Mitochondrial respiratory chain composition and organization in response to changing oxygen levels

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Abstract

Mitochondria are the major consumer of oxygen in eukaryotic cells, owing to the requirement of oxygen to generate ATP through the mitochondrial respiratory chain (MRC) and the oxidative phosphorylation system (OXPHOS). This aerobic energy transduction is more efficient than anaerobic processes such as glycolysis. Hypoxia, a condition in which environmental or intracellular oxygen levels are below the standard range, triggers an adaptive signaling pathway within the cell. When oxygen concentrations are low, hypoxia-inducible factors (HIFs) become stabilized and activated to mount a transcriptional response that triggers modulation of cellular metabolism to adjust to hypoxic conditions. Mitochondrial aerobic metabolism is one of the main targets of the hypoxic response to regulate its functioning and efficiency in the presence of decreased oxygen levels. During evolution, eukaryotic cells and tissues have increased the plasticity of their mitochondrial OXPHOS system to cope with metabolic needs in different oxygen contexts. In mammalian mitochondria, two factors contribute to this plasticity. First, several subunits of the multimeric MRC complexes I and IV exist in multiple tissue-specific and condition-specific isoforms. Second, the MRC enzymes can coexist organized as individual entities or forming supramolecular structures known as supercomplexes, perhaps in a dynamic manner to respond to environmental conditions and cellular metabolic demands. In this review, we will summarize the information currently available on oxygen-related changes in MRC composition and organization and will discuss gaps of knowledge and research opportunities in the field.
Keywords: Hypoxia, Mitochondrial OXPHOS, MRC, hypoxia-inducible factors (HIFs)

1. Introduction

Eukaryotic cells have developed complex molecular signaling pathways to maintain homeostasis upon changing environmental and nutritional conditions. Oxygen is a crucial determinant for cellular aerobic energy transduction, and its deficit is linked to the pathogenesis of severe human diseases, such as stroke, myocardial infarction, and chronic obstructive pulmonary disease. Therefore, cells present multiple oxygen sensing and response pathways to minimize cellular damage caused by hypoxia; that is, a decrease in the availability of oxygen. The main regulators of the rapid and efficient response to hypoxia are a family of transcription factors known as hypoxia-inducible factors (HIFs). HIFs are heterodimeric factors, composed of an oxygen-regulated alpha subunit (HIF-α) and a constitutively-expressed beta subunit (HIF-β). The alpha subunit is synthesized continuously in the cytosol and degraded by the proteasome in the presence of oxygen, whereas the beta subunit is proteolytically stable [1-3]. As the oxygen levels drop (threshold levels are tissue-specific [4-6]), the alpha subunit is stabilized, and the HIF complex is formed and translocated to the nucleus of the cell, where it binds to conserved regions, named hypoxia-responsive elements (HRE), in the promoter or the enhancer sequences of the hypoxia-regulated genes [2]. In

this way, hypoxia-responsive genes are transcriptionally activated to adapt to low oxygen conditions. Many genes activated in a HIF-dependent manner belong to functional clusters involved in oxygen homeostasis modulation and metabolic regulation [7, 8]. Simultaneously, a set of genes, mostly involved in cell proliferation [9], undergo an indirect decrease in their expression by transactivation of genes encoding chromatin-modifying enzymes [10, 11], transcriptional repressors [12, 13], or microRNAs [14].

Mitochondria are the major oxygen-consuming organelles of the cell because it houses the oxidative phosphorylation (OXPHOS) reaction, the process that couples oxygen consumption by the mitochondrial respiratory chain (MRC) with energy transduction to the chemical form of adenosine triphosphate (ATP). Upon oxygen deprivation, anaerobic energy transduction needs to take control to maintain the minimum cellular needs and, therefore, there are major molecular changes to shift from oxidative mitochondrial metabolism to glycolysis [15, 16]. The cellular adaptation to hypoxia also involves a decrease in energy demand by inhibition of processes, such as general protein synthesis and cell division [17]. Intracellularly, mitochondria undergo distinct modifications in their morphology and distribution to adapt their function to low oxygen levels (reviewed in [18, 19]). These changes include a decrease of mitochondrial mass, both by suppression of mitochondrial biogenesis [20, 21] and induction of mitophagy [22]; changes in intracellular mitochondrial distribution towards a perinuclear accumulation [23]; and modifications of mitochondrial morphology, promoting either fission in acute hypoxia [24], or fusion under chronic hypoxia to protect from apoptosis [25].

