deletion of the Q-respirasome [35]. As pointed by Moreno-Loshuertos and colleagues [15], the optimization of the use of available substrates through complexes and supercomplexes implies the definition of partially dedicated pools of mobile electron carriers.

5.1. Segmentation of the CoQ pool

One of the strongest arguments in support of this is that the existence of a pure single pool necessarily follows the Loss of Memory (LOM) principle. The LOM principle implies that very differentiated upstream metabolic pathways would flow into the unique pool of CoQ and the information on the identity of the donor should be lost (Fig. 2).

Seminal kinetic and molecular data strongly indicated that the LOM principle does not occur [60]. It was also challenged by two articles which, however, raised opposite conclusions on the partition of the CoQ pool [61,62]. The first article [61] showed that both oxygen consumption and cytochrome reduction by the mETC become complete only after addition of both CI and CII substrates, and that individual addition of any of the substrates yielded only fractional respiration/cytochrome reduction (see discussion in Ref. [12]). The second article used AOX from *Trypanosoma brucei brucei* to evaluate the possibility of having a dedicated CoQ pool into the N-respirasome by incorporating AOX recombinant protein in a reaction mixture with SMPs. This ingenious approach attempted to provide a competing pathway for CoQ oxidation and test for channelling. The authors showed that NADH oxidation increased upon AOX addition to the reaction mixture, only after CIV was inhibited [62]. Importantly, whether electrons are derived to AOX in normal respiring conditions was not tested. This approach, being clever, present some caveats: (1) AOX recombinant protein was added into the reaction mixture, together with the SMPs from bovine heart. Given the nature of AOX, an adequate incorporation into the membrane to prevent protein aggregation in the solvent and to yield proper function and activity is necessary, and this could not be warranted. (2) The specificity of CI activity for NADH oxidation was not tested (e.g. rotenone-sensitive NADH oxidation); this is relevant since there are several NADH oxidases in the IMM that can recapitulate the increase in NADH oxidation observed after AOX addition as they do not assemble into supercomplexes. (3) Only NADH was monitored and not oxygen consumption. Recently, a similar idea, solving most of the caveats, analysed the coupled and uncoupled respiratory rates in AOX-expressing mouse heart mitochondria (in this case AOX was derived from *Ciona intestinalis*). When respiring on CI substrates little-to-no engagement of AOX depending respiration could be recorded [63]. On the contrary, AOX was contributing to the overall oxygen consumption when respiring in CII substrates [63]. In these experiments the LOM principle was once

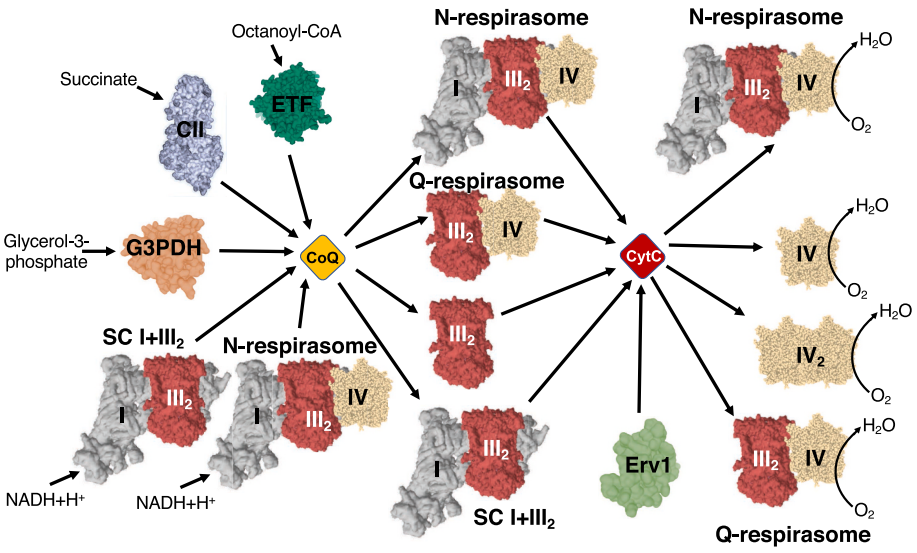

Fig. 2. - Loss of Memory (LOM) principle adapted to the mETC. Schematic representation of the LOM principle showing that all metabolic pathways, including fatty acid oxidation, glucose metabolism, TCA cycle and redox homeostasis would derive electrons to a common CoQ and cyt *c* sink. I: Complex I; CII: Complex I; III_2: Complex III; IV: Complex IV; ETF: Electron-transferring flavoprotein; G3PDH: Glycerol-3-phosphate dehydrogenase; SC I + III_2: supercomplex I + III_2; Q-respirasome: supercomplex III_2 +IV; N-respirasome: supercomplex I + III_2+IV.