In this manuscript, we aim to briefly review the current literature on hypoxia-induced adjustments in MRC biogenesis and organization, and their biological significance. Specifically, we will discuss the switch of several MRC complex subunits to hypoxic isoforms, and modifications in the organization of MRC complexes into individual entities or assembled as macromolecular enzymes called supercomplexes (SCs), when cells and tissues are exposed to low oxygen levels.

2. Oxygen utilization by the mitochondrial respiratory chain

The MRC facilitates electron transfer from reducing equivalents (NADH and $FADH_2$) to molecular oxygen, a process coupled to the generation of a proton gradient across the mitochondrial inner membrane that is used by the F_1F_o-ATP synthase, to catalyze the phosphorylation of ADP to ATP. The MRC is composed of four multiprotein enzymatic complexes (CI to CIV) and two mobile electron carriers (ubiquinone and cytochrome *c*). CI, CIII, and CIV are responsible for the proton-motive gradient between the mitochondrial matrix and the intermembrane space. The MRC CIV, or cytochrome *c* oxidase (COX), is the terminal oxidase of the pathway, to whose catalytic center molecular oxygen binds and is sequentially reduced to H_2O. Consequently, CIV presents a high affinity for oxygen [26] and is considered the major cellular consumer of this molecule. During the MRC functioning, electrons can escape the pathway prematurely and contribute to the generation of reactive oxygen species (ROS) [27]. Although, in most cases, small-molecule electron carriers such as NADH or $CoQH_2$ (reduced coenzyme Q) do not react with O_2 to generate superoxide anion O_2^-, it can take place at redox-active prosthetic groups within MRC proteins, or when electron carriers such as $CoQH_2$ are bound to proteins [28]. Specifically, CI and CIII are considered main sites of ROS production in the MRC [28]. ROS can cause severe damage to lipids, proteins, and DNA within the cell. Thus, mitochondria contain several antioxidant defense systems to protect from those free radicals that are generated during the oxidative metabolism, such as detoxifying enzymes or glutathione. Excessive ROS production by mammalian mitochondria underlies oxidative damage in an array of human pathologies [29-33]. However, physiological ROS

generation contributes to retrograde redox signaling from the organelle to the cytosol and nucleus and may play an important role in the adaptation to an array of stressors, including hypoxia [34-38].

The MRC enzymatic complexes are located in the inner mitochondrial membrane; however, their organization has been a matter of intense debate over the last 50 years. Two opposed models have been historically proposed to explain the MRC organization. Whereas the solid-state model poses that MRC complexes are organized in a single rigid macromolecular assembly, the fluid-state model views the individual complexes freely diffusing in the mitochondrial inner membrane. Based on extensive data, the existence of mitochondrial supercomplexes (SCs) is today accepted and the prevalent model for the MRC organization is known as the plasticity model [39], in which supercomplexes of variable composition dynamically coexist with individual complexes presumable to facilitate fast adaptation to changes in cellular metabolism [40, 41]. The composition and abundance of these SCs vary depending on the cell type, tissue, organism, and even the environmental and nutritional state of the cell. In human cells, apart from CIII and CIV associations (SC $CIII_2$-CIV), CI can bind to $CIII_2$ (SC CI-$CIII_2$), and together to CIV, forming the so-called respirasome (CI-$CIII_2$-CIV_{1-n}). Recently, cryo-EM structures of mammalian SC CI-$CIII_2$ [42], mammalian respirasome [43-46], and human megacomplex [47] have been obtained, confirming their existence and facilitating the study of their function. The MRC organization into supercomplexes has provided a rationale for the fact that mutations directly affecting a single complex of the MRC frequently result in multienzymatic deficiencies in patients suffering from mitochondrial diseases. Besides, the disruption of these SCs is associated with a large number of mitochondrial disorders and age-associated human diseases [48-50]. However, despite the physiological and biomedical relevance of this MRC organization, little is known about the regulation of SC dynamics, the factors involved, and the functional benefits that the SC organization might provide.