more broken. AOX, which does not super-assemble neither with CI nor with CII, was able to discriminate whether the electrons reducing CoQ were provided by NADH or by succinate. This results fully discard the existence of a homogenous and unique CoQ single pool. Importantly, in agreement with Fedor et al. [62], AOX was only able to respire under CI substrates after pharmacological inhibition of CIII or CIV. However, Fedor and Szibor results provided important discrepancies. In one side Fedor reported that the NADH oxidation capacity provided by AOX after CIII-CIV inhibition was much higher than that of the non-inhibited mtETC [62]. On the other, Szibor et al. found that the CI dependent respiration after CIII-CIV inhibition was similar to that reached by CIV [63]. This discrepancy strongly suggests that a significant portion of the NADH oxidation in the experiment by Fedor et al. may be due to dehydrogenases other than complex I.

In parallel to Szibor, Calvo et al. [21] tested the potential partition of CoQ pools using AOX (AOX derived from *Emericella nidulans*). In this case: (1) AOX was ectopically expressed in the IMM of mouse heart, muscle and fibroblasts; (2) NADH oxidation and (3) respiration sensitivity to both rotenone and piericidin A was measured. In addition, due to the effects of AOX on preserving CI stability in the absence of CIII or CIV [51], the influence of electron delivery from CI to AOX when superassembled or not superassembled into supercomplexes could be investigated. Thus, $CIII^{KO}$ + AOX cells have all CI free of interactions with CIII and CIV^{KO} + AOX cells allows CI expression mainly in the form of CI + $CIII_2$. In parallel, the delivery of electrons from CII to AOX was monitored. In both contexts, CI was able to deliver electrons to AOX as there was oxygen consumption in both cases and both cell types could be grown in the absence of uridine. However, when not superassembled, CI + AOX respiration rate was higher and there was competition between CI + AOX and CII + AOX respiration. In accordance, NADH oxidation was diminished when succinate was present. Contrarily, in the presence of CI + $CIII_2$+AOX respiration rate was lower while CII-AOX was not affected. In fact, an additive effect of CI and CII substrates in oxygen consumption was revealed, while no detectable competition with succinate could be seen on NADH oxidation [21]. Interestingly, in agreement with Szibor results [63], neither CI-AOX respiration nor NADH oxidation was detected when using wild-type cells expressing AOX unless CIII or CIV inhibitors are applied. However, in contrast with Szibor, AOX in Calvo et al. was only partially capable of using CI substrates after CIII or CIV inhibition, but never reached the level achieved without inhibitors [21]. This difference may be due to the use of AOX from different origin between the two groups.

In summary, all the experiments from different laboratories allow to explain the apparently contradictory results in a working model which is now able to accommodate all of them (Fig. 3). Thus, under full blockage of the electron flux at CIII or CIV, and in agreement with the structural data showing that the CoQ binding sites of CI and CIII are not closed by protein barriers within the structure of the supercomplex [16,18–20], CoQ can diffuse out and be oxidized outside the supercomplex (Fig. 3B) [17,21,61–63]. However, under normal circumstances, the super-assembly between CI and CIII generate a CoQ fraction within the SCs functionally dedicated NADH oxidation (Fig. 3A) [17,21,63]. What is fully in agreement with the seminal suggestions by Lenaz and co-workers [60]. By the same token, in the abnormal situation in which all CIII is superassembled with CI, the ubiquinol generated by CII or G3PDH can diffuse in and be oxidized by the N-respirasome and CI + $CIII_2$ [15]. However, under physiological conditions CIII fractions attached and non-attached to CI co-exist, and the ubiquinol generated by CII or G3PDH is mainly oxidized by the CIII non-attached to CI fraction [15].

Results by Protasoni et al., using AOX overexpression in human WT or $CIII^{KO}$ cells, resembled those commented above by Calvo et al. but using human cells [21]. In 2019, the structure of supercomplex CI + $CIII_2$ was solved and a series of elegant experiments were carried out to test channelling [17]. The authors found that: (1) native CoQ_{10} is bound to the structure of the supercomplex; (2) the purified supercomplex CI + $CIII_2$ is able to perform the NADH-cyt c oxidoreductase activity without the addition of any external ubiquinone; thus, native CoQ_{10} inside the supercomplex is able to perform electron transfer between CI and CIII; (3) the purified supercomplex CI + $CIII_2$ shows a ~10% of its total NADH oxidase activity which can be reached upon the addition of a CoQ analogue, irrespectively of its concentration, meaning that despite CoQ has no protein mediated physical barriers preventing its diffusion out of the SC, it cannot easily diffuse in and out of the supercomplex I + III_2. To note, since purified CI + $CIII_2$ was maintained in amphypols rather than in native membranes the physiological signification of this observation could not be established. In this respect, unpublished results from our group using enriched N-respirasome fractions and CI + $CIII_2$ fractions in their native lipid environment showed their insensitiveness to the external addition of CoQ analogues.

Moreover, it was recently demonstrated that Na^+ can modulate IMM fluidity through its interaction with phospholipids [32]. The increase of Na^+ in the mitochondrial matrix decrease membrane fluidity and diminish CoQ transfer between CII and CIII, but not between CI and CIII. This observation, performed in bovine aortic endothelial cells which have virtually all CI into supercomplexes, reveal that the CoQ responsible for the delivery of electrons between CII + CIII diffuse through the membrane and therefore is sensitive to Na^+. However, the CoQ involved in CI-CIII electron flux is not affected by alterations in membrane fluidity and therefore insensitive to Na^+. This bivalency observed *in vivo* reveal the signification of the functional segmentation of the CoQ by the superassembly between CI and CIII.

In summary, though the initial scepticism, structural, kinetic and regulatory evidence support the idea of that CoQ is partially and functionally partitioned in the IMM and that supercomplexes use a dedicated pool for their catalysis. However, the question remains of how the functional segregation is achieved in the absence of physical barrier impeding CoQ exchange between these superstructures and the bulk

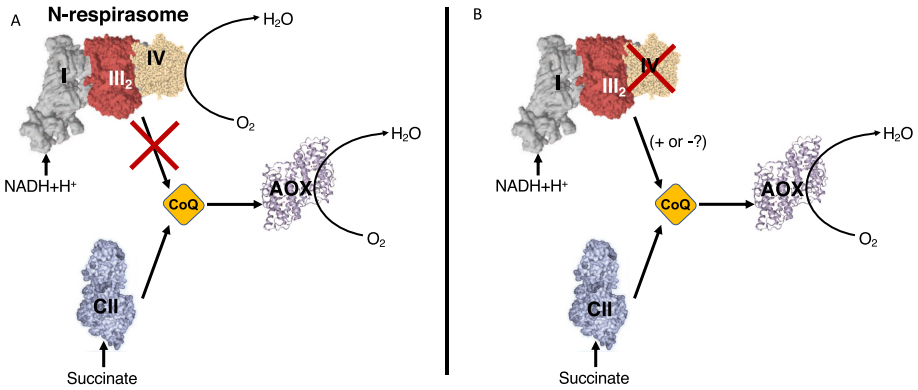

Fig. 3. - N-respirasome functionally segment the CoQ pool from the bulk IMM CoQ pool, unless CIV becomes inhibited. A) Schematic representation of IMM overexpressing AOX. The N-respirasome, constituted by CI (grey), CIII (red) and CIV (ochre) does not provide reduced CoQ molecules to the bulk IMM CoQ pool (yellow) which is, however, reduced by CII (blue-grey) activity, among others. AOX oxidizes only CoQ from the bulk IMM CoQ pool. B) As the N-respirasome becomes inhibited it is able to provide reduced CoQ to the bulk IMM CoQ pool which will be, in turn, oxidized by AOX. The extent to which the N-respirasome shares its CoQ pool varies among studies which probably depends on AOX origin (depicted by "+ or -?"). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IMM [16–20]. In one side, the vicinity of the catalytic sites of CI and CIII inside the supercomplex could promote a quick use of the CoQ juxtaposed to the supercomplex structure, preventing their diffusion to the bulk IMM. It is also possible that the particular composition of phospholipids around the supercomplex hinder CoQ diffusion to the rest of the IMM, as if the supercomplex was into a microdomain with a specific phospholipid composition. As mentioned above, IMM may be laterally compartmentalized in microdomains with functional roles [8]. Thus, mutations in Tafazzin, a phospholipid acyltransferase involved in maturation of cardiolipin, affects the stability of supercomplex I + III_2 and the N-respirasome [64]. In addition, reduction in mitochondrial PE through the deletion of PS decarboxylase (PSD) blunted the assembly of respiratory supercomplexes and decreased respiration [29]. Thus, if specific phospholipids are necessary for the stability of supercomplexes, it is plausible that the accumulation of certain specific phospholipids in the surroundings of the supercomplex impose a semi-diffusible barrier for the transit of CoQ to/from the bulk IMM.