There are several hypotheses about the functional advantages of the MRC organization in SCs (reviewed in [51-53]), including substrate channeling, decreased ROS production, stabilization of CI, regulation of MRC activity, and/or prevention of protein aggregation in the inner membrane. Despite numerous investigations, until now, only the hypothesis of quinone channeling as a kinetic advantage of respiratory SCs has been discarded based on experiments using an alternative quinol oxidase [54] and evidence provided by structural approaches [42]. A recent theoretical modeling approach, however, has indicated that forming respiratory supercomplexes may provide a kinetic advantage by linking complexes III and IV. This model has shown that the electron flux through these complexes can be limited by diffusion of cytochrome *c,* and, therefore, minimizing the distance between these complexes is kinetically advantageous [55].

In support of the relevance of SCs for CI assembly and stabilization, it was recently shown in a *Ndufs4*-KO mouse model [56] and in a homoplasmic *MTCYB*-deficient human cell line, that the incorporation of the NADH module to CI was deficient in the absence of CIII [57]. Several lines of evidence also reinforce the ROS hypothesis. Maranzana and colleagues also provided the first direct demonstration that the disruption of SC I+III_2 leads to an increase in superoxide generation from CI [58]. Additional observations have linked SC dissociation with higher ROS production: (i) lymphoblasts from Barth syndrome patients, biochemically characterized by reduced concentration and altered composition of the mitochondrial lipid cardiolipin, which leads to destabilized SCs, showed higher production of superoxide compared to control cells [59, 60]; (ii) a decreased stability of SCs in a mouse model lacking catalytic CIII subunit UQCRFS1 (or RISP) was associated with increased ROS levels [61]; (iii) K-RAS transformed fibroblasts presented a lesser

amount of SCs, correlated with higher ROS production [62, 63]; and (iv) knockdown of the CI subunit NDUFS1 in neurons led to decreased incorporation of CI into SCs, impaired oxygen consumption, and increased ROS levels [49]. Nevertheless, several of these hypotheses may be simultaneously correct, even if in a tissue- or condition-specific context and, therefore, further investigations are needed to establish the validity of these hypotheses and the functional relevance of MRC organization into SCs.

3. Mitochondrial respiratory chain and hypoxia

3.1. Changes in the composition of MRC complexes

When oxygen levels decrease, the activation of HIF signaling to enhance anaerobic ATP production is accompanied by a simultaneous downregulation of MRC biogenesis. However, during the transition to these adaptations, at the physiological level, a slower MRC electron transfer activity in the presence of decreasing oxygen levels also increases the chances of ROS generation, especially the superoxide anion O_2^- [64, 65]. Stimulation of ROS production under hypoxia mostly occurs at the CI, CII, and CIII sites [28, 66, 67], which concurrently activates several antioxidant defense pathways within the cell. An adaptation to minimize ROS production involves changes in the expression of tailored isoforms of several MRC complex subunits. The most targeted complexes for these adaptations are CI, a dominant acceptor of electrons, and CIV, the terminal MRC oxidase that regulates the overall electron flow.

CI undergoes several changes to decrease its activity. Hypoxia provokes a CI conformational change from an active to a dormant form, which prevents a burst of ROS generation following reoxygenation [68, 69]. The dormant form is a Na^+/H^+ antiporter, independent of energy transduction, and formed spontaneously by a lack of substrates [70]. Under chronic hypoxia, HIF-1α induces the degradation of the transmembrane CI assembly factor TMEM126B, which attenuates functional complex I assembly and reduces the cellular respiratory capacity [71]. Moreover, a hypoxia-responsive microRNA, miR-210, attenuates the expression of iron-sulfur cluster assembly proteins 1 and 2 (ISCU1/2) [72-74], affecting the maturation of mitochondrial iron-sulfur containing proteins, such as NDUFS in CI and SDHD in CII [75], and hence affecting MRC complex assembly.