5.2. Segmentation of the cyt c pool

Similarly, to CoQ, cyt *c* was proposed to be functionally segmented in mammalian mitochondria [15]. The experiments leading to this conclusion comprised the discovery of a bona fide supercomplex assembly factor, SCAF1 which function is to assemble CIII and CIV into the Q-respirasome and to provide physical linkage between CIII and CIV inside the N-respirasome [21]. SCAF1 thus induces a structural segmentation of CIV that organizes it into functionally separate subpopulations, monomer free, dimer free, Q-respirasome and N-respirasome. Interestingly, the absence of functional SCAF1 does not cause major bioenergetic problems and animals lacking functional SCAF1 are fertile and healthy, although they have lost regulatory options for the fine-tuning of their bioenergetic performance [21,35]. Thus, when cells are grown in DMEM, total cell respiration (driven by glucose, pyruvate and glutamine) was significantly higher in cells lacking functional SCAF1, whereas CIV respiration driven by TMPD in intact cells was similar regardless of SCAF1 expression. Respiration and the rate of ATP production were normal, or even increased with either substrate (pyruvate/malate or succinate) in permeabilized cells with non-functional SCAF1; however, to achieve maximal respiration both substrates need to be added simultaneously in WT, something that does not occur in non-functional SCAF1 cells (98). Similar results were obtained when comparing liver mitochondria from C57BL6/J (SCAF1 deficient) mice with those purified from CD1 mice (SCAF1 functional). These observations indicate that LOM principle only applied to SCAF1 deficient animals but, in this case, at the level of cyt *c*. These results allow to propose that superassembly define two subsets of cyt *c* and CIV molecules dedicated to receiving electrons coming from either NADH (N-respirasome) or FAD dependent enzymes (Q-respirasome), and a third CIV subpopulation able to receive electrons from both donors (Fig. 4). This third subpopulation of free CIV seems to be the most abundant form, and since it can receive electrons from both sources and requires free cyt *c*, its presence would explain the predominant interpretation of a pool behavior for cyt *c* (163). Recently, SCAF1 ablation was found to impair both adaptation to highly demanding physical work in mice [21] and to food restriction in both mouse and Zebrafish [35], indicating the physiological relevance of superassembly between complexes III and IV.

The same question was addressed in *Saccharomyces cerevisiae* [65]. Contrarily to mammalian, yeast CIII and CIV are only found superassembled and cyt *c* can only be oxidized by the supercomplex. In addition, *S. cerevisiae* lacks CI and the analysis, therefore, relates only to assemblies containing CIII and CIV; thus, its mtETC has lost plasticity. In this study the authors defined two populations, 16% pre-bound to the supercomplex and the remaining not bound to it. In this context, Trouillard and co-workers designed a series of experiments aimed to determine if superassembly impacts on the kinetic of cyt *c* oxidation [65]. To do that, a method was stablished to assess the ability of CIV to oxidize the cyt *c* pool which required the presence of carbon monoxide (CO) in the gaseous phase. Photoactivation of CIV after one laser shot was performed under different oxygen concentrations; however, a low oxygen concentration was chosen to assess cyt *c* compartmentalization to make oxygen binding a poor competitor with CO binding. It was that cyt *c* oxidation could be achieved completely only after two or more flashes. Interestingly, they described two well defined kinetics: fast, ascribed to the bound cyt *c*, and slow, ascribed to the non-bound cyt *c*. The authors concluded that since CIV could be accessible to both fractions of cyt *c* it was conclusive of cyt *c* not being compartmentalized into the IMM. Unfortunately, the study did not investigate the kinetics when CIII and CIV are dissociated but functional. However, their results predict that in these conditions the oxidation of cyt *c* would be significantly delayed although the physiological relevance of this needed to be stablished.

Recently, Berndtsson and colleagues addressed the same problem in a more comprehensive experimental set up [66]. After developing a high-resolution model of the yeast Q-respirasome structure the authors identified the key residues leading to critical contacts between CIII and CIV in the supercomplex. Mutations in such key residues in the CIII-subunit Cor1, both in wild type (WT) or strains devoid of cardiolipin synthase (*ΔCRD1*), showed impaired Q-respirasome assembly without relevant variations in neither CIII or CIV assembly. These novel cellular models allowed them to show that lack of supercomplex assembly delays the diffusion of cyt *c* between CIII and CIV [66]. As a consequence, Cor1 mutant yeast strain had lowered coupled respiration on both NADH and succinate oxidation which, importantly, could be recovered by the addition of external cyt *c*. Of note, mitochondrial membrane potential and respiratory control ratio were maintained similar to WT levels,

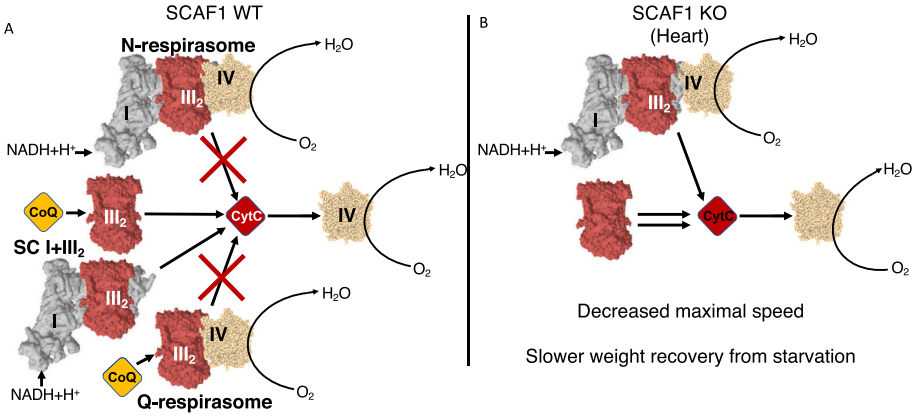

Fig. 4. - N- and Q-respirasomes do not share their functional cytochrome *c* pools under normal conditions. A) WT cells have the N-respirasome, constituted by CI (grey), CIII (red) and CIV (ochre) and the Q-respirasome, constituted by CIII (red) and CIV (ochre), which work with their own functionally segmented cytochrome *c* pools. Only SC I + III_2 and free CIII reduce cytochrome *c* from the bulk IMM cytochrome *c* pool (red) which is subsequently oxidized by free CIV molecules. B) ablation of SCAF1 promotes disassembly of the Q-respirasome and lack of physical bridge between CIII and CIV in the N-respirasome, providing a lower maximal speed and slower recovery from starvation in mice, and severe phenotypic changes in zebra fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

probably due to an enhancement of CIV activity in Cor1 mutant. Thus, this detailed study reveals that physical distance between CIII and CIV catalytic sites is a critical factor determining the efficiency of electron transfer between CIII and CIV. Indeed, recent cryo-electron microscopy structure and kinetics have revealed that electron transfer between CIII and CIV in yeast Q-respirome resemble substrate channeling [67]. Very importantly, the absence Q-respirasome becomes disadvantageous for yeast in a competitive environment due to the fact that more cyt *c* is needed to maintain electron transfer at the level of wild type cells.

In summary, studies in mouse, zebrafish and yeast provide kinetic and structural evidence on the existence of functionally segmented cyt *c* pools in mitochondria, which may exist by bringing together CIII and CIV in close vicinity, and that its absence brings critical metabolic consequences for their fitness. Its relevance may by more complex in vertebrate mitochondria than in yeast since the latter have all CIV in a single form. Yet to be explained is whether inhibition of CIV in supercomplexes would promote cyt *c* oxidation by individual CIV molecules, as occurs with CoQ in AOX-expressing mitochondria. These studies reveal the critical role of functional supercomplexes and that the partial segmentation of cyt *c* has important consequences in physiology and disease.