CIV is also modified to respond to changing oxygen levels by undergoing post-translational modification of its subunits [76-78], or by changing its composition [79, 80], to regulate energy production. Mammalian CIV contains a COX4 subunit, which exists in a pair of normoxic/hypoxic isoforms: COX4-1 and COX4-2. COX4-2 is expressed under hypoxic conditions [81], and its presence enhances the CIV catalytic constant, thereby increasing overall MRC efficiency [82]. Concurrently, under low oxygen levels conditions, the Lon protease is upregulated by HIF-1 and degrades the COX4-1 subunit [83]. By regulating the proportion of each isoform, cells can adjust the MRC electron transfer rate to oxygen availability, thereby increasing the efficiency of electron transfer while minimizing ROS generation when oxygen availability is reduced. Paradoxically, mammalian COX4-2 is mostly expressed in the lung, a tissue exposed to the highest concentration of oxygen. By ensuring a higher CIV efficiency, the presence of COX4-2 could act as a mechanism of protection and energetic adaptation to minimize ROS in highly oxygenated tissues [81, 84, 85]. A Hypoxia-Inducible Gene Domain family (HIGD) protein, HIGD1A, was similarly described to be induced under hypoxia and to bind CIV around its heme *a* active center to ensure optimal CIV activity, exerting a protective function under hypoxia [86]. In addition, the CIV subunit NDUFA4 is a target of miR-210 and is decreased in a HIF-dependent manner [87]. The decrease of NDUFA4 coincides with the increase of its hypoxic isoform NDUFA4L2, which lowers CI and CIV activities and, thus, mitochondrial respiration, preventing excessive ROS production and maintaining the mitochondrial membrane potential [88]. NDUFA4L2 expression

was recently correlated with poor patient survival in hepatocellular carcinoma [89]. Finally, the CIV assembly factor COX10, also a target of miR-210, and the CIV subunit COX5B [90] are decreased in a HIF-dependent manner, attenuating CIV assembly under low oxygen levels.

In summary, cells undergo a decrease in the abundance of MRC CI and CIV (see **Figure 1**), whereas the expression of hypoxic subunit isoforms from these complexes are activated to decrease ROS production and optimize electron transfer and energy production under hypoxic conditions.

3.2. Changes in the MRC organization into supercomplexes

The plasticity model of the MRC organization postulates that individual respiratory complexes dynamically associate into SCs to adapt to changing environmental and nutritional conditions [91]. Several studies have shown that SCs are increased in human [92] and rat [93] mitochondria after exercise, participating in the antioxidant effect of physical activity. Similarly, ER stress also increases the rate of SC formation in mitochondria in human cell lines [94]. On the contrary, after starvation [39], during aging [95], or in multiple human diseases [96], the abundance of respiratory SCs is decreased. All these studies point towards a link between MRC supercomplex formation and the metabolic and/or nutritional state of the cell. However, these investigations are based on the steady-state levels of MRC complexes and SCs analyzed by Blue Native PAGE (BN-PAGE), which cannot provide information regarding the capacity of MRC complexes to dynamically associate and dissociate. There have been several attempts to overcome these methodological challenges, as reviewed in [97]. The development of proximity-dependent labeling techniques (e.g., BioID) has allowed identifying transient or weak protein-protein interactions, even in the case of insoluble complexes [98]. Crosslinking mass spectrometry also supported the existence of SCs in intact tissue or mitochondria [99-101]. Both techniques may reflect the organization of the proteins into SCs in a more native manner than BN-PAGE because there is no extraction of the MRC complexes from the mitochondrial inner membrane. But again, their results only provide information in a steady-state manner. The recent use of GFP-FRET technique to determine SCs formation in live cells [102] has better prospects regarding dynamism information. The authors analyzed in this study fluorescent sensor proteins located in the MRC SCs by fluorescence lifetime imaging microscopy (FLIM) to monitor SC assembly and plasticity in live cells. The use of this technique could be useful to establish relationships between MRC organization and cellular nutritional and environmental conditions.

Nevertheless, there is a particular paucity of investigations on the rearrangements that the mitochondrial SCs might undergo under low oxygen levels conditions. In plants, CI becomes destabilized and dissociates from SCs after prolonged hypoxic conditions [103] (**Figure 1**). It was proposed that this disruption of CI-containing SCs is due to a transition from an active to a dormant form of CI, alongside with activation of alternative oxidases that could prevent the inhibition of the glycolytic pathway because of a deficiency of NAD^+. A different scenario was described in freshwater turtles *Trachemys scripta*, known to tolerate severe hypoxia and reoxygenation without suffering from heart damage. Heart mitochondria from these turtles contain highly stable CI-containing SCs, which remain intact even in the presence of the detergent dodecyl maltoside, contrary to what is observed in mammalian SCs from other species [104]. Although ROS generation was found attenuated in heart mitochondria from anoxia-acclimated turtles, the authors could not establish a direct relationship between SC formation or stability and protection against hypoxia.