6. CoQ segmentation in signalling and disease

Hypoxia is involved in many physiological and pathophysiological scenarios [68–70]. Paradoxically, it was observed long ago that hypoxia triggered the production of mitochondrial ROS [71–74] and, though their involvement in chronic adaptation to hypoxia is not yet clear [75], they have been clearly shown to be necessary for the acute pulmonary and carotid body oxygen sensing [76–80]. The mechanism by which ROS increase during hypoxia has been recently solved [32]. Mitochondrial CI undergoes the active/deactive (A/D) transition and this promotes a slight acidification of the mitochondrial matrix, sufficient to partially dissolve the calcium phosphate precipitates in this compartment. Ca^{2+} is released and activates the mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX) in the IMM, which extrudes Ca^{2+} and enters Na^{+}. Na^{+} into the matrix interacts with phospholipids in the inner leaflet of the IMM, forming aggregates with the stoichiometry 3:1 (phospholipid:Na^{+}) which decrease the IMM fluidity. CoQ transfer between CII, G3PD and CIII is decreased, but not between CI and CIII. Reduction in CII + III activity is accompanied by an increase in ROS production which, in turn, mediate hypoxic pulmonary vasoconstriction. Besides being an evidence of the existence different functional pools of CoQ, these results demonstrate that the existence of different CoQ pools allows redox signalling while maintaining sufficient respiration capacity, and that the supercomplexes and CoQ pools are involved in signalling in physiological adaptation to hypoxia.

As mentioned above, the composition of phospholipids in the IMM is critical for the stability of supercomplexes. Mutants of several key enzymes in phospholipid biogenesis are involved in disease. Tafazzin is involved in maturation of cardiolipin, a fundamental phospholipid in the IMM, and mutations in its coding sequence have been linked to Barth syndrome, an X-linked mitochondrial disease affecting cardiac and skeletal muscle, and neutrophils [81,82]. Defective Tafazzin and impaired cardiolipin conversion have been associated not only with impaired assembly of supercomplexes [28,64], but also with increased ROS production [28,83] and with decreased coupled respiration [28,84]. Defects in mitochondrial phospholipid composition or clustering have been also associated with prevalent diseases. In particular, changes in PE are associated with Alzheimer's [85,86], Parkinson's [85,87] and liver disease [88,89]. In agreement with human data, defects in mitochondrial PE content by PSD knock-out mice diminished respiratory capacity, increased ROS production and blunted the assembly of supercomplexes [29]. Stomatin-like protein-2 (SLP-2) associates with cardiolipin and is involved in IMM spatial organization [90]. Overexpression of SLP-2 compensates the mitochondrial defects triggered by loss of Parkin, pointing to a role of SLP-2 in Parkinson's [91]. SLP-2 promotes the assembly of supercomplexes [92,93] and its knock-out has been shown to decrease respiration and raise mitochondrial ROS levels [31,92]. The same phenomenon occurs after decreasing the levels of SLP-2 homologous proteins, such as prohibitins [94,95], which have been also shown to interact with cardiolipin [96,97].

Given that supercomplex formation is associated with decreased production of ROS [21,26] and that Na^{+} can affect CoQ transfer between non-superassembled complexes and enhance ROS production [32], it is possible that reducing the stability of supercomplexes (e.g. Mutations in Tafazzin, SLP-2, etc.), disrupted the existence CoQ segmentation, particularly in supercomplexes, and this rendered CoQ transfer more susceptible to variations in IMM fluidity (e.g. by Na^{+}), increasing ROS production and lowering the electron transport in the mETC. This is particularly important as the recovery of supercomplex assembly and CoQ pool segmentation act as modulators or respiratory capacity and ROS production and points them out as potential therapeutical targets in disease.

7. Cyt *c* segmentation in signalling and disease

The discovery of SCAF1 lead to the identification of functionally segmented cyt *c* pools in vertebrate mitochondria. However, another unexpected conclusion was that respirasome assembly at the level of CIII + CIV is not required for life in mice [15]. However, the phenotypic consequences of SCAF1 ablation and, therefore, lack of Q-respirasome assembly and interaction between CIII + CIV in the N-respirasome, have not been studied until recently. García-Poyatos et al. studied the role of SCAF1 in zebra fish and showed that SCAF1 knock-out individuals, which specifically lacked Q-respirasome, were smaller in size, showed abnormal fat deposition and decreased female fertility. Very interestingly, doubling food supply rescued the phenotype of the mutant zebra fishes [35], pointing to a metabolic defect behind the phenotypic alterations. As high fat diet did not rescue the phenotype and respiration on CI substrates at the level of SCAF1 WT, it is possible that anabolism-related pathways, such as pyrimidine synthesis or folate pathway are affected by the specific loss of the Q-respirasome. Supporting the role of the Q-respirasome in anabolism, it has been shown that specific downregulation of SCAF1 in pancreatic ductal adenocarcinoma (PDAC) dampened growing rate and oxidative mitochondrial metabolism in severely hypoxic cells [98].