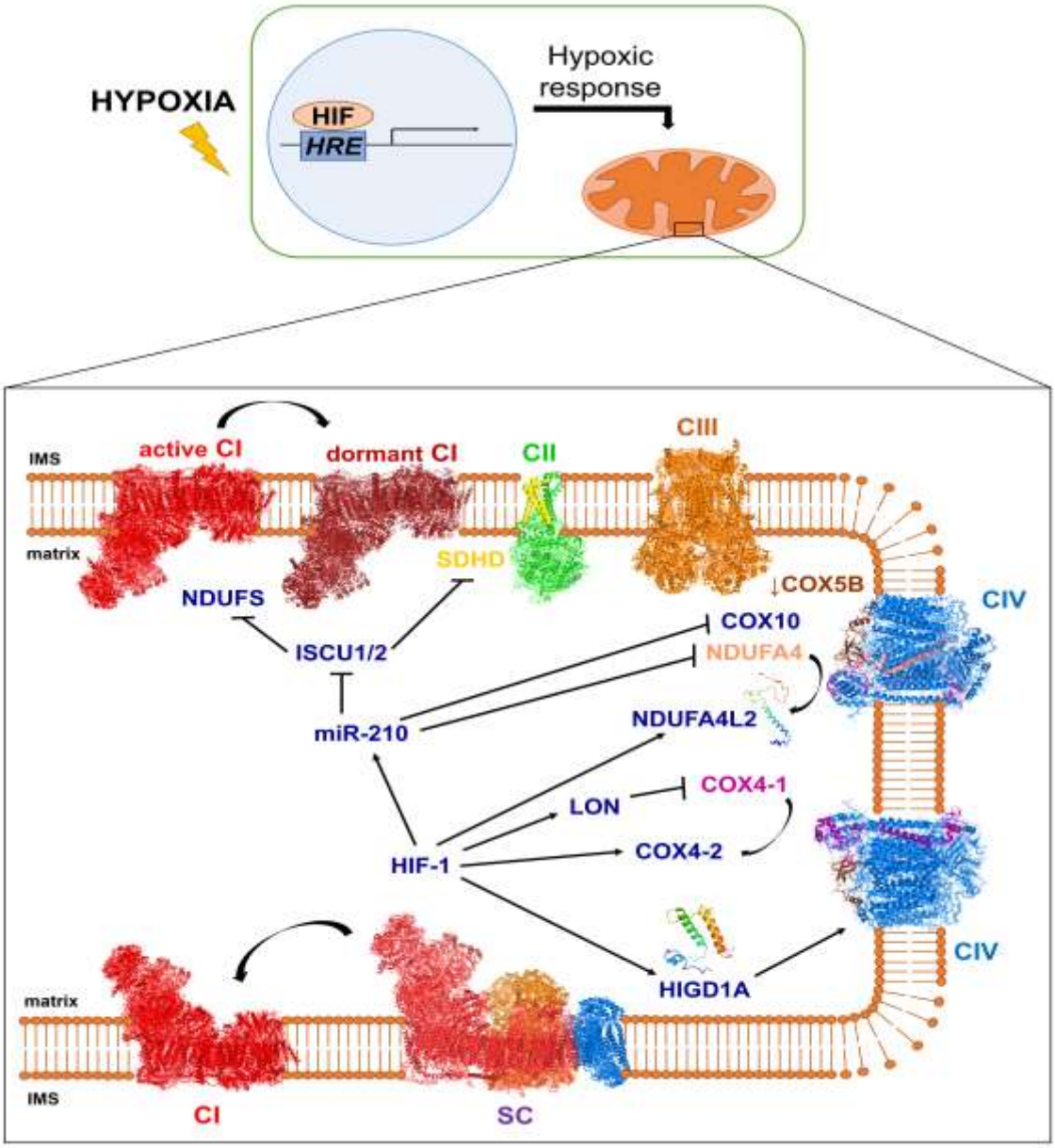

Figure 1. Schematic representation of known modifications in the mitochondrial respiratory chain composition and organization during hypoxia.

Changes induced in complexes and supercomplexes (SCs) of the mitochondrial respiratory chain under low oxygen levels. CI undergoes a switch from its active to its dormant form, which has been related to destabilization of CI in the SCs in plants. HIF1 induces the expression of hypoxic isoforms of subunits of CIV NDUFA4L2 (SWISS-MODEL Q9NRX3) and COX4-2, while upregulating LON protease that degrades COX4-1 isoform of CIV. There is also a HIF1-dependent induction of HIGD1A (RCSB PDB 2LOM), to generate a more efficient CIV, and a decrease of the CIV subunit COX5B. Concurrently, miR-210 is activated and downregulates ISCU1/2, decreasing the levels of NDUFS subunits of CI and SDHD of CII. CIV subunit NDUFA4 and CIV assembly factor COX10 are also targets of miR-210, lowering their levels.

CI, complex I (dormant state RCSB PDB 5O31; active state RCSB PDB 5XTD). CII, avian complex II (RCSB PDB 1YQ3). CIII, complex III (RCSB PDB 5XTE). CIV, complex IV (RCSB PDB 5X62). SC, respiratory supercomplex $I+III_2+IV$ or respirasome (RCSB PDB 5GPN). HIF, Hypoxia Inducible Factor. HRE, Hypoxia Responsive Element. IMS, Intermembrane Space.

The single mammalian SC assembly factor described so far, COX7A2L (COX7RP, or SCAFI), is involved in the assembly of $CIII_2$-CIV and in higher-order structures (CI-$CIII_2$-CIV_{2-n}) [39, 105-107]. COX7A2L was discovered in mice (named COX7RP) simultaneously by two different groups. Lapuente-Brun and colleagues performed a screening for proteins present in SCs but not in free complexes, showing that COX7A2L promotes interactions between $CIII_2$ and CIV [39]. However, it is now accepted that COX7A2L not only associates with SCs but also independently interacts with both $CIII_2$ and free CIV to promote SC III_2+IV_1 stabilization, without affecting the formation of the respirasome or SC I+III_2+IV_1. Lapuente-Brun and colleagues also reported that some wild-type mouse strains (e.g., C57BL/6J and BALB/c) express only a short, unstable COX7A2L isoform that failed to support CIV association into SCs, thereby promoting differences in mitochondrial respiration rates and ATP production [39]. However, although the degree of respirasome instability varies among tissues [108], the functional deficits originally claimed remain under debate [51]. Concurrently, Ikeda and co-workers generated a *COX7A2L*-KO mouse, after the identification of this protein as estrogen-sensitive [109] with a homologous sequence to COX7A subunit; and described an impairment in SC formation in muscle [110].

Expression of COX7A2L was shown to be upregulated in a tissue-specific manner in human cell lines during heat-shock and acute oxidative stress; however, the SC organization was not altered in these conditions [105]. Levels of COX7A2L were also increased after carbon source switch from glucose to galactose, which stimulates mitochondrial energy metabolism [111], in several cell lines [94, 105]. However, Lobo-Jarne and colleagues did not find any evident impact in MRC biogenesis and mitochondrial bioenergetics in the absence of COX7A2L in HEK293T or U87 cells [105], whereas Balsa and colleagues described a decrease in SC levels and oxygen consumption in human U2OS cells and mouse fibroblasts lacking COX7A2L in the presence of galactose [94]. Its homolog protein in mice was shown to play a role in glucose metabolism [112]. These differences in the results could be due to a tissue- or species-specificity of SC rearrangements or to technical issues; e.g., varying experimental conditions (use of isolated clonal KO cell lines vs. pools of clones, whole cell vs intact mitochondria analysis, or membrane solubilization conditions) and/or methods (polarography or high-resolution respirometry vs. Seahorse Bioanalyzer measurements) between groups. Therefore, the role of COX7A2L under nutritional stress remains to be fully resolved.

Recently, however, overexpression of COX7A2L in human cells was described to promote cell growth during hypoxia, inhibiting the hypoxia-induced generation of mitochondrial ROS [113]. Furthermore, the authors observed stimulation of SCs CI-CIII-CIV_n and $CIII_2$-CIV_2 assembly and stabilization during hypoxia when COX7A2L was overexpressed, together with an increase in the maximum respiratory rate of the cells [113]. This could indicate a possible function of mitochondrial respiratory SCs in the adaptation to different environmental conditions, conceivably by enhancing respiratory efficiency while decreasing ROS production. However, more investigations are necessary to study SC biogenesis and organization under hypoxic conditions, in order to understand the possible role of these macrostructures and their significance in the adaptation to hypoxia.