In addition, SCAF1 has been also shown to perform physical interaction between CIII and CIV in the N-respirasome (Fig. 4) [21]. Mice without functional SCAF1 reached lower maximal speed in the treadmill, indicating a lower capacity of oxidizing CI respiratory substrates, as evidenced by a lower NADH oxidation rate of the N-respirasome without SCAF1. Importantly, it has been shown that exercise promote the increase of N-respirasome in humans [99]. In addition, male mice with active SCAF1 exposed to periodic starvation were able to maintain, or even gain weight during the first days, in contrast to male mice without active SCAF1 which rapidly lost weight. In this line, cells subjected to glucose deprivation and endoplasmic reticulum (ER) stress stimulate the biogenesis of supercomplexes and enhanced respiration by increasing the expression of SCAF1. In fact, SCAF1 ablation is sufficient to decrease mitochondrial respiration, ATP levels, and impair proliferation in the absence of glucose [59].

As mentioned above, a mutant yeast strain with impaired Q-respirasome assembly showed lowered respiration due to increase of distance in the catalytic sites of CIII and CIV [66]. This led to severely reduced competitive fitness which could be restored by overexpression of cyt *c*. This evidences that Q-respirasome formation allows a more efficient use of energy as cyt *c* levels do not need to be put in excess, which would also potentially impact on the signalling to apoptosis. In addition, in a competitive environment which can be found by yeasts by encountering other yeast and bacteria, the pathways using resources more efficiently would prevail and become selected. Therefore, the existence of the

Q-respirasome represents an evolutionary advantage by bringing closer CIII and CIV which allows the more efficient use of cyt *c* in the surroundings of the supercomplex (i.e. functional segmentation). In this line, downregulation of SCAF1, and ablation of the Q-respirasome, in PDAC promoted a lower respiratory metabolism which led to decrease growing rate in severe hypoxia [98]. Indeed, cell growth rates after dihydroorotate dehydrogenase (DHODH) inhibition or in conditions of limited nucleotide availability, depend upon CIII and CIV superassembly [100]. All together, these results, reinforce the idea of that supercomplex assembly is required for an efficient use of oxygen and other respiratory substrates by the mETC.

Besides its role in transferring electrons between complexes III and IV, cyt *c* interact with other mitochondrial and non-mitochondrial components exerting a variety of roles that are modulated by its redox status. All together those conform the redox interactome of cyt *c* [101]. Cyt *c* can act as prooxidant as well as an antioxidant. Thus, oxidized cyt *c* scavenge superoxide radical ($O_2^{\bullet-}$) by removing unpaired electrons and thus regenerating O_2 [102,103]. Cardiolipin and other phospholipids can interact preferentially with oxidized cyt *c* leading to lipid peroxidation, an early stage in mitochondrial induced apoptosis [104]. The cytochrome b_5/cytochrome b_5 reductase system has a wide subcellular distribution including the outer mitochondrial membrane, facing the inter membrane space [105]. Cytochrome *b*5 reductase system catalyze a NADH:cyt *c* oxidoreductase activity that may be coupled to the inner membrane cyt *c* oxidase activity using intermembrane cyt *c* as an electron shuttle [106,107]. This reaction may stimulate an NADH-dependent production of superoxide [108]. Cyt *c* can also be reduced by the enzyme ALR, responsible for the reoxidation of Mia40 in the mitochondrial disulfide relay protein import pathway. Alternatively, ALR electrons may be passed directly onto molecular oxygen, yielding H_2O_2 [109]. When released to the cytoplasm reduced and oxidized cyt *c* interact with additional partners.

The rich and complex cyt *c* interactome make also unlikely the existence of a unique pool. A single cyt *c* pool may cause that competing reactions requiring cyt *c* could enter in conflict. In this regard, the N- and Q-respirasomes bound cyt *c* may define its preferential utilization to shuttle electrons between complexes III and IV, which can eventually be released to participate in other functions included the pool of cyt *c* released to induce apoptosis. The regulation of the proportion of oxidized/reduced cyt *c* may play a role in apoptosis. Thus, by controlling the oxidoreduction of a cyt *c* pool, segmentation could prevent the accumulation of excessive amounts of oxidized cyt *c* and, potentially, the induction of apoptosis. Indeed, cells exposed to galactose showed lower apoptosis when SCAF1 was present [110].