The existence of other SC assembly factors involved in the adaptation of the MRC to different conditions is also plausible. The HIGD1A and HIGD2A proteins, homologous of the yeast Respiratory superComplex Factor Rcf1, are good candidates to perform a role in SC assembly and regulation during hypoxia. Rcf1 was proposed to have a role in the assembly and stability of yeast SCs [114, 115]. Rcf1 and its homologous HIGD proteins belong to the Hypoxia Inducible Gene Domain 1 family and, therefore, are overexpressed under hypoxic conditions, increasing cell survival [86, 116, 117]. In hypoxia, HIGD1A was shown to interact with CIII and CIV [86, 116], and in standard cell culture

conditions, complexome analysis showed HIGD1A to associate with SCs [118]. HIGD2A was able to promote CIV and CIV-containing SCs formation in a yeast deletion model of Rcf1 [114]. Recently, our group created HEK293T *HIGD*-KO cell lines to study the role of HIGD proteins under standard cell culture conditions. HIGD2A was described as a CIV-assembly factor, controlling and coordinating modular assembly of isolated and supercomplexed CIV [119]. Differently, HIGD1A has a function in the incorporation of the CIII catalytic subunit UQCRFS1, regulating the kinetics of CIII and CIII-containing SCs. HIGD1A also binds to CIV subunits COX4-1 and COX5A and, when overexpressed, is able to suppress the CIV biogenetic and respiratory defect of *HIGD2A*-KO cells. Therefore, we concluded that both HIGD proteins play independent and overlapping roles in the biogenesis of respiratory complexes and SCs in physiological conditions [119]. However, the mechanism of action and the role in MRC assembly and organization of these HIGD proteins in hypoxic conditions remain to be investigated.

4. Concluding remarks

Oxygen is a critical molecular component of the mitochondrial energetic metabolism, and eukaryotic cells have evolved to adjust to different levels of this molecule to survive. HIF complexes are responsible for orchestrating the cellular response to low oxygen levels, activating and suppressing the expression of diverse genes. Mitochondria are one of the main targets in the hypoxic adaptive response. Recently, multiple studies are showing that remodeling of the MRC organization into SCs is part of the adaptation of cellular metabolism to changing environmental and nutritional conditions. Exposure to hypoxia has been proposed as a candidate therapeutic option to treat mitochondrial diseases. Several studies in mice and cellular models of Leigh syndrome, an encephalomyopathy associated with MRC malfunction, showed that hypoxia attenuated the symptoms and increased the life span of the disease models. Accordingly, the authors of this review speculate that hypoxic conditions could induce modifications in the MRC, not only at the composition level but also at the organizational level. The relationship between hypoxia and human diseases accentuates the interest to explore the molecular mechanisms that modulate the MRC upon low oxygen levels, as a way to increase our knowledge of the pathogenesis of mitochondrial diseases.

Several technical and conceptual open questions remaining, some of which are listed here, are a priority for future work. For example, an optimized and standardized method to evaluate MRC function at different oxygen tensions is necessary to provide consistent results within the scientific community. For this purpose, high-resolution respirometry has gained momentum as a rigorous and accurate approach [105]. The discovery of new SC assembly factors is also required for the understanding of the molecular pathways leading to SC formation and its plasticity upon changing environmental and nutritional conditions. New structural information, screening for proteins detected in SCs, or Complexome Profiling Alignment (COPAL) to elucidate novel proteins co-migrating with SCs could lead to the detection of new proteins involved in SC assembly and biogenesis. Also, with the structural information available, it is feasible to engineer yeast strains and human cell lines containing fully functional MRC complexes but unable to superassemble, as well as lines in which the MRC complexes are permanently linked. Furthermore, the field requires the development of new methodologies to analyze, in live cells, MRC SCs formation and disruption, to understand whether and how the process changes in a dynamic-manner to respond to nutritional or environmental stressors. Approaches such as fluorescence lifetime imaging microscopy, explained earlier, could be useful to provide answers to some of the remaining questions in the field.

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