All together, these results evidence the importance of SCAF1, the interaction of CIII and CIV and, in particular of the cyt *c* pool functional segmentation under stress conditions through the more efficient use of respiratory substrates.

8. Conclusions and future perspectives

The functional segmentation of the CoQ and cyt *c* pools and the functional relevance of supercomplexes have been challenged for years. Recent structural, kinetic and regulatory evidence demonstrate the existence of these pools in yeast and vertebrate mitochondria. The existence of supercomplexes allows the more efficient use of substrates by mitochondria, particularly under stress conditions, following the principles of the plasticity model (Fig. 5), while they are not strictly necessary for CI stability. In addition, superassembly between CI and CIII, and also between CIII and CIV, are necessary to maintain normal levels of ROS.

As suggested by some studies, it is possible that specific supercomplexes are dedicated to particular pathways. In this regard, deficiencies in supercomplex assembly could potentially lead to defects in signalling and development of diseases after breaking the functional segmentation of the CoQ and cyt *c* pools, which can lead to the LOM principle at the level of the mETC, potentially leading to upstream metabolic alterations and phenotypic alterations, as evidenced by the absence of SCAF1. Now, the identification of factor(s) regulating the superassembly of $I + III_2$ is demanding, as it would allow the study of the metabolic and phenotypic consequences of ablating the functional segmentation of the CoQ pools.

In summary, the functional segmentation of mobile electron carriers is the very consequence of the existence of supercomplexes as the catalytic sites between individual complexes are brought together. Yet, the exploration of their role in signalling and disease, together with the consequences of their modulation, is still in its dawn.

Funding

This study was supported by MINECO: SAF2015-65633-R, RTI2018-099357-B-I00, HFSP (RGP0016/2018) and CIBER (CB16/10/00282). The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the Ministerio de Ciencia, Innovación y Universidades (MCNU) and the Pro

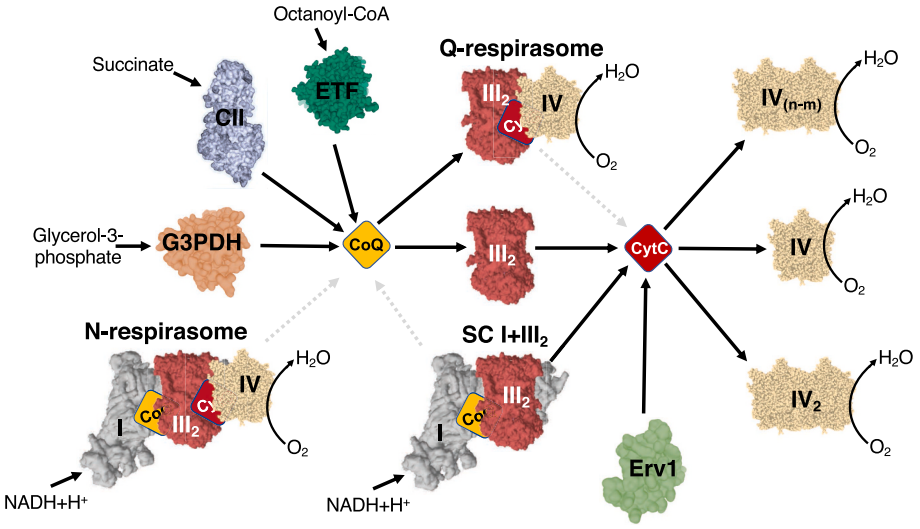

Fig. 5. - Schematic representation of the plasticity model. The plasticity model showing the partial functional segmentation of the CoQ and cytochrome *c* pools in the IMM. Functional segmentation of the mobile electron carriers allows the mitochondria to fine-tune the transfer of electrons in the respiratory chain to the specific metabolic needs of the cell.

CNIC Foundation and is a Severo Ochoa Center of Excellence (SEV-2015-0505). his research has been financed by Spanish Government grants (ISCIII and AEI agencies, partially funded by the European Union FEDER/ERDF).

Declaration of competing interest

The authors declare no competing interest

Acknowledges

The authors thank the whole GENOXPHOS group for suggestions and discussions. Figures created with BioRender.com.

